Università degli Studi di Cagliari

DOTTORATO DI RICERCA

Scienze e Tecnologie per l'Innovazione

Ciclo XXX

Biology and Immunogenetics of Immune Effectors: Applications in Hematology and Cell Therapy

Settore scientifico disciplinare di afferenza

MED-15

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Esame finale anno accademico 2016 – 2017

Tesi discussa nella sessione d'esame Marzo 2018

A Fede

Summary

Abstract

Immune effectors are the cells responsible for host response to cancer cells and germs. The key to understand how to defeat cancer and infectious disease is probably hidden in biology of immune effectors. Most of recent advances in cancer therapy are based on drugs that use immune effectors abilities to kill neoplastic cells. Unfortunately, no similar advances have been observed in infectious disease therapy. So, there is a stringent need to understand immune effectors biology to improve infectious disease management, particularly for patients affected by deep grade immunosuppression. During my research, I chose to investigate the role of Glucose-6-Phosphate Dehydrogenase (G6PD) on immune effectors biology, to evaluate its relevance for response to infectious agents. G6PD is a key enzyme of pentose-phosphate pathway, producing reducing power in the form of NADPH, that is essential for oxidative stress protection machinery. G6PD deficiency is the most common human enzyme defect, affecting over 400 million people worldwide. The typical manifestations of enzyme deficiency are acute hemolytic anemia (AHA) following exposure to drugs or food and neonatal jaundice. The enzyme is expressed in all cells, but typical manifestations are related to enzyme deficiency in red cells. G6PD deficiency impact on white cells is less studied; some authors postulated that enzyme deficiency confers susceptibility to infections, but evidences are controversial. I tried to study the role of this enzyme defect in patients with hematologic malignancies, a population characterized by deep grade immunosuppression. Bacterial infections are the leading cause of morbidity and mortality in hematologic patients with chemotherapy induced neutropenia. Fluoroquinolone prophylaxis was proved to be the only effective strategy to reduce febrile neutropenia, but the safety of this class of agents in patients with G6PD deficiency is questionable because of a claimed association with AHA. I retrospectively analyzed 242 patients treated with 628 intensive chemotherapy courses; 59 patients presented G6PD deficiency. All patients underwent fluoroquinolone prophylaxis and were transfused according to “single unit”

transfusion policy. The primary endpoint was incidence of AHA. Secondary endpoints were incidence of febrile neutropenia, microbiologically and clinically documented infections and incidence of Gram Positive or Gram Negative infections. No episode of AHA was observed in the entire cohort. Incidence of microbiologically and clinically documented infection was similar, but incidence of invasive fungal disease (IFD; p<0.0001, HR 11.4, 95%CI 3.5-37.05) and Candida sepsis (p=0.008, HR 37, 95% CI 2.01-680.9) was higher in patients with enzyme deficiency. Interestingly, I observed a reduced incidence of febrile neutropenia in patients with G6PD deficiency (p=0.01, HR 0.46, 95%CI 0.25-0.8). These data suggest that fluoroquinolone prophylaxis in patients with G6PD deficiency, treated with intensive chemotherapy, is feasible and safe. Also, data about IFD incidence and febrile neutropenia suggest that G6PD may be important in susceptibility to opportunistic pathogens and host response in neutropenic patients. Next, I evaluated G6PD deficiency role in susceptibility to IFD in patients affected by acute myeloid leukemia (AML) undergoing intensive chemotherapy, that are the patients at higher risk of fungal infections. I investigate this point in a cohort of 108 patients; 28 harbored G6PD deficiency, whereas 80 were normal. The incidence of IFD was significantly higher in patients with G6PD deficiency compared to normal patients (35.7% vs 5%, p=0.0002, OR=10, 95%CI=2.96-37.5). Higher risk of mold infections (17.9% vs 5%, p=0.048, OR=4.1, 95%CI=1.0-16.6) and Candida sepsis (17.9% vs 0%, p=0.0009, OR=37.68%, 95%CI=2.0-707.1) was observed in patients with G6PD deficiency. These data suggest that the evaluation of G6PD activity may help to identify AML patients at higher risk of IFD, allowing to design more intensive surveillance and therapeutic strategies. The identification of G6PD deficiency as a risk factor for IFD in patients with AML results in an urgent need for strategies to properly manage this kind of patients at high risk of invasive mycoses. Next, I proposed an algorithm for correct identification, prophylaxis and treatment of IFD in patients with G6PD deficiency undergoing intensive chemotherapy for AML.

Introduction

Immune effectors could be defined as activated cells that defend the body in an immune response. The immune response machinery is essential for host response to infectious disease and cancer cells. The cells involved in cancer surveillance and response to infections are neutrophils, monocytes, lymphocytes, natural killer lymphocytes and several subgroups of tissue resident antigen presenting cells. The biology of immune response is a field of active investigation, but there are many points that still have to be elucidated.

In recent years, the most relevant advances in oncology have been made through the introduction of new drugs that interact with immune effectors, using their ability to selectively target cancer cells. However, these advances have not been observed in infectious diseases; in fact, strategies for infectious disease management are still based on direct administration of antibiotic, antifungal and antiviral chemotherapy. Most drugs used for treating infectious diseases were introduced more than one decade ago. This point has potentially a great relevance; in fact, infectious diseases are mediated by germs that could achieve resistance to any established treatment, despite its efficacy. Moreover, this resistance ability could be transmitted to other strains, leading to broad resistance to previously efficacious drugs.

With improvements in health conditions, introduction of vaccines and efficacious antibiotics, the mortality attributable to infectious disease drastically decreased in general population. However, some subgroups are still exposed to great morbidity and mortality due to infectious diseases. In fact, hematologic patients suffer from disease and chemotherapy induced immunosuppression, and are greatly exposed to infectious diseases. The pathogens causing infectious diseases in hematologic patients are germs frequently harmless in immunocompetent patients; however, these germs became virulent and often lethal in immunocompromised hosts.

Little is known about biology of immune effectors and infectious disease control. There is a stringent need to describe the machinery that underpins infection control in the hematologic patients, with the aim to identify new targets to improve outcomes. I decide to focus my research on biology of leukocytes involved in immune response to infections in hematologic patients. I chose to investigate the role of a well-studied enzyme, Glucose-6-Phosphate Dehydrogenase, that in my opinion could be greatly involved in this scenario. The setting of hematologic patients could be the ideal situation to investigate the role of this enzyme in infections control. During my research, I investigated the role of G6PD in susceptibility to infections and its interaction with the drugs administered for prophylaxis according to international guidelines. The aim was to identify the eventual association between enzyme deficiency and susceptibility to infections and to discriminate if patients with G6PD deficiency could safely be exposed to fluoroquinolones, with the hope to elaborate new strategies for the proper management of hematologic patients undergoing chemotherapy.

Infectious Disease in Hematology: a survey of the problem

Infectious diseases currently represent an important cause of morbidity and mortality for patients affected by hematological malignancies. In the last decades, the introduction on clinical practice of new chemotherapeutic agents and of new strategies, as hematopoietic stem cell transplantation (HSCT), determined a decrease in mortality attributable to hematological malignancies. However, unfortunately, most of these therapeutic strategies may worsen and prolong immunosuppression, during which patients are highly exposed to infectious diseases.

Therefore, despite improvements in terms of long term overall survival, infectious diseases are still a common complication secondary to cancer chemotherapy and are responsible for most of the deaths during cancer treatment [1]. It was estimated that almost a third of oncohematological patients suffer from an infection during their clinical history [2], frequently requiring admission to intensive care units, invasive diagnostic work-up and high cost intravenous drugs administration.

During last years, we learned several aspects about factors predisposing to infectious diseases, and now we have sophisticated diagnostic instruments and efficacious drugs. However, the increasingly number of immunocompromised patients, the variability of epidemiology and the outbreak of multi-drug resistant germs make infectious disease a persistent and unsolved problem in oncohematology [1]. The identification of risk factors for infectious diseases is one of the fields of active investigations in hematology [3]. The knowledge of the variables involved in determining the risk of infectious diseases is fundamental to identify patients at high risk of infections, for whom it's mandatory an adequate surveillance strategy and a prompt antimicrobial therapy. Likewise, is important to identify patients at low risk of infection, for whom a less aggressive approach is acceptable, saving days of hospitalization and allowing an appropriate use of resources.

In the last years, several studies [4,5] investigated the epidemiology of infectious diseases in many subgroups of hematological patients and the relative risk factors, especially in those affected by

acute myeloid leukemia and in those undergoing allogeneic HSCT. These studies, although contributing to describe the problem of infectious disease in hematological patients, were limited because of their retrospective design. Only recently the incidence of infectious diseases in the oncohematological patient has been prospectively described, with the Hema E-Chart, an online registry [2]. This study provided important data about epidemiology of viral, bacterial and fungal infections in all subgroups of oncohematological patients, confirming that patients affected by acute myeloid leukemia (AML) presented the highest risk of infectious diseases, followed by those with acute lymphoblastic leukemia (ALL). The risk of infectious diseases was instead lower for patients affected by other hematological malignancies as Multiple Myeloma (MM), Hodgkin Lymphoma (HL), non-Hodgkin Lymphoma (NHL), myeloproliferative syndromes and chronic lymphoproliferative disorders [2].

Pathogens responsible for infectious disease in cancer patients have changed during last fifty years. In '60- '70 years, Gram Negative bacteria, especially Escherichia Coli, Klebsiella species and Pseudomonas Aeruginosa, were responsible for most of microbiologically documented infections in oncohematological patients. Since half of '80 years it was observed a progressive increase of Gram Positive bacterial infections, especially mediated by Coagulase Negative Staphylococci and Staphylococcus Aureus. In the last decades it was registered an outbreak of other Gram Positive bacteria, rapidly emerging as relevant pathogens. Within this group of "emerging" bacteria, the most relevant are Viridans Group Streptococci and Enterococcus species [1].

Recent studies showed that the percentage of infections mediated by Gram Positive bacteria increased from 62% observed in 1995 to 76% in 2000. In contrast, the rate of infections sustained by Gram Negative bacteria decreased in the same time frame from 22% to 15% [6].

The reasons proposed for this epidemiological shift are several: widespread use of central venous catheters, common antibiotic prophylaxis with fluoroquinolones, routine use of proton pump

inhibitors, aggressive chemotherapy regimens causing severe and long-lasting oral mucositis. However, it should be noted that most of epidemiological studies reported only documented bacteremias [6]; in fact, only 15-25% of patients with neutropenia develop bacteremia, mostly because of Gram Positive infections. In contrast, most infections involving other sites as upper and lower airways, urinary tract, gastrointestinal system, are caused principally by Gram Negative bacteria [1]. This observation has been confirmed by prospectively data from Hema E-Chart study, where the percentage of documented infections attributable to Gram Negative bacteria reaches 51%. This finding suggests that probably the Gram Negative/Gram Positive infection incidence ratio is near one [2].

Another important aspect, often ignored on epidemiological studies, is the incidence of polimicrobial infections, in which is possible to isolate more than one etiological agent.

According to recent studies, incidence of polimicrobial infections doubled during last 30 years, and actually it's estimated that up to 23% of all documented infections in hematological patients are sustained by more than one germ [7]. In about 80% of these infections is involved a Gram Negative bacterium, with a percentage of 33% of polimicrobial infections sustained only by Gram Negative germs [7]. Less frequent, but anyway of relevance, are the infections mediated by viruses, parasites and fungi. Systemic fungal infections represent 3.7% of all infectious diseases affecting patients with hematological malignancies; a fungus is identified as the pathogen responsible in the 14% of febrile events [2]. The species more frequently involved are Candida and Aspergillus. Other common pathogens are Fusarium, Tricosporon, Acremonium and Scedosporium. Moreover, after the introduction of highly immunosuppressive agents as Fludarabine and Clofarabine, it was observed a return of infections mediated by Criptococcus, dimorphic fungi and Pneumocystis Jirovecii [1].

Herpes Simplex 1 and 2, Herpes Zoster, Cytomegalovirus, Epstein Barr Virus and Human Herpes Virus 6 are the most common pathogens involved in viral infections; Toxoplasma Gondii is the responsible of most of parasite infections [1].

Gram Positive Bacterial Infections

Coagulase negative Staphylococci are actually the most frequently isolated pathogens on blood cultures from hospitalized patients. The high incidence of opportunistic infections mediated by these germs in immunosuppressed patients is related more to host susceptibility than to virulence of the bacteria. In fact, Coagulase negative Staphylococci are weak pathogens; however, their impact on cumulative incidence of infection is relevant, in particular if considered the increase on median length of hospitalization and the augmented need for antibiotic use, especially Vancomycin. Coagulase negative Staphylococci are the main component of human cutis normal bacterial flora. These germs are the principal pathogen involved in bacteremia, intravascular device and prosthesis infections. Most of these staphylococci are resistant to Methicillin, and therefore Vancomycin and Teicoplanin are the drugs of choice for these infections [8].

Staphylococcus Aureus is present on nasal mucosa about in 30% of healthy population. The infection mediated by this germ results from synergy of bacterial intrinsic virulence factors, as toxins production, tissue-invading enzymes and rapid acquisition of antibiotic resistance machinery, with host immune defenses decrease. The most important factors causing increased infection risk are mucosal and cutis integrity loss, neutropenia, white cells functional defects and presence of intravascular devices or prosthesis. Staphylococcus Aureus is the most common cause of vascular catheter infections, and could provoke central nervous system or soft tissue infections through hematogenous spread or contiguous structures dissemination. Lungs are another possible infection site, invaded through aspiration of bacterial flora from upper respiratory tract. Some strains of Staphylococcus Aureus are responsible for two distinct clinical pictures: Toxic shock syndrome and Burn Skin Syndrome. In most centers, more than 90% of Staphylococcus Aureus produce Beta-Lactamases, and it was estimated that in several hospitals a percentage about 40% of the strains is

resistant to Methicillin. Here again, Vancomycin and Teicoplanin are the drugs of choice [9]. During last years, several strains of Glycopeptide-resistant Staphylococci have been described, probably because of common use of this class of drugs in clinical practice. For this reason, two new drugs are being introduced in the management of this kind of infections: Linezolid, belonging to Oxazolidinones class, and Quinupristin/Dalfopristin, belonging to Streptogramins [1].

Between Enterococcus species, Enterococcus Faecalis and Enterococcus Faecium are the most relevant in terms of pathogenicity for humans. In hematological patients, these germs are frequently responsible for infections in those who develop mucositis, or where the normal bacterial flora equilibrium has been altered by antibiotic treatment. Enterococci are frequently involved in intravascular devices infections. These germs are resistant to all Cephalosporins, and most strains are Streptomycin resistant. Historically, Enterococcus infections have been treated with Carbapenemes and Glycopeptides, but actually is frequent the isolation of Vancomycin resistant strains. Also in this case, Linezolid and other recently introduced drugs, as Daptomycin, seem to play an important role as salvage therapy in case of resistance to conventional treatment [10]. Within Streptococci, Viridans group strains are those most frequently involved on infections affecting hematological patients. These are α-hemolytic strains, usually resident in oropharynx and in gastrointestinal tract. The principal risk factors are high dose chemotherapy, especially with Cytarabine, development of mucositis and Fluoroquinolone or Trimetoprim-Sulphametoxazole prophylaxis. Interestingly, Endocarditis, a common complication of Viridans Streptococci observed on immunocompetent patients, is rarely associated with Streptococcus bacteremia in neutropenic patients. The drug of choice is Vancomycin, because of the high incidence of resistance to Beta-Lactam antibiotics. Neutropenia and immunodeficiency, associated with malnutrition, are also well described risk factors for infections mediated by another Streptococcus species, as Streptococcus Pneumoniae. This germ is present in the rhinofarynx of 5-10% of adult population, and is responsible

for pneumonia, sinusitis, otitis and central nervous system infections. The drugs of choice are Beta-Lactam, but also Fluoroquinolones and Macrolides have a good activity against this bacterium [1,11].

Gram Negative Bacterial Infections

The Gram Negative bacteria most frequently responsible for infections in hematological patients are Pseudomonas Aeruginosa and Enterobacteriacae; within Enterobacteriacae species, Klebsiella and Escherichia Coli are the most relevant pathogens.

Pseudomonas Aeruginosa is a facultative anaerobic bacillus, ubiquitary in the environment and detectable on soil, water, plants or animals. In humans, is occasionally resident on cutis, external ear, upper respiratory tract or gut of healthy people. This germ is responsible for bacteremias, pneumonia and infections of cutis, urinary tract, eye and central nervous system. It represents an important cause of morbidity and mortality in hematology, especially because of the high incidence of multi-drug resistant strains, that makes hard to find efficacious antibiotic treatments. Pseudomonas Aeruginosa infections treatment is constituted by Penicillin and third-fourth generation Cephalosporins, Carbapenemes, Aminoglycosides, Fluoroquinolones, often combined to prevent resistant strains breakthrough [12].

Within Klebsiella species, Klebsiella Pneumoniae is the most important pathogen. This germ colonizes gut and oropharynx mucosa in 5-35% of healthy population. Klebsiella Pneumoniae is responsible for pneumonia and infections of urinary tract, gut and cutis; other complications are intravascular devices infections. Also Escherichia Coli is emerging as opportunistic pathogen for hematological patients. Escherichia Coli infections may affect the gut, often mediated by commensal strains, or the urinary tract: in this last case, the infection is provoked by extra-intestinal strains. Moreover, Escherichia Coli may cause also pneumonia, bacteremias and intravascular device infections. Historically, Fluoroquinolones, Carbapenemes, Aminoglycosides and Cephalosporins are considered active drugs against Enterobacteriacae, but Extended Spectrum Beta-Lactamase (ESBL) producing strains and fluoroquinolone resistant strains are acquiring clinical

relevance. For these reasons, the management of these infections of immunocompromised hosts in clinical practice is extremely complex [13].

Fungal Infections

Fungal infections are one of the most complex and relevant problem in the management of hematological patients. The diagnosis of invasive mycoses is often complicated. The consequent delay in starting adequate therapy makes even now very difficult to obtain acceptable results in the management of this kind of infections. Aspergillus species and Candida species are responsible of about 95% of invasive fungal infections. Candida species provoke several clinical manifestations, that may range from superficial infections involving cutis and mucosae to disseminate infections, potentially affecting every district. Candida species are part of normal endogenous flora, and could gain access to circulation through damaged areas of anatomical barriers, as frequently happens during mucositis. Damaged gut epithelium is the principal way of Candida access to circulation in patients undergoing chemotherapy regimens with mucosal toxicity. The most important risk factors are broad spectrum antibiotic therapy, presence of a central venous access, total parenteral nutrition and exposure to immunosuppressive drugs as corticosteroids. Candidemia is the forth cause of septicemia in United States, and is associated to high rate of mortality, up to 47% in several studies. Candida Albicans is the most common agent of Invasive Candidiasis (IC), followed by Candida Tropicalis, Candida Glabrata, Candida Krusei and Candida Parapsilosis. However, the large usage of Fluconazole prophylaxis contributed to modify fungal infections epidemiology; according to several studies, we can actually observe an inversion of the Candida Albicans/Non Albicans infection rate.

In superficial infections, the diagnosis is based upon fungal detection with light microscopy and subsequently isolation with culture. For systemic infections, the diagnosis is based upon histological examination or culture of blood, cerebrospinal fluid or other biological fluids. Concerning therapy, beyond the classical Fluconazole, new drugs have recently been introduced in clinical practice, as

Voriconazole, Caspofungin, Anidulafungin, Micafungin and Liposomal Amphotericine-B (L-AMB), that have great importance for the management of resistant cases and advanced infections. Of note, drugs belonging to Echinocandin class as Caspofungin, Anidulafungin and Micafungin are emerging as gold-standard choice for invasive infections treatment. When disseminated infection and septic shock are diagnosed, removal of central venous access, if present, is strongly recommended [14].

Aspergillus infections have been historically considered as a rare finding in immunocompetent patient; however, these infections are one of the most important causes of mortality and complications in immunocompromised hosts. Invasive Aspergillosis (IA) is the main responsible for lung-infectious related death in patients undergoing HSCT, and is a common cause of respiratory tract and disseminated infection in many subgroups of immunocompromised patients. Aspergillus species pathogens for humans are ubiquitary, and spore inhalation is a frequent event. Aspergillus Fumigatus is the more frequently isolated pathogen, followed by Aspergillus Flavus, Aspergillus Niger and Aspergillus Terreus. Aspergillosis are traditionally classified as invasive, saprophytic or allergic. IA group is constituted of infections involving lungs, central nervous system and paranasal sinuses. Pulmonary Aspergillosis is the most frequent clinical presentation in immunocompromised hosts. Subsequently to spore inhalation, the infection in a first time is localized to lower respiratory tract; next, because of the ability of the fungus to invade blood vessels, Aspergillus spread through circulation towards other organs, most frequently to central nervous system and more rarely to cutis, hearth, kidney and liver. Blood cultures are rarely positive, and cultures of sputum or bronchoalveolar lavage have a sensibility of 50% in lung focal lesions. For these reasons, diagnosis is often based upon indirect systems, as determination of fungal specific antigens as serum and bronchoalveolar lavage Galactomannan, or upon radiological evaluation with High-resolution Chest TC [15]. Chest TC is more sensitive than conventional radiography; the earliest radiological sign of

IA is pulmonary nodule. The so-called “halo sign” is a large nodule surrounded by a perimeter of ground glass opacity, a marker of blood vessel invasion, and is suggestive of IA in patients with compatible host factors. Other radiological findings associated with IA are consolidation, wedge-shaped infarcts, and cavitation, a lesion that is observed typically after neutrophil recovery [15].

The introduction in clinical practice of these techniques allows for a diagnosis of probability, that is what is called call a “probable infection” [16]. The diagnosis of a probable fungal infection requires the presence of almost a clinical criterion (e.g. halo sign on Chest TC), a host criterion (e.g. recent history of neutropenia) and a mycological criterion (e.g. serum Galactomannan positive). [16]

The diagnosis of proven fungal infection is based upon histopathological examination of a sample obtained from biopsy or needle aspiration, or upon recovery by culture from a sterile site [15]. The drug of first choice for IA treatment is Voriconazole, with L-AMB and Echinocandins playing a role on second line therapy and combination therapy [16]

Risk Factors

In immunocompromised hosts, as hematological patients, infectious diseases are favored by altered interactions between patient defenses, immune-mediated or not, and exogenous and endogenous microbial flora. Factors interfering with microbial flora equilibrium have substantial influences on pathogen germs. These factors include antimicrobial therapy, trauma or invasive procedures, ingestion or inhalation of infected substances and hospitalization. Risk factors interfering with host defenses are several and may coexist in the same patient. Some of these risk factors are related to the malignancy, other to interventions that are required during diagnosis and therapy [1]. During antineoplastic treatment, cytotoxic and immunosuppressive drugs are frequently administered, as corticosteroids or radiotherapy. Several chemotherapy agents as Methotrexate, Cyclophosphamide, 6-Mercaptopurine and Azathioprine have a detrimental action on cell-mediated immunity. Several of this drugs have negative effects also against other components of immune response, as humoral immunity, and may determine quantitative defects of granulocytic line.

Fludarabine administration, first-line therapy in chronic lymphocytic leukemia, causes a deep and prolonged deficiency of cell-mediated immunity, increasing susceptibility to infections from Pneumocystis Jirovecii, yeasts and Herpes Virus. Similarly, monoclonal antibodies like Rituximab and Alemtuzumab increase the risk of infectious diseases. Corticosteroid treatment causes susceptibility to infections; the degree of immunosuppression and the relative infections risk are related to dose and duration of corticosteroid treatment. Steroids main effect on granulocytes function is to impair chemotactic activity. This finding explains why symptoms and signs of infection may be masked or greatly reduced in patients receiving steroid treatment. Moreover, corticosteroids increase infection risk because of detrimental effect on tissue-repairing ability, cutaneous frailty and lymphocyte and monocyte function. Moreover, steroids administration affect

cytokines production, causing inefficacious humoral responses [1]. Radiotherapy was associated to deficiency of granulocyte function and to delay in wound healing. Cell mediated immune deficiency is observable up to a year after intensive radiotherapy or total body irradiation for the preparing regimen to HSCT. Also Interleukine-2 (IL-2) administration was associated to impairing of immune defenses. Specifically, following IL-2 administration, granulocytes presented a reduced production of superoxide anion with reduced chemotactic ability, showing susceptibility to Gram Positive infections [17].

As stated before, malignancies frequently represent the main cause of immune deficiency, exposing patient to infections. Infectious diseases are more frequently observed in patients affected by acute leukemias, but also other hematological malignancies in some way favor opportunistic infections. In Multiple Myeloma, neoplastic plasma cells undergoing clonal expansion compete against normal plasma cells for vital space in the hematopoietic niche, compromising long term immunological memory. Moreover, neoplastic cells secrete cytokines and express aberrant ligands inhibiting NK cell function, limiting host ability to react to infections. The presence of a monoclonal protein is often associated to immunoparesis, with effective hypogammaglobulinemia: this event makes hosts susceptible to infections from germs that require efficacious opsonization for their clearance, as Streptococcus Pneumoniae and other capsulated bacteria [18].

Similar mechanisms have been described on Chronic Lymphocytic Leukemia, as the result of unbalanced immunoglobulin synthesis with subsequently hypogammaglobulinemia. Infection risk is related to dosage of serum immunoglobulins, especially IgG fraction, and with duration of disease, reflecting probably a deeper immunosuppression related to treatment [19].

Presence of central venous catheter [CVC] is another important infection risk factor in hematological patients. CVC are usually used in hematology, both for chemotherapy administration and for

supportive therapy. Despite strategies for prevention of infectious diseases, these devices are still one of the main sources of bacteremia and septicemia in neoplastic patient.

CVC related infections are several, and are constituted by:

- Catheter colonization, that means a significant growth of germs by a catheter segment without clinical signs of infection
- Infections localized at catheter emergency site
- Sub-cutaneous tunnel Infections
- Sub-cutaneous niche Infections in totally implanted catheters
- Bacteremia and Sepsis

Coagulase Negative Staphylococci, Staphylococcus Aureus, Gram Negative aerobic bacilli and Candida Albicans are the germs more frequently involved. In every patient harboring a CVC, the presence of signs and symptoms of infection, in absence of other likely source, must to be considered as a suspected CVC related infection [20].

Febrile Neutropenia

During the natural history of some hematological disorders, as Aplastic Anemia and Acute Leukemias, and often after chemotherapy treatment for several hematological malignancies, the presence of an absolute or deep neutropenia is frequently observed. Using the most accepted definition, a neutropenia episode is defined as the finding of a neutrophil count below 500/mm3, or superior to 500/mm3 but with an expected decrease below this threshold during the following 48 hours. A neutropenia is defined “deep”, or of IV grade, when the granulocyte count is below 100/mm3; usually this value is confirmed through a manual examination of peripheral blood smear [21]. Since 1966, Bodey et al evidenced the inverse correlation between neutrophil count and infection incidence in patients undergoing chemotherapy for acute leukemia [22]. In this paper, neutropenia was associated with several kind of infections. In fact, the study documented localized process as pneumonia, disseminated infections and bacteremias, but also episodes of fever in absence of other signs and symptoms of infection. For these reason, with the aim to include every possible manifestation, the definition of “Febrile Neutropenia” was introduced. In fact, fever is often the first, principal and only sign of infection in neutropenic patient. Febrile Neutropenia could be diagnosed when, in a patient with a granulocyte count below that over cited, is detected a body temperature of 38.3°C, or a body temperature major or equal than 38°C for at least on hour [21].
Patients with febrile neutropenia substantially may have three principal clinical presentations [2]:

- Clinically documented infection, observed about in 30% of the cases, in which clinical and instrumental exams detect a site of infection, without identifying etiological agent
- Microbiologically documented infection, observed about in 30% of the cases, in which cultural exams allow to isolate the pathogen responsible of infection
- Fever of unknown origin (FUO), observed about in 30-50% of cases

Fever in a neutropenic patient requires prompt initiation of several diagnostic and therapeutic strategies, to identify rapidly the etiological agent and to start quickly adequate empirical treatment with a drug active against the probable pathogens responsible for the infection [21].

Despite modern strategies and adequate antibacterial and antifungal treatments, fever lasts for over 4-5 days in a percentage of patient up to 30%, without any correlation with etiological agent [23].

Another relevant finding is that the insurgence of FUO seems to be not related to degree of neutropenia. These considerations suggest that fever is not necessarily linked to an infectious event, and that the machineries underlying fever development are several and complexes. It was proved that fever is a non-specific response to several trigger factors, and that fever is only one of these. Germs and structural motifs of cell membranes of several microorganisms, the so called “pathogen associated molecular patterns” (PAMP), are potent inducers of cytokine release [24,25]. A similar role has been proposed for “damage associated molecular patterns” (DAMP), released from damaged tissues [26].

In literature, several diseases that are substantially a manifestation of an altered response of host to a damage have been described [27]. Most of these diseases involve districts where mucosae epithelium is colonized by microorganisms. Immune responses are strictly regulated at mucosal level, to prevent infections and at the same time excessive immune response [28]. Patients developing neutropenia after cytotoxic therapy contextually have damages to gastrointestinal mucosa, potentially altering this equilibrium. Injuries of the so called “mucosal barrier” (MBI) also play a role in the insurgence of fever and infections. Lesions of gastrointestinal mucosa represent an attractive site for germs to determine local infections and bacteremias. Moreover, damaged mucosae are unable to play their immune regulatory action against microbial flora. Several studies

suggested that MBI is more important than neutropenia as risk factor for the development of infectious complication after chemotherapy and HSCT.

MBI develops through five phases [20]:

- Activation of “Nuclear Factor-kB” (NF-kB), directly by chemotherapy and radiotherapy, and indirectly through oxygen reactive species generation and DNA damage
- Production and release of cytokines (IL-1β, IL-6, IL-8, TNF-α, IL-23, IFN-γ) by tissue macrophages, dendritic and epithelial cells
- TNF-α positive feedback, epithelial cells apoptosis and augmented mucosal permeability
- Germs and PAMP translocation, with worsening of inflammation
- Mucosal healing with restitutio ad integrum

Therefore, MBI is essentially an inflammatory disorder arising from alteration of normal mucosal innate immune system, that provokes a pathological interaction between microbial flora and host [27]. Animal models showed that chemotherapy provokes an initial sterile inflammatory response at level of intestinal mucosa an, followed by epithelial alterations with cryptic hypoplasia and villi atrophy, and then the generation of ulcers [31].

MBI starts before translocation of germs and PAMP. These factors induce and enforce resident immune cell response, determining an inflammatory systemic response that manifests essentially as fever. Moreover, the scenario is worsened by DAMP release chemotherapy/radiotherapy exposed dying cells. DAMPS are represented by molecules as ATP, HMGB1 proteins and Heat-Shock proteins. Fever observed during febrile neutropenia is probably the result of cytokine release from residual immune and stromal cells, as epithelial cells, that recognize microorganism and specific molecular motifs through their “pattern recognition receptors” (PRR).

Indeed, what actually is defined as “febrile neutropenia”, probably should be called properly “febrile mucositis” (Figure 1); As in febrile neutropenia, under the definition of febrile mucositis are recognized all the clinical situations that causes fever in neutropenic patients. It is probable that only in a percentage of cases fever is caused by a real infection; in other circumstances, fever is sustained by other mechanisms, especially pathways involving innate immunity [32].

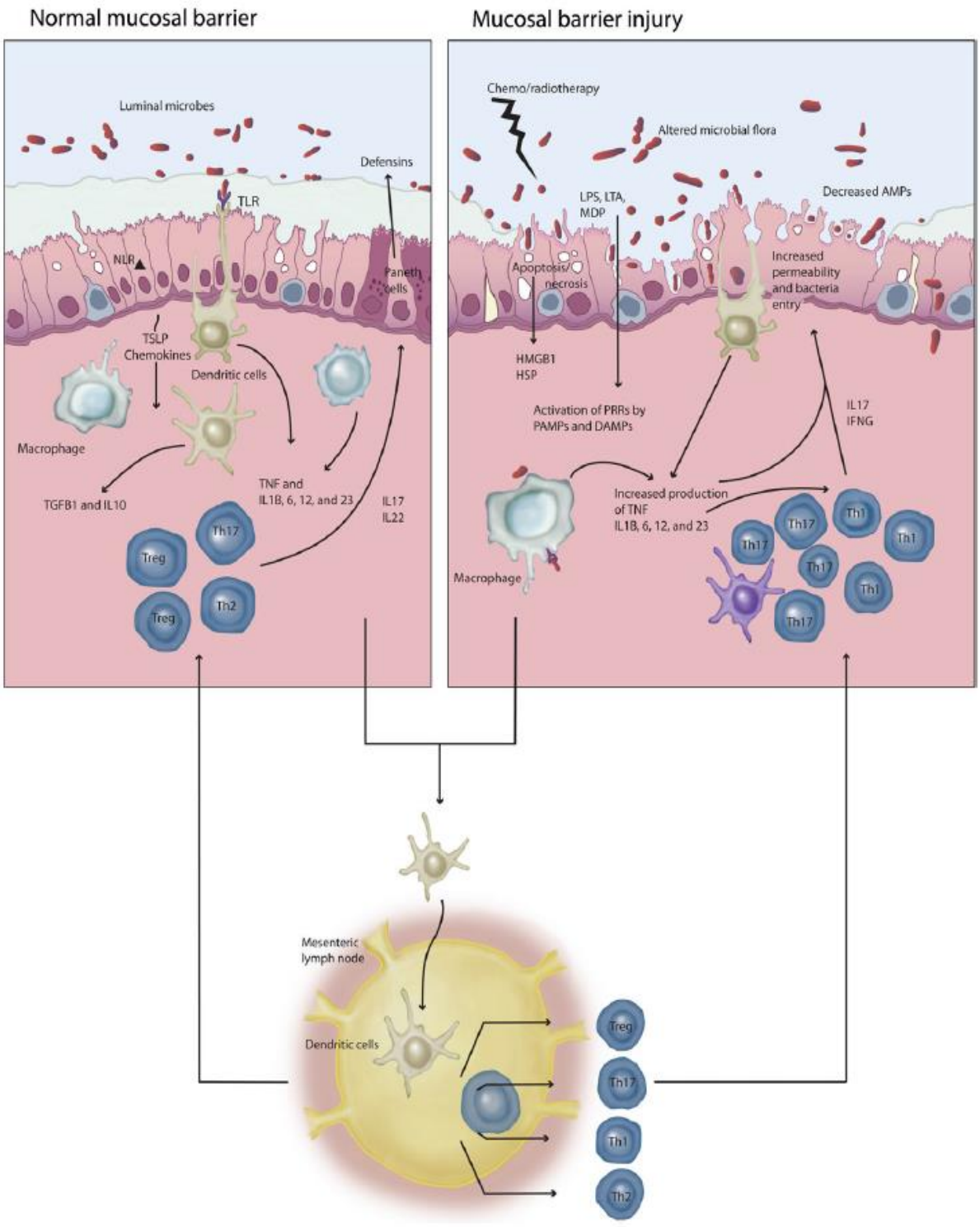

Figure 1: Physiopathology of febrile mucositis in patients undergoing chemotherapy [32]

The concept of febrile mucositis may have important consequences on clinical management of patients suffering from chemotherapy induced neutropenia. Current guidelines for the treatment of the fever in patients with chemotherapy induced neutropenia derive from a vision that consider fever as equivalent of infection. Most recommendations share the suggestion to administer fluoroquinolone prophylaxis to all patients undergoing chemotherapy for whom the expected neutropenia is more than 7 days; when fever is detected, if a germ is not yet identified by cultures, is mandatory to modify prophylaxis therapy, introducing parenteral empirical treatment.

If fever persists for over 96 hours, a relatively common event, guidelines suggest another change in therapy, with the introduction of empirical antifungal treatment [33, 21]. Considering recent findings on febrile mucositis, while fluoroquinolone prophylaxis seems to be anyway indicated in all patients with MBI, switching toward empirical therapy could not be necessary in all neutropenic patients with fever.

At the same time, it seems not sustained by evidence, in presence of only fever without other signs of infections, the introduction of other antimicrobial drugs in clinically stable patients. However, actually is not possible to discriminate neutropenic patients with FUO with a real infection, that could have benefits from empirical antimicrobial therapy, and so these considerations appear to be speculative [32]. Therefore, there is a stringent need to find new markers to identify patients affected by febrile neutropenia that are really suffering from an infectious disease; the purpose is to administer targeted therapies and save useless and expensive treatments, often with relevant side effects. Another objective of similar importance is to identify, within hematological patients undergoing chemotherapy, the risk factors for the different kind of infectious diseases and their complications. If identified, these factors allow to “design” a surveillance strategy and an adequate empirical therapy for the single patient. Actually, the only tools able to predict the risk of

complications are scores as the Multinational Association for Supportive Care in Cancer Risk Index (MASCC risk score), that unfortunately could be used only at fever insurgence.

Glucose 6-Phosphate Dehydrogenase Deficiency

Glucose 6-Phosphate Dehydrogenase (G6PD) is an enzyme that catalyzes the first reaction of the pentose-phosphate metabolic pathway, generating oxide-reductive potential for other cells in the form of NADPH, that is the reduced form of Nicotinamide Adenine Dinucleotide Phosphate. NADPH allows cells to save Glutathione in reduced form and counterbalances oxidative stress, that is provoked by several oxidants. G6PD deficiency is the most common enzyme defect worldwide, and it was estimated that this mutation is present in over 400 million people [34]. We have knowledge of the presence of G6PD deficiency in pre-Christian age, with the description of a disease related to fava bean ingestion. Some tales described how the Greek philosopher and mathematic Pythagoras prohibited to his students to eat fava beans, because of their possible toxic effect. These tales were considered as popular commonplace since to XX century, when was scientifically described the disorder that actually we know as Favism [35]. However, the effects of fava bean ingestion were attributed, also because of their unpredictability, to allergic reactions or chemical toxicity. The discovery of G6PD and the pathological consequences of the deficiency is certainly related to studies on hemolytic anemia observed in patients after the administration of the antimalarial drug Primaquine. Only in 1956, with a trial executed on healthy volunteers, a group of prisoners of an Illinois prison, was proved that patients that presented hemolytic anemia after Primaquine administration had a low level of erythrocyte G6PD enzyme activity [36]. As stated before, G6PD catalyzes the first reaction on pentose-phosphate pathway, in which glucose is converted on pentose sugars fundamental for glycolysis and other biosynthesis reactions. Pentose-phosphate pathway produces reductive power in form of NADPH through G6PD and 6-phosphogluconate dehydrogenase activity. NADPH acts as electrons donor in several enzyme reactions fundamental for biosynthesis metabolic pathways, and its production is essential to guard cells from oxidative

stress. G6PD is necessary also to regenerate reduced Glutathione, through donation of a NADPH electron. Reduced Glutathione is essential to reduce hydrogen peroxide and free radicals, and to maintain Hemoglobin (Hb) and other red cells proteins in a reduced form (figure 2) [34].

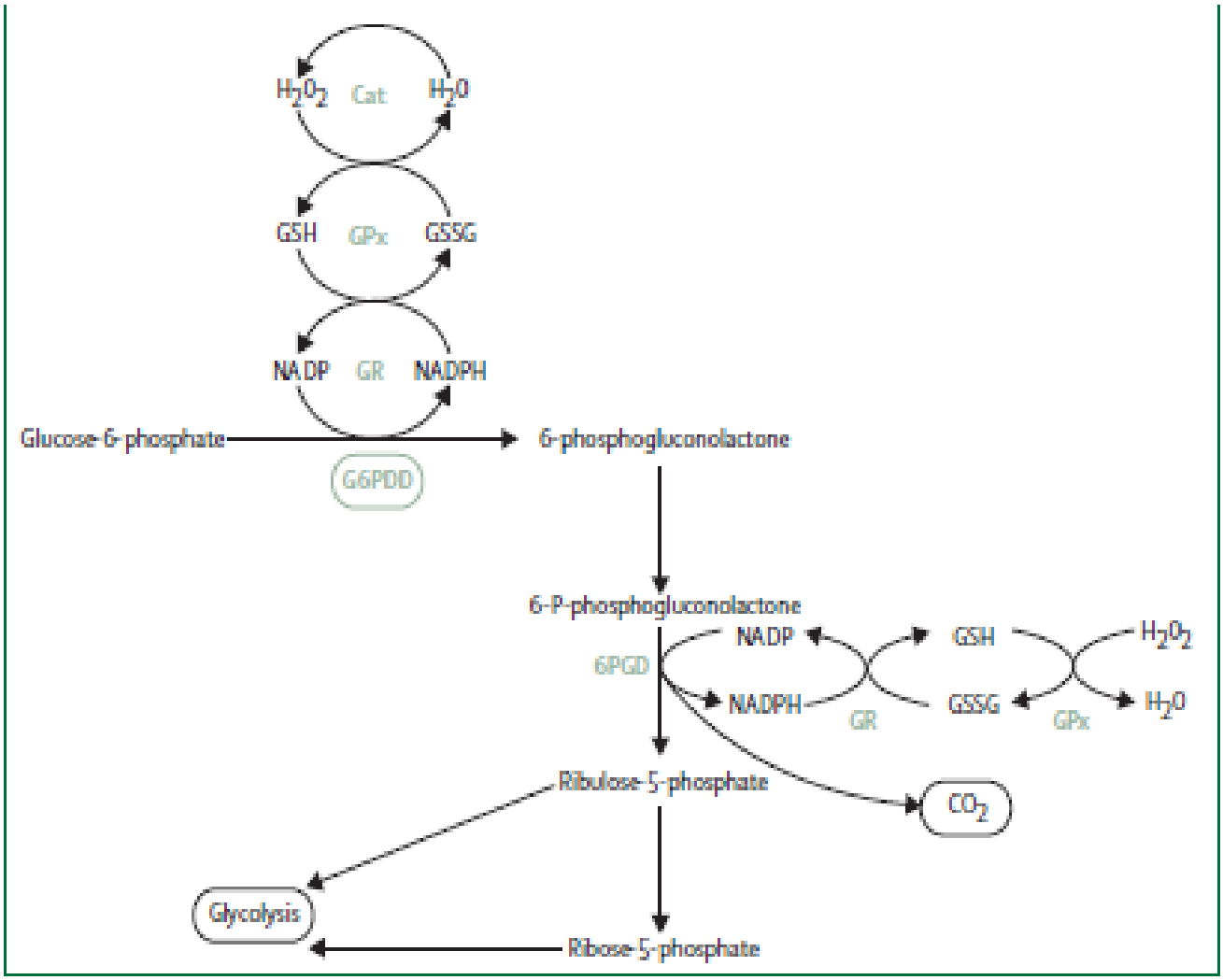

Figure 2: Pentose-Phosphate Metabolic Pathway [34]

G6PD monomer is constituted by 515 amino acids, with a molecular weight of 59kDA. The enzyme is active as a tetramer or a dimer, in an PH dependent equilibrium. Every monomer has two domains: The N-terminal domain, composed by amino acids 7-200, with a dinucleotide binding site

β-α-β constituted by amino acids 38-44, and a larger domain β+α. This second domain is constituted by a 9 filament antiparallel sheet. The two domains are linked through an α-helix structure containing a peptide-binding-site of 8 highly conserved residues, that acts as substrate binding site. The stability of quaternary structure is fundamental for enzyme activity (figure 3)

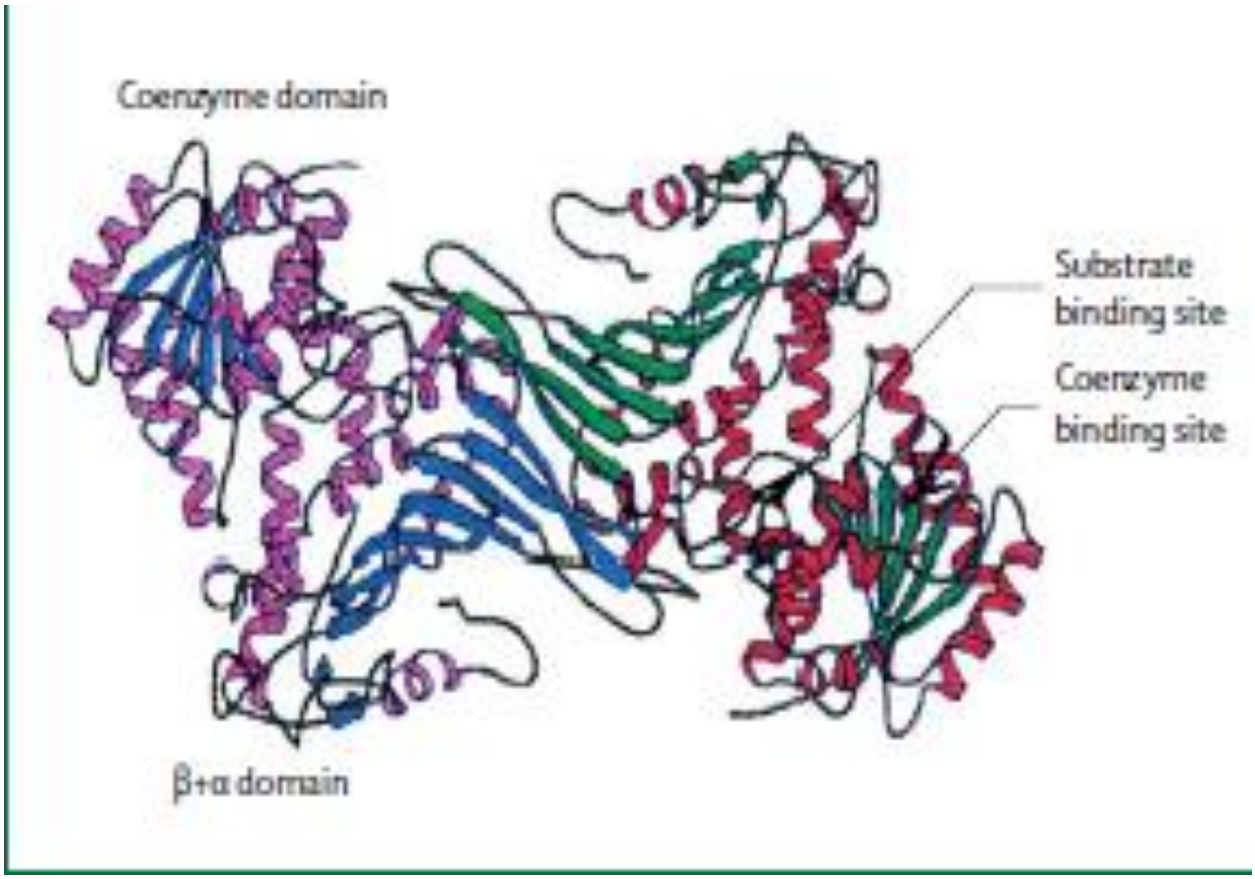

Figure 3: G6PD enzyme structure [34]

G6PD enzyme is present in all cells; its concentration however is variable within different tissues. In red cells, the enzyme works usually at 1-2% of its potential; therefore, there is a relevant functional reserve in terms of reducing power. This reserve is substantially reduced in patients with deficiency, with possible pathological consequences [34]. Phenotypic expression of G6PD is variable; more than 180 gene mutations have been identified, and this finding suggest a relevant genetic heterogeneity [37]. This heterogeneity was described since 60' years, when was proposed a classification of G6PD

variants based on phenotype severity. The variants analysis was executed with enzyme activity determination and electrophoresis mobility. In particular, were classified several kind of deficiency:

- Class I, with severe enzyme deficiency, associated with non spherocytic hemolytic anemia. Variants included in this class are G6PD Volendam and Durham
- Class II, with severe enzyme deficiency, with activity <10% than normal. Mediterranean variant is included in this class
- Class III, with moderate enzyme deficiency, with activity between 10-60% than normal. The most frequently variant belonging to this class is the A-
- Class IV, with weak or absent enzyme deficiency. The clinical phenotype is silent and not associated to hemolysis. The main variants included in this class are A+ and B+
- Class V, with increased enzyme activity, more than two times than normal. Variants belonging to this class are rare. One of the most common is G6PD Hektoen

Wild type enzyme is defined as G6PD B. In African population were described two mutations: G6PD A, an enzyme with normal activity and rapid electrophoresis mobility, and G6PD A-, with the same mobility but reduced activity. In Mediterranean populations, the enzyme was initially defined as G6PD B-, and then renamed Mediterranean G6PD. This variant has reduced activity in confront to G6PD A- (5% vs 13%) [38].

G6PD hereditariness shows a typical X-linked pattern, that was suggested since first description because of the different incidence of Favism, more present in males. Males are hemizygous for G6PD gene, and therefore may be totally deficient or with a normal activity. By contrast, females may have two copies of the gene or more frequently a single copy, that is defined as heterozygous status. In some populations in which G6PD deficiency is endemic, is not rare to observe homozygous

females. Heterozygous females are a typical example of genetic mosaicism, and could have pathological manifestations in some situations.

G6PD gene is localized on telomere region of Chromosome X long arm (Xq28 band), near to Hemophilia A and Congenital Dyskeratosis. The gene was cloned only in 1986, and is constituted by 13 exons and 12 introns, for a total length of about 20 kilobases (kb).

The gene codifies for 515 amino acids; the 5' non traduced region of Rna messenger (mRNA) coincides with exon 1 and part of exon 2. In the promoter region are present several transcription factor binding sites (figure 4)

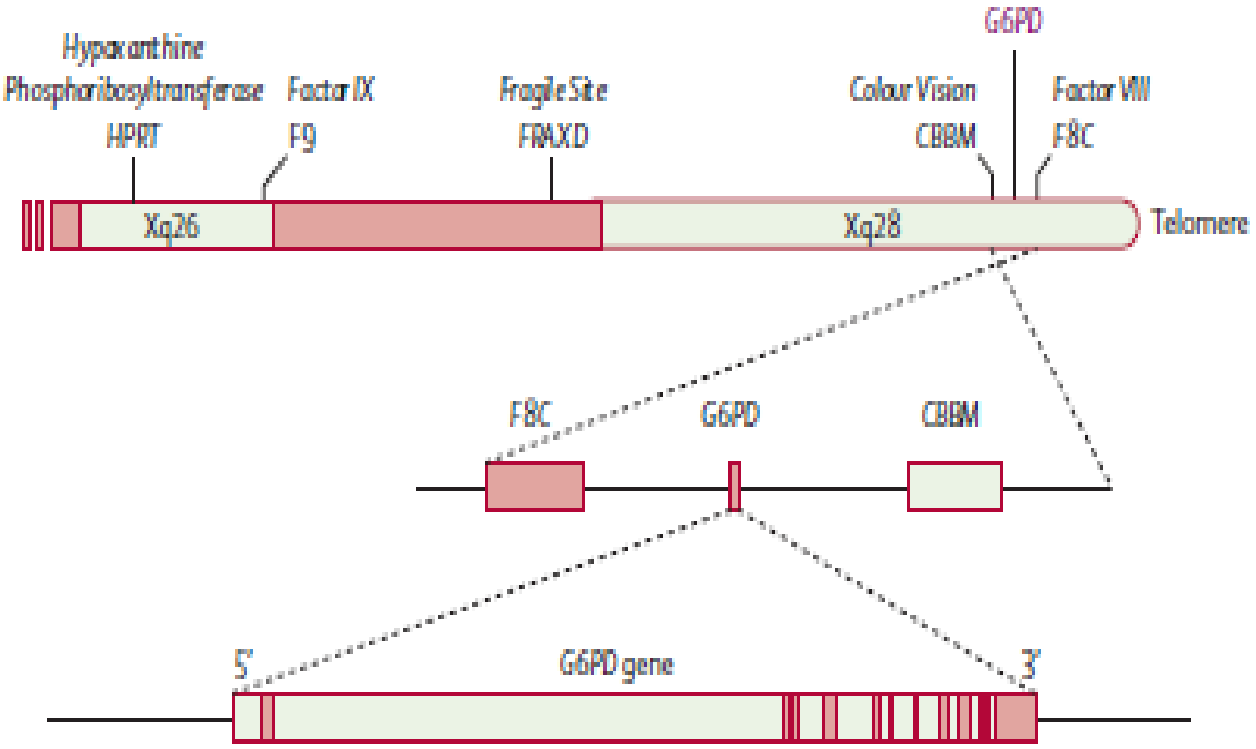

Figure 4: Structure of G6PD gene [34]

All the described mutations that cause enzyme deficiency are located within codifying sequences. Most of these mutations are single base substitutions that provokes an amino acid change. Rarely,

a second mutation is present *in cis*, while deletions are an unusual event. The promoter region has been well characterized, but no mutations were observed [34]. G6PD deficiency causing mutations have been described worldwide. The most elevated incidence is reported in Africa, Mediterranean Europe, Asian South East and in islands of Central and Meridional Pacific (figure 5). G6PD epidemiology is surprisingly similar to that of malarial endemicity. Malaria, with its hard tribute in terms of morbidity and mortality, played a role as powerful selective agent during human evolution. With elegant experiments, Luzzatto et al proved that malaria is the responsible for a balanced polymorphism; in fact, malaria infection maintained within population a high incidence of G6PD causing mutations because of their protective effect against Plasmodium [39].

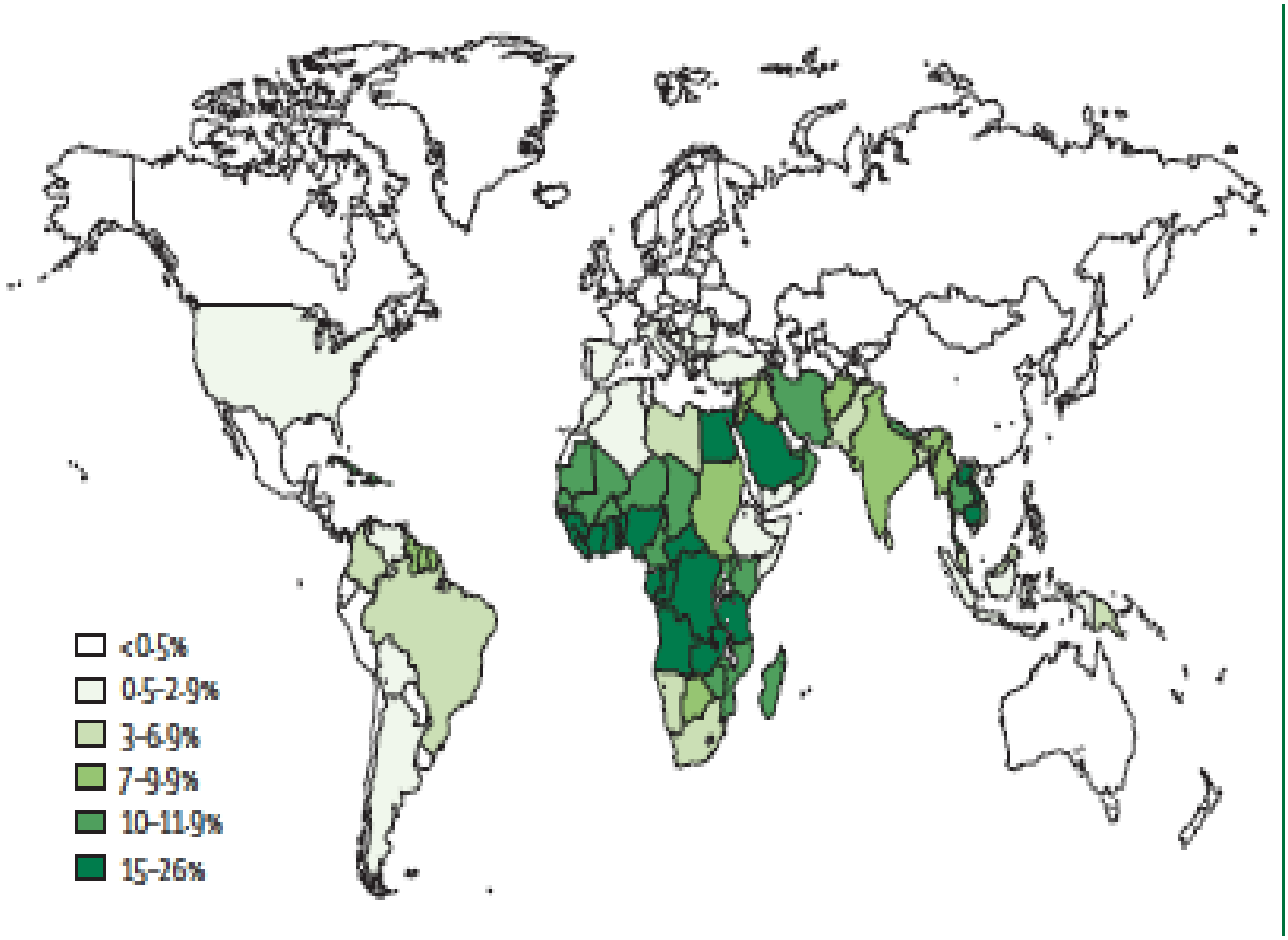

Figure 5: G6PD deficiency epidemiology [34]

The definite diagnosis of G6PD deficiency is based upon enzyme activity determination, that is performed with quantitative spectrophotometric analysis of NADPH production from NADP. Some difficulties may be present when the enzyme activity is evaluated during acute hemolysis or when is present an elevated reticulocyte count. In fact, enzyme activity is higher in young red than in mature cells, and this phenomenon could cause false negative results. Similar difficulties have been described on determination of enzyme activity in newborns, that obviously have large population of young red cells. At now, there isn't available any screening test that is able to detect heterozygosis in female, and perhaps is mandatory a molecular analysis to confirm the carrier status of G6PD deficiency in women [34]. Fortunately, most G6PD deficiency carriers are asymptomatic for all their life, often unaware of their status. G6PD deficiency seems to not have any impact in general population in terms of overall survival and quality of life of affected individuals. The presentation of the disease is typically acute hemolysis, that usually happens when red cells are exposed to oxidative stress triggered by drugs (e.g. fluoroquinolones, drugs of choice for febrile neutropenia prophylaxis), infections or fava bean ingestion. The other classical syndromes associated to G6PD deficiency are neonatal icterus or non spherocytic chronic hemolytic anemia. The exact mechanism of hemolysis subsequent to augmented oxidative stress sensibility has still to be fully understood [34]. G6PD deficiency causes red cells inability to reverse Hb and membrane lipids oxidation and to maintain enzyme and membrane proteins thiol groups in reduced form. When oxidized, Hb is converted to meta-Hb, and this event contributes to increase oxygen radicals induced damage of cell membranes. When instable, Hb is prone to precipitate with subsequent formation of the so called Heinz Bodies, that are detectable in blood smear with optic microscopy, after Crystal-violet coloration. These bodies appear as 0,2-2µ included corpuscles inside red cells (figure 6).

Red cells with Heinz Bodies are sequestered in splenic filter. Before being released into circulation, these red cells are modified, with the removal of precipitates (splenic pitting); these phenomenon has a detrimental effect on membrane damage.

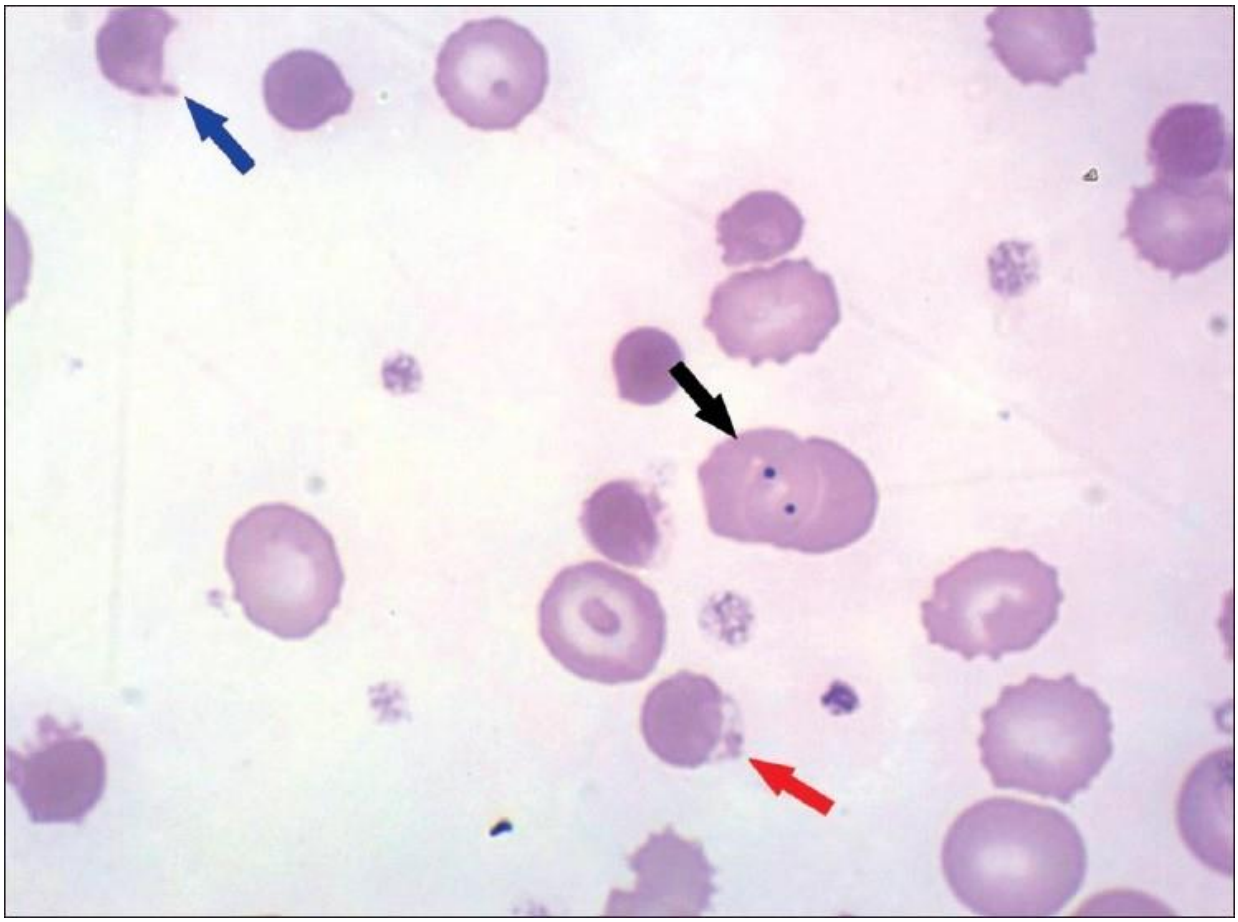

Figure 6: Modifications of red cell morphology during acute hemolysis in patient with G6PD deficiency. In this picture are detectable Heinz Bodies, bite cells and red cell anisopoikilocytosis.

Therefore, even if in absence of acute stress condition, the deficient enzymatic activity determines a minor ability of red cells to resist to oxidative damage, with reduction of lifespan. So, damaged red cells are removed from circulation by reticuloendothelial system before the classical threshold of 120 days. In A- and Mediterranean variants, in which red cells persist in circulation for 90-100 days, erythropoietic activity is augmented to balance the spontaneous early hemolysis; This

compensation is not sufficient in several variants that are characterized by enzyme activity reduction after only 20 days (e.g. New York variant or Oklahoma). This phenomenon causes a chronic hemolytic anemia; the severity of this complication is related to the enzyme variant involved. As stated before, G6PD is codified by the same gene in leucocytes, red cell and in all cell in which is expressed; in fact, it was proved the exact correspondence between G6PD electrophoretic abnormalities in all these cell lines. Despite this, important differences in terms of enzyme activity between leucocytes and red cells have been reported. Typically, G6PD deficiency is less evident in leucocytes than erythrocytes, probably because of the reduced lifespan of white cells respect to red cells [34]. Despite G6PD deficiency was reported to be linked to protection from malaria, some authors reported that enzyme deficiency is a risk factor for other kinds of infection. The rationale for this hypothesis is that the alteration of pentose-phosphate pathway, with subsequently NADPH decrease, provokes a reduction of neutrophil oxidative burst potential. The association between G6PD deficiency and infections has been described in few case reports, suggesting a susceptibility of patients with enzyme defect and particular germs [40], but these findings have not been confirmed in controlled clinical trials. Several observational studies reported an association between enzyme deficiency and bacterial infections in particular subgroups as newborns and trauma patients [41,42]. By contrary, other authors not evidenced any difference between in terms of neutrophils function between healthy controls and G6PD deficiency carriers [43]. Some animal models were proposed, however with controversial results. These studies didn't show any differences in terms of sepsis survival between rats with enzyme deficiency and wild type controls. However, in the same experiment, rats with G6PD deficiency presented an augmented mortality following Lipopolysaccharide administration, suggesting that complex factors, as G6PD, are involved in inflammatory response [44].

Moreover, recently another potential mechanism involving G6PD in susceptibility to infections has been described. In fact, Siler et al reported their analysis on three brothers with severe G6PD deficiency showing impaired neutrophil extracellular trap (NET) formation. This finding was previously evidenced only in patients affected by Chronic Granulomatous Disease (CGD), who have a high risk of bacterial and fungal infections. The authors hypothesized that defective NET production could be one of the mechanism through which G6PD deficiency contributes to infection susceptibility [45].

Therefore, the role of G6PD deficiency in susceptibility to infections is still controversial, and deserves further investigation.

Aim of the Study

The management of infectious diseases in hematology is one of the field of active investigation in biomedical research. In particular, identify those patients at higher risk of infections is one of the most stringent needs. In fact, despite the introduction of new antimicrobial agents, morbidity and mortality related to infectious disease are still high and unacceptable. Moreover, new drugs are more efficacious but often very expensive, and the identification of high risk patients would allow for a better resource allocation. For these reasons, I chose to investigate the role of G6PD deficiency in hematological patients, a subgroup intrinsically exposed to opportunistic infections. G6PD deficiency role in determining susceptibility to infectious disease is controversial, and there are no prospective studies clearing this point. There are many points that deserve further investigation regarding the role of G6PD deficiency in this scenario. First, fluoroquinolone prophylaxis is universally recommended in patients undergoing chemotherapy with an expected neutropenia duration of at least 7 days [21]. However, fluoroquinolones have been associated with acute hemolytic anemia (AHA) in patients with G6PD deficiency, and have been included by several authors among drugs to be avoided in this patient setting [46], although there aren't guidelines addressing these patients' management. Therefore, it would be very interesting to investigate if this supposed association between G6PD deficiency and AHA following fluoroquinolone exposure is confirmed, or instead also patients with enzyme deficiency could beneficiate from this prophylaxis. Similarly, also infections have been described as a trigger for AHA in G6PD deficient patients, and my aim was to verify also this association. Second, G6PD deficiency interferes with NADPH production, and NADPH is essential for the activity of neutrophils and to oxidative killing machinery. My hypothesis was that the enzyme defect could impair host defenses, leading to increased infection rate in patient with G6PD deficiency. If this hypothesis was true, I would expect to find a higher incidence of infection in G6PD deficient patients. Obviously, the identification of a biomarker

predicting the risk of infection in hematological patients would be of great importance, and will open perspectives on different strategies for surveillance, prophylaxis and treatment.

Part 1: Safe fluoroquinolone prophylaxis in blood cancer patients with chemotherapy-induced neutropenia and Glucose-6-Phosphate-Dehydrogenase Deficiency

Background

Bacterial infections are the major cause of morbidity and mortality in blood cancer patients with chemotherapy induced neutropenia [2]. More than 80% of these patients are expected to develop fever after one or more chemotherapy cycles, with a mortality rate of almost 10%. A single episode of fever usually requires 7-12 days of drug treatment, with an approximate daily cost of more than 1500 USD [53]. A randomized clinical trial showed the efficacy of antibiotic prophylaxis with levofloxacin in reducing the incidence of bacterial infections and febrile events [54], and most relevant international guidelines recommend antibacterial prophylaxis with fluoroquinolones for patients at high risk of neutropenia [21, 33, 55, 56], defined as expected neutropenia lasting more than 7 days [21]. Fluoroquinolones toxicity profile is characterized by gastrointestinal disturbances, cutaneous rash and tendinitis [57]. Moreover, several authors have reported an association of quinolone exposure with AHA in patients with G6PD deficiency [58, 59]. G6PD deficiency, as stated before, is the most common human enzyme disorder, affecting more than 400 million people worldwide. It's an X-linked hereditary genetic defect due to mutations in the G6PD gene which presents as many different biochemical and clinical phenotypes. The most frequent mutations are the so-called A- variant, frequently observed in tropical regions of South America and Africa, and the Mediterranean variant, present in Italy, Spain, Portugal and the Middle East [34]. There are no international guidelines listing drugs to avoid in patients with G6PD deficiency (G6PD-) [46], whereas a recent review excluded these drugs from a list of seven compounds to avoid in patients harboring enzyme defects [60]. There are no drugs other than quinolones that have been shown to be effective

in prophylaxis of febrile neutropenia. Clinicians prescribing chemotherapy to G6PD- patients have to face a difficult choice when deciding whether to administer an efficacious drug with a claimed risk of a serious adverse event such as AHA, or to prescribe a possibly safer drug for which there is no evidence of efficacy in febrile neutropenia. The aim of this study was to evaluate the safety and efficacy of fluoroquinolone prophylaxis in a large cohort of patients with G6PD deficiency, affected by blood cancer, undergoing intensive chemotherapy and at high risk of neutropenia.

Methods

Study design

I retrospectively collected the clinical data of patients undergoing intensive chemotherapy or autologous/allogeneic HSCT in Bone Marrow Transplantation Unit, R. Binaghi Hospital since January 2012 to January 2015. Patients enrolled in the study were affected by AML, ALL, MM, NHL and HL. Each chemotherapy cycle with an expected neutropenia major or equal than 7 days was classified as intensive chemotherapy. The following chemotherapy regimens were considered associated with neutropenia duration ≥7 days: "3+7" (AML induction regimen with Idarubicin 13 mg/m2 on days 1-3 and Cytarabine 100 mg/m2 on days 1-7); "high dose ARA-C" (Cytarabine 3 g/m2 for 3 days as consolidation cycle in patients with AML); "FLAI" (Fludarabine 30 mg/m2 from day 1 to day 5, Cytarabine 2g/m2 from day 1 to day 5, Idarubicin 13 mg/m2 on days 1,3,5);"G-CLAC" (Clofarabine 30 mg/m2 from day 1 to 5, Cytarabine 2g/m2 from day 1 to 5); "Hyper-CVAD" (Cytarabine 3000mg/m2 twice daily on day 2,3 and Methotrexate 1000 mg/m2 on day 1, alternating to Cyclophosphamide 300mg/m2 twice daily on day 1,2,3, Vincristine 2 mg on day 4 and 11 and Doxorubicin 50 mg/m2 on day 4); "FEAM"(Fotemustine 150 mg/m2 on day 1,2, Etoposide 200 mg/m2 and Cytarabine 400 mg/m2 on day 3,4,5,6 and Melphalan 150 mg/m2 on day 7) as autologous HSCT conditioning regimen; "TBF" (Thiotepa 5 mg/kg on day 1 and 2, Fludarabine 50 mg/m2 on day 3,4,5, Busulphan 3.2 mg/kg on day 3,4,5) as allogeneic HSCT conditioning regimen. Patients were treated with fluoroquinolone prophylaxis with Levofloxacin 500 mg/die or Ciprofloxacin 500 mg twice daily from the first day of chemotherapy until neutrophil recovery with an absolute neutrophil count (ANC)>1000. Prophylaxis for patients with AML consisted of Posaconazole on remission induction courses. During consolidation chemotherapy, the prophylaxis for AML patients with no history of invasive fungal disease (IFD) was represented by Fluconazole. Fluconazole prophylaxis was administered to patients undergoing HSCT conditioning

chemotherapy, whereas Posaconazole prophylaxis was the choice for patients with active disease at transplantation or developing acute or chronic graft versus host disease (GvHD). Patients with ALL underwent Fluconazole prophylaxis. All the patients with a previous episode of IFD were treated with secondary prophylaxis with Voriconazole, Posaconazole or Echinocandins according to clinical guidelines [21, 55, 56]. An infection in which the etiological agent was isolated by culture or biopsy was classified as microbiologically documented infection (MDI), whereas an infection in which was not possible to identify the etiological agent but a site of infection was determined was classified as clinically documented infection (CDI). Blood cultures were processed using the automated BACTEC system (Becton Dickinson Diagnostic Instruments, Sparks, MD, USA). Blood unit transfusions were documented with analysis of clinical data, and confirmed through analysis of databases of Transfusion Centers. The transfusion policy was “single unit”, that means that below a determined threshold (hemoglobin 8 g/dl in this study) the patient is transfused with only one blood unit. Febrile neutropenia analysis was performed considering only the first episode of neutropenia for patient. Neutropenia duration was calculated from the first day with ANC ≤1000/mm3 to the first day with ANC ≥1000/mm3.

G6PD determination

G6PD activity was determined with G6PD/6GPD Automatic Analyzer (KUADRO), Nurex SRL. G6PD activity was expressed as the ratio between G6PD and 6PDG activity. Patients with enzyme activity <10% were classified as G6PD deficient (G6PD-). Following a potential interference between G6PD activity assay and hyperleucocytosis (frequently recorded at diagnosis in AML and ALL), patients with white cell count (WBC) >20000/mm3 and G6PD activity between 10% and 85% were evaluated a second time for G6PD activity after chemotherapy treatment and normalization of WBC count to assess the real G6PD status. Female with heterozygous status because of X-chromosome inactivation and G6PD activity ≥10% were considered as individuals not carrying enzyme defect and classified as G6PD wild type (G6PD+).

Statistics

The study was a retrospective analysis performed on a cohort of patients affected by hematologic malignancies followed on Bone Marrow Transplantation Unit of R. Binaghi Hospital. The cohort was stratified according to G6PD status, and the differences between G6PD+ and G6PD- were evaluated. The principal endpoint was the incidence of AHA. Secondary endpoints were the incidence of febrile neutropenia, MDI, CDI, Gram-positive or Gram-negative infections. Significant differences were calculated using Fisher's two-sided exact test or Pearson's chi-squared test, as appropriate. Only P-values of less than 0.05 were considered to be statistically relevant.

Results

Patients

Two hundred and sixty-five patients were analyzed over a period of 42 months. Twenty-one patients with G6PD deficiency were excluded from the study because they were not treated with fluoroquinolone prophylaxis, according to physician choice. Two more patients were excluded because early death occurred in acute promyelocytic leukemia. Overall, 23 patients were not included in the study and the analysis was performed on the remaining 242 patients. The clinical features of the patients are reported in Table 1. No statistically significant differences were observed between patients with G6PD- and G6PD+ in terms of sex, age, hematological disease, number of chemotherapy courses for patient, relapsing/refractory disease, duration of neutropenia and fluoroquinolone prophylaxis.

Table 1. Clinical characteristics of the patients enrolled in the study

		G6PD- N=59	G6PD+ N=183	p-value
Age				
	median	59	59	ns
	range	18-72	20-75	
Sex				
	male	34 (57.6)	105 (57.4)	ns
	female	25 (42.4)	78 (42.6)	ns
Disease				
	AML	28 (47.4)	80 (43.7)	ns
	MM	17 (28.8)	55 (30.05)	ns
	NHL	6 (10.1)	25 (13.6)	ns
	ALL	6 (10.1)	17 (9.2)	ns
	HL	2 (3.4)	6(3.3)	ns
Chemotherapy courses for patient		2,6	2,6	
Total Intensive Chemotherapy courses		152	476	
	AML	100	309	
	MM	33	98	
	LNH	6	25	
	LAL	12	38	
	LH	1	6	
Relapsing\Refractory disease > 2 lines chemotherapy		48 (81.3)	151 (82.5)	ns
Auto-HSCT		24(40.7)	86 (47)	ns
Allo-HSCT		9 (15.2)	40(21.8)	ns
Mean Neutropenia duration (days)		17.2	16.4	
Mean Quinolone prophylaxis (days		16.1	15.6	
Median Follow up (months)		33	34	

AML=acute myeloid leukemia
MM=Multiple Myeloma
NHL=Non Hodgkin Lymphoma
ALL=Acute Lymphoblastic Leukemia
HL=Hodgkin Lymphoma
HSCT=Hematopoietic Stem Cell Transplantation

Acute Hemolytic Anemia and Blood Transfusions

Over 152 courses of intensive chemotherapy, no episodes of AHA were reported in patients with G6PD-; following expected aplasia, 947 blood units were transfused in these patients, with a mean number of 6.23 blood units transfused per cycle of cancer treatment. Patients with G6PD+ were transfused with 2957 blood units over 476 chemotherapy courses, with a mean number of 6.21 blood unit transfused per cycle of cancer treatment. No statistically significant were observed between the two subgroups, even when the analysis was performed according to the type of chemotherapy cycle (e.g. conventional chemotherapy, autologous HSCT, allogeneic HSCT). These data are resumed on Table 2.

Table 2. Acute Hemolytic anemia and Transfusion support

		G6PD- n=59	G6PD+ n=183	p-value
Acute Hemolytic anemia		0	0	ns
Administered RBC unit	Total	947	2957	
	Chemotherapy	796	2478	
	Auto-HSCT	75	232	
	Allo-HSCT	76	247	
RBC unit/cycle		6,23	6,21	

RBC=red blood cell unit

HSCT=Stem cell Transplantation

Infections

No statistically significant differences were observed in terms of bacterial MDI incidence, CDI infections, Gram Positive or Gram Negative infections. Interestingly, I observed a reduced incidence of febrile neutropenia in patients with G6PD- (p=0.01, HR=0.46, 95%CI 0.25-0.8). Another interesting finding was that in patients with G6PD- was observed a higher incidence of IFD (p<0.0001, HR 11.4, 95%CI 3.5-37.05). Significant differences were present also in terms of mold infections (p=0.005, HR 6.0, 95%CI 1.69-21.3) and Candida sepsis (p=0.008, HR 37, 95%CI 2.01-680.9). These data are resumed on Table 3.

Table 3. Characteristics of documented infections

	G6PD - N=59	G6PD+ N=183	p-value
Febrile Neutropenia	21 (35.6)	99 (54.1)	0,01
Bacterial MDI	12 (20.3)	37(20.2)	ns
Gram Positive	7 (11.8)	24 (13.1)	ns
Gram Negative	5 (8.5)	13 (7.1)	ns
CDI	27 (45.8)	79 (43.1)	ns
IFD	12 (20.33)	4 (2.2)	< .0001
Mold Infection	7 (11.8)	4 (2.2)	.005
Candida Sepsis	5(8.5)	0 (0)	.008

MDI=microbiological documented infection
CDI=clinically documented infection
IFD=Invasive fungal disease

Discussion

In this retrospective analysis, two hundred and forty-two patients were analyzed to evaluate the impact of G6PD deficiency in terms of safety, efficacy and feasibility of fluoroquinolone prophylaxis in patients undergoing chemotherapy for hematological malignancies. To my best knowledge, this is the first study investigating these points, and, interestingly, some findings may have a great impact on clinical practice.

I didn't find any evidence of AHA in G6PD- patients, over 152 chemotherapy courses. This finding is quite unexpected, as historically several authors reported about association between G6PD deficiency and AHA after fluoroquinolone exposure [58,59]. Some considerations may explain these results. The "single unit" transfusion policy, with a hemoglobin threshold of 8 g/dl, may have played a role in protecting patients from AHA. In fact, it is likely that blood supply from patients with no enzyme deficiency might have been protective against drug-induced AHA. Fresh red cells, containing normal or high levels of G6PD, may balance the detrimental effect of enzyme deficiency in hosts.

It could be argued that transfusion support may mask subclinical hemolysis; if this hypothesis was true, I would expect to find evidence of an increased blood supply in G6PD- patients in comparison to G6PD+. However, the mean number of blood units transfused for cycle in patients with G6PD- was similar to patients with G6PD+. This value is however lower than that reported by other centers using a single-unit transfusion policy [61]. This finding may be explained by differences between the two cohorts analyzed. In fact, my cohort included more patients undergoing autologous HSCT, who usually have a lower need for blood transfusion in comparison with patients undergoing conventional intensive chemotherapy or allogeneic HSCT. Another possible cause may be our prophylactic platelet transfusion policy, using a threshold of 10.000 platelet/mm3. In fact, it is possible that the reduced incidence of hemorrhagic complications might have had an impact on total blood transfusion requirement in the cohort.

Overall, these findings suggest that fluoroquinolone prophylaxis in hematologic patients undergoing intensive chemotherapy, associated with a transfusion threshold of hemoglobin 8 g/dl, is feasible and safe, even with a single-unit transfusion policy.

The most relevant implication of this research is however the potential economic impact of the access of patients with G6PD- to efficacious antibiotic prophylaxis. In fact, fluoroquinolones have proved to reduce febrile neutropenia and bacterial infections in patients undergoing chemotherapy cycles [54]; the implementation of this strategy in patients with G6PD- may reduce the mean length of stay and other costs associated with febrile neutropenia. I didn't observe any difference between G6PD- and G6PD+ in terms of bacterial MDI, Gram Positive or Gram Negative infections and CDI. However, a surprisingly higher incidence of IFD was observed in G6PD- patients in comparison to G6PD+. The difference was statistically significant even when calculated for mold infections and for Candida infections. These findings suggest a possible association between G6PD deficiency and susceptibility to IFD. It is possible that enzyme deficiency interferes with normal oxidative killing machinery of neutrophils, exposing affected host to fungal infections. Another interesting finding of this study is that the incidence of febrile neutropenia was unexpectedly lower in G6PD- patients, although the two subgroups didn't present any difference in terms of documented infections. It is possible that G6PD deficiency reduces cytokine release and pyrogenic response, but this hypothesis is speculative and deserves to be evaluated with in vitro studies.

My analysis has some limitations. First, the retrospective design might have reduced the ability to detect some associations, and might overestimated some findings. These observations, in particular the relation between G6PD deficiency and IFD, should be confirmed in prospective studies. In fact, the population included in the analysis was not homogenous, comprising patients at different risk of infectious disease and IFD. In addition, no molecular data on G6PD mutations were available; however, patients with G6PD deficiency were classified as G6PD- only if presenting enzyme activity

<10%, and therefore this cohort includes only patients harboring class I or II mutations associated with the Mediterranean variant [62].

In conclusion, this is the first study analyzing the safety and feasibility of fluoroquinolone prophylaxis in a large cohort of patients with G6PD deficiency and blood cancer. These findings suggest that fluoroquinolone prophylaxis is safe, apparently with no risk of AHA in the treatment of patients with G6PD-. Moreover, data emerging from incidence of IFD and febrile neutropenia suggest that the G6PD enzyme plays a significant role in susceptibility to opportunistic pathogens and immune response in neutropenic patients, and should be investigated with further studies.

Part 2: Glucose-6-phosphate Dehydrogenase Deficiency and risk of invasive fungal disease in patients with acute myeloid leukemia

Background

IFD represent a leading cause of morbidity and mortality for patients affected by hematologic malignancies [63]. As stated before, patients with highest risk of IFD are those affected by AML receiving remission induction chemotherapy [2] and those undergoing allogeneic HSCT [64]. In AML patients, invasive fungal infections are substantially mediated by Candida species or Aspergillus species. IFD incidence in AML patients is around 12%, with an incidence of IC and proven/probable Aspergillosis of 2.6-4.4% and 6-8% respectively [50, 65]. The risk of developing IFD in immunocompromised hosts is influenced by several variables, such as genetic and environmental factors, patient performance status and comorbidities [65]. However, the exact role of these factors still remains to be elucidated and is currently under active investigation. Only recently, a paper by Caira et al [50] described prospectively risk factors for IFD in AML, identifying body weight, recent house renovation, kind of job and chronic pulmonary obstruction as the variables involved in susceptibility to fungal infections.

G6PD, as stated before, is the most common human enzyme defect, affecting more than 400 million people worldwide. G6PD is the key enzyme in the pentose-phosphate pathway, and it is essential for the production of NADPH. This machinery protects cells from oxidative stress and promotes neutrophil oxidative burst responses [62]. The inheritance of G6PD deficiency shows a typical X-linked pattern, in which males are hemizygous and can therefore have normal gene expression or be G6PD deficient.

The clinical manifestations of G6PD deficiency are neonatal jaundice and acute hemolytic anemia episodes secondary to exogenous agents, as drugs and food [46]. The role of G6PD deficiency in

susceptibility to infections is controversial, with some reports suggesting an increased risk for newborns or trauma patients to develop bacterial infections [41,42]. However, these observations have not been confirmed by data arising from prospective clinical trials [66,67]. To my best knowledge, the relationship between IFD and G6PD deficiency has never been investigated. Because of the interesting preliminary findings of my retrospective analysis about G6PD deficiency and infections, I decided to investigate the role of G6PD deficiency in IFD onset in a cohort of AML patients undergoing chemotherapy or HSCT.

Methods

Study design

Patients diagnosed with AML from January 2012 to January 2015 at Bone Marrow Transplantation Unit of R. Binaghi Hospital in Cagliari and at University Hospital Clinic in Sassari were enrolled in this study. The study protocol was approved by the institutional review board of each participating center. The treatment algorithm was based on European Leukemia Net guidelines [68]. Patients were defined as having relapsing/refractory AML if relapse occurred during follow-up or in cases of induction chemotherapy refractory AML. Performance status was graded by the Eastern Cooperative Oncology Group (ECOG) scale [69]. Proven/probable IFD were defined according to the revised EORTC/MSG definitions published in 2008 [16]. Briefly, proven infection was diagnosed only with histologic evidence of Aspergillus or Candida by biopsy from a sterile site, or positive culture from a sterile site; probable infection was instead diagnosed in presence of at least one clinical (e.g. halo sign), one host (e.g. neutropenia) and one mycological criteria (e.g. recovery of Aspergillus species from culture of non-sterile site as BAL or sputum). Diagnostic workup was identical in the participating centers and included the following: nasal, pharyngeal and rectal swabs at the time of admission; blood cultures and chest X-rays at onset of fever; Galactomannan assays twice a week, and computed tomography (CT) scan on the 4th to 7th day of fever non responding to appropriate antibiotic therapy without positive cultural tests. Additional examinations were based on clinical indications, for example abdominal ultrasound scan, sinus or brain CT, skin biopsy, bronchoalveolar lavage, fundus examination.

The induction therapy was administered with one or more courses of “3+7” regimen, containing Idarubicin (13 mg/m2 from day 1 to 3) and Cytarabine (100 mg/m2 from day 1 to 7). Patients were considered primary refractory if blasts were detected on bone marrow examination after two cycles of “3+7” regimen. Consolidation therapy was performed in patients obtaining complete remission

after induction with high dose Cytarabine (3 g/m2 for 3 days). Primary refractory or relapsing patients were treated according to the FLAI scheme (Fludarabine 30 mg/m2 from day 1 to day 5, Cytarabine 2g/m2 from day 1 to day 5, Idarubicin 13 mg/m2 on days 1,3,5);" G-CLAC" (Clofarabine 30 mg/m2 from day 1 to 5, Cytarabine 2g/m2 from day 1 to 5). Additional courses of chemotherapy included G-CLAC scheme (Clofarabine 30 mg/m2 from day 1 to 5, Cytarabine 2g/m2 from day 1 to 5). Patients undergoing allogeneic HSCT were conditioned with the "TBF" regimen (Thiotepa 5 mg/kg on day 1 and 2, Fludarabine 50 mg/m2 on day 3,4,5, Busulphan 3.2 mg/kg on day 3,4,5). All patients undergoing remission induction chemotherapy received Posaconazole prophylaxis. During consolidation chemotherapy, patients with no history if IFD underwent prophylaxis with Fluconazole; patients with a previous episode of IFD underwent secondary prophylaxis with Voriconazole, Posaconazole, Echinocandins, according to clinical guidelines [21].

Antibacterial prophylaxis was performed according to IDSA guidelines [21]. Briefly, all patients underwent prophylaxis with Levofloxacin 500 mg/die or Ciprofloxacin 500 mg twice daily from the first day of chemotherapy until neutrophil count >1000/mm3.

Microbiologically documented infection was defined as isolation of the etiologic agent by culture or biopsy. Blood cultures were processed using the automated BACTEC system (Becton Dickinson Diagnostic Instrument Sparks, MD). Clinically documented infection was defined as a site of infection on physical examination without isolation of the etiologic agent.

The median follow-up was 32 and 34 months for patients with G6PD deficiency and normal, respectively. I assessed the infectious outcomes at the end of each hospitalization, and every 2 months for patients at home.

G6PD determination

G6PD activity was determined before starting treatment using the G6PD/6PGD Automatic Analyzer (KUADRO, Nurex SRL, Rome, Italy). G6PD activity was expressed as the ratio between G6PD and 6PDG activities. Patients with enzyme activity level <10% were classified as G6PD deficient (G6PD-). In consideration of the potential interference between the G6PD activity assay and hyperleucocytosis (frequently observed at diagnosis in AML), patients with a white cell count >20.000/mm3 and G6PD activity between 10% and 85% were evaluated a second time for G6PD activity after chemotherapy and normalization of white cell count to assess the real G6PD status. Because of X chromosome inactivation in females, heterozygous females with G6PD activity ≥10% were considered to be without the enzymatic defect and classified as G6PD+.

Statistics

Incidence of IFD was the primary endpoint of the study. Secondary endpoints were the impact of G6PD deficiency on the incidence of Candida sepsis, mold infections, bacterial MDI, Gram Positive or Gram Negative infections, CDI, leukemia-free survival, overall survival (OS, infection-related death. The independent variables such as sex, age, G6PD activity, ECOG performance status, recent house renovation, BMI≥30, chronic obstructive pulmonary disorder (COPD) and diabetes were analyzed in univariate analysis with the chi-squared test. Significant differences were calculated using Fisher's two-sided exact test or Pearson's chi-squared test, as appropriate. Only p-values of less than 0.05 were considered to be statistically significant.

Results

Patients

Overall, 130 consecutive patients with a diagnosis of AML were analyzed over a period of 42 months. Four patients for whom G6PD activity at diagnosis was unavailable because of a technical problem were excluded from the study. Other two patients were excluded because of acute promyelocytic leukemia and early death. In all, 16 patients were excluded because they are not eligible for intensive chemotherapy. Altogether, 22 patients were excluded; analysis was performed on the remaining 108 patients. Of these patients, 28 (26%) were G6PD- and 80 (74%) were G6PD+. No significant differences were found between two groups in terms of sex, age, courses of chemotherapy, disease evolution and median follow up. The clinical features of the patients are reported in Table 4. Overall, 14/108 (13%) proven or probable IFD were recorded: five cases (4.6%) of Candida Sepsis were identified. Nine (8.3%) mold infections were also observed. Additionally, 24 cases (22.2%) of bacterial MDI were reported: 14 (13%) were caused by Gram Positive and 10 (9.2%) by Gram Negative bacterial strains. Furthermore, 39 (36.1%) infections were recorded as CDI. Overall, 68 infectious events occurred in 64 patients, with a cumulative incidence of 59.2%.

Table 4 Clinical characteristics of patients enrolled in the study

		G6PD - (n=28)	G6PD + (n=80)	
Clinical Variables		N(%)	N(%)	p-value
Age				
	median	63	62	ns
	range	25-82	23-84	
Sex – num (%)				
	Male	18 (64.3)	48 (60.0)	ns
	Female	10 (35.7)	32 (40.0)	
Chemotherapy + Allo-HSCT		4 (14.3)	19 (23.7)	ns
Median Follow up (months)		32	34	ns
Relapsing/refractory disease ≥2 lines of therapy		20(71.4)	57(71.2)	ns
ECOG performance Status				
	<2	27(96.4)	75(93.7)	
	>2	1(3.6)	5(6.3)	ns
Hyperleucocytosis (wbc>40.000/mm^3)		8(28.6)	27(33.7)	ns

HSCT=Stem Cell Transplantation
G6PD -/+ : Glucose-6-phosphate dehydrogenase deficiency or normal

Invasive fungal disease and G6PD status

No association was found between IFD and COPD, diabetes, age, sex, performance status, recent house renovation or BMI. A modest trend, although not statistically significant, was observed for IFD and relapsing/refractory disease (p=0.06). The incidence of IFD was significantly higher in G6PD- patients compared to G6PD+ patients (35.7% vs 5%, p=0.0002, OR=10.5, 95%CI =2.96-37,5). This difference was not influenced by the allogeneic stem cell transplantation procedure. This is supported by the fact that also analysis performed before HSCT showed a significantly higher incidence of IFD in G6PD- patients (p=0.0006, OR=9, 95%CI =2.5-32,4). Interestingly, I observed only one case of IFD after HSCT in a G6PD- patient transplanted from a G6PD deficient sibling donor. These findings are resumed in Table 5.

Table 5 Incidence of Invasive fungal disease in acute myeloid leukemia patients

Risk Factors	Total number	IFD (N=14) N(%)	Non-IFD (N=94) N(%)	p-value
Diabetes				
yes	26	3(11.5)	23(88.5)	
no	82	11(13.4)	71(86.6)	ns
Age				
<50	11	1(9.1)	4(66.7)	
>50	97	13(13.4)	90(89.8)	ns
ECOG ≥2				
yes	6	2(33.3)	4(66.7)	
no	102	12(10.2)	90(89.8)	ns
Relapsing/refractory disease				
no	31	1(3.2)	30(96,8)	
yes	77	13(16.8)	64(83.1)	ns
COPD				
yes	4	0(0)	4(100)	
no	104	14(13.5)	90 (86.5)	ns
House renovation				
yes	3	0(0)	3(100)	
no	105	14(13.3)	91(86.6)	ns
BMI<30	92	12(13.0)	80(87.0)	
BMI>30	16	2(12.5)	14(87.5)	ns
g6pd +	80	4(5.0)	76(95.0)	
g6pd -	28	10(35.7)	18(64.3)	p= .0002
Censoring analysis pre-HSCT				
G6PD+	80	4(5.0)	76(95.0)	
G6PD-	28	9(32.1)	19(67.9)	p= .0006

IFD=Invasive fungal Disease; ECOG= Eastern Cooperative Oncology Group performance status score; COPD=Chronic Obstructive Pulmonary Disorder; BMI= Body Mass Index; HSCT=Allogeneic Hematopoietic Stem Cell Transplantation

Species of fungal and bacterial infections

No differences were observed between the two cohorts of G6PD- and G6PD+patients for the incidence of microbiologically documented bacterial infections. Also the incidence of clinically documented infections was similar in the two groups. These data are illustrated in Table 6.

I found five cases of Candida sepsis in the G6PD- cohort and no cases of Candidemia in the G6PD+ cohort (17.9% vs 0%, p=0.0001, OR=37.6, 95%CI=2.0-707.1). Focusing on mold infections, I found a higher incidence in G6PD- compared to G6PD+ patients (17.9% vs 5%, p=0.048, OR=4.1, 95%Ci=1.02-16-67). All isolated yeasts were non-Albicans Candida species (Candida Tropicalis, Candida Guillermondi, Candida Glabrata and Candida Krusei in two patients). Clinical data concerning Candida sepsis and other IFD, as well as clinical treatment, are resumed in Table 7.

All patients who developed IFD were under Posaconazole prophylaxis, except patient 10, who was diagnosed with probable Aspergillosis after consolidation chemotherapy. Three of the five patients with IC died of Candida sepsis. The only two patients who survived to IC had been treated with G-CSF and early catheter removal. Kaplan-Meyer estimates of Candidemia-free survival during follow up showed a clear increase in risk for G6PD- patients (94.9% vs 64.3%, P<0.0001), as illustrated in Figure 7.

Table 6. Characteristics of Infections documented in the Study

	G6PD- N(%)	G6PD+ N(%)	
Candida Sepsis	5(17.9)	0(0)	p= .0001
no Candida Sepsis	23(82.1)	80(100)	
Candidemia only	*3*	*0*	
Multivisceral involvement (Liver+Kidney)	*1*	*0*	
Brain involvement	*1*	*0*	
Mold Infection	5(17.9)	4(5.0)	p= .048
No mold	23(82.1)	76(95.0)	
Aspergillus Pneumonia	*2*	*2*	
Fungal Sinusitis-Mastoiditis (not identified)	*1*	*0*	
Fungal Pneumonia (not identified)	*2*	*2*	
Bacterial MDI	6(18.7)	18(19.6)	ns
No Bacterial MDI	26(81.3)	74(80.4)	
Gram Positive	3(50)	11(61.1)	ns
Gram Negative	3(50)	7(38.9)	
CDI	12(37.5)	27(29.3)	ns
No CDI	20(62.5)	65(70.7)	

MDI=microbiologically documented infection; CDI=clinically documented infection

Table 7. Clinical features of IFD diagnosed in the study

PN	Age /Sex	G6PD status	Treatment	DP	DN	IFI	Level of Certainty	CR/days from infection	Treatment	G-CSF	FRD
1	69/M	-	Re-induction (FLAI)	16	13	C.Krus	Prov	no	Caspo; 2nd line L-Amb+ Anidul	no	yes
2	46/F	+	Re-induction (FLAI)	18	35	Asp	Prob	no	Voric	yes	no
3	65/M	+	Re-induction (FLAI)	16	23	Asp	Prob	no	Voric; 2nd line L-Amb	no	yes
4	53/M	-	Re-induction (G-CLAC), post HSCT relapse	5	10	NI	Prob	no	L-Amb; 2nd line Voric	yes	yes
5	68/M	-	Induction (3+7)	15	20	C.Krus	Prov	yes/12	Caspo	yes	no
6	64/F	+	Induction (3+7)	17	29	NI	Prob	no	L-Amb; 2nd line Voric	no	no
7	62/M	-	Induction (3+7)	24	36	C.Trop	Prov	no	Anidul	no	yes
8	25/M	-	Induction (3+7)	25	32	NI	Prob	no	L-Amb	no	no
9	53/F	-	Re-induction (G-CLAC)	14	25	C.Guill	Prov	no	Caspo; 2nd line L-Amb+ Anidul	no	yes
10	55/M	-	Induction (3+7)	19	25	Asp	Prob	no	Voric	yes	no
11	47/M	-	Induction (3+7)	16	23	Asp	Prob	no	Voric	yes	no
12	71/M	-	Induction (3+7) follow up post 1 consolidation	25	33	NI	Prob	no	L-Amb; 2nd line Voric	no	yes
13	51/F	+	(HD-ARA-C)	0	0	NI	Prob	no	Voric	no	no
14	63/F	-	Induction (3+7)	25	64	C.Glab	Prov	yes/10	Caspo	yes	no

PN= Patient number; G6PD=Glucose-6-Phosphate-Dehydrogenase; DP=Duration of Antifungal Prophylaxis; DN=Duration of Neutropenia; IFD= Invasive Fungal Disease; CR=Catheter removal; G-CSF=Granulocyte stem cell factor; FRD= Fungal related death; M=Male; F=Female; FLAI=Fludarabine, Cytarabine, Idarubicin; HD-ARA-C= High Dose Cytarabine; G-CLAC=Clofarabine, Cytarabine; HSCT=Allogeneic hematopoietic stem cell transplantation; C.Krus=Candida Krusei; NI=Not identified; C.Trop=Candida Tropicalis; C. Guill=Candida Guillermondi; C.Glab=Candida Glabrata; Asp=Aspergillus Species; Prov=Proven; Prob=Probable; Caspo=Caspofungin; Anidul=Anidulafungin; L-Amb=Lyposomal Amphotericin-B; Voric=Voriconazole

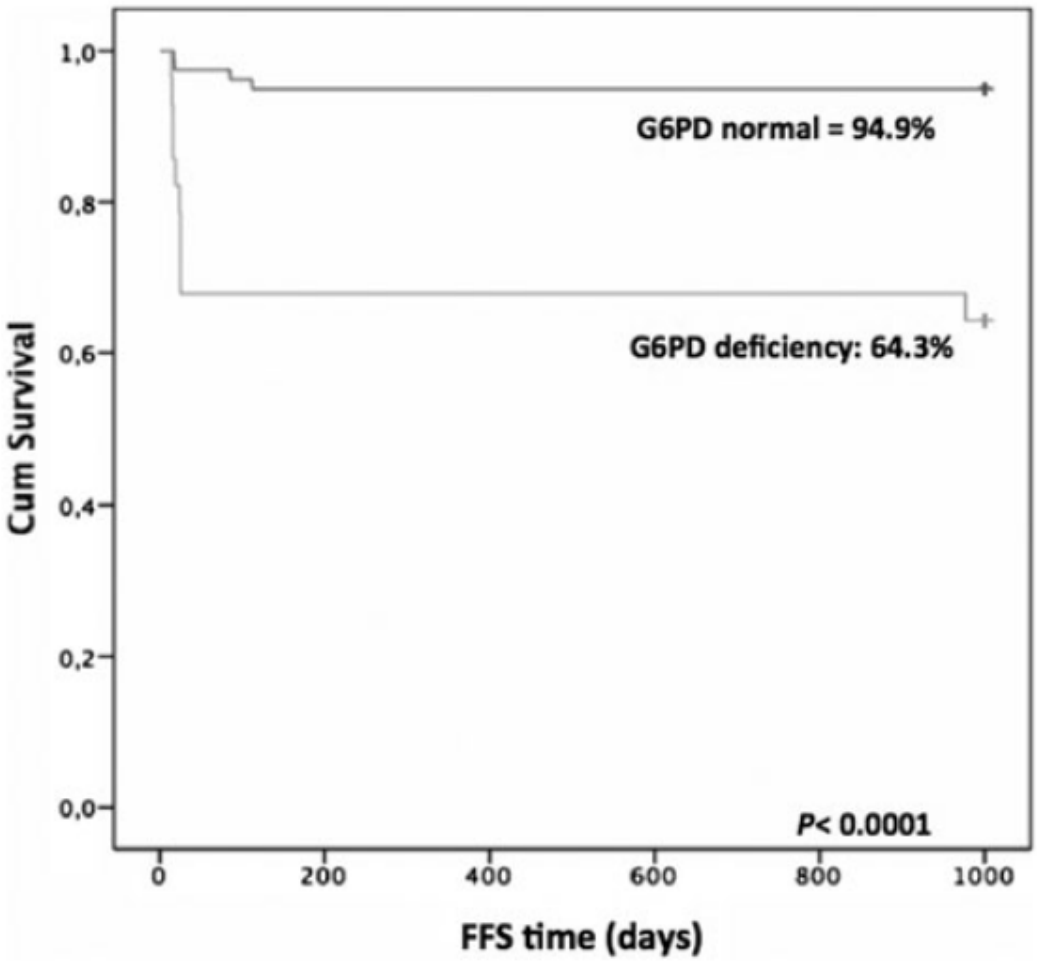

Figure 7: Fungal free survival (FFS) in two groups of acute leukemia patients according to G6PD status

Clinical outcome

In this cohort of AML patients, 2-year leukemia-free survival (LFS) was 28.7%. No differences between G6PD- and G6PD+ patients were found for overall survival (OS), 2 year LFS and infection-related death. These data are resumed in Table 8.

Table 8. Clinical outcome of patients enrolled in the study

	G6PD-	G6PD+	
Overall survival	10(35.7)	28(52.2)	ns
LFS	8(28.6)	23(28.8)	ns
IFRD	6(21.4)	10(12.5)	ns

LFS=Leukemia Free Survival; IFRD=Infection related death; G6PD +/-: glucose-6-phosphate dehydrogenase deficiency or normal

Discussion

To the best of my knowledge, this is the largest cohort of AML patients investigated for association between fungal infections and G6PD deficiency. I observed that G6PD activity influenced the risk of developing IFD. In particular, G6PD enzyme deficiency seems to significantly increase the risk of Candida sepsis and mold infections. Several studies have identified risk factors for developing IFD in hematological disorders: patients with AML or undergoing HSCT were the categories with the highest risk [2, 21, 63, 70]. Several modifiable and non-modifiable risk factors are associated with an increased risk for IFD [63], but only recently a prospective study identified body weight, recent house renovation, kind of job and COPD as the variables associated with mold infections [50].

I didn't find a correlation between these pre-chemotherapy variables and the onset of IFD. In this study, the cumulative incidence of proven/probable IFD was 11.3%, which is lower in comparison to the reports of other authors [50]. This discordance may be attributable to differences in the design of the studies (multicentric vs monocentric), clinical approach, radiological evaluation and local epidemiology. The most interesting finding in this study was the higher incidence observed for Candida sepsis, with all cases diagnosed in G6PD- patients. It is important to underline that Candida sepsis is a proven infection, and that the isolation of the yeast is an automated process. It follows that the higher incidence observed in this cohort with respect to other studies [50, 63, 65] cannot be explained by differences in clinical approaches or radiological evaluation. Instead, it is possible that this difference may be related to differences in immune response against fungal species. The G6PD enzyme catalyzes the first reaction in the pentose phosphate pathway, thereby providing reducing power to cells in the form of NADPH, that is essential to NADPH oxidase enzyme activity [62]. It has been shown that patients affected by Chronic Granulomatous Disease (CGD), an inherited immunodeficiency disorder characterized by defective functioning of NADPH oxidase enzyme in phagocytes, are exposed to recurrent infections by catalase-positive organisms [71].

Candida and Aspergillus species both express catalase enzymes; hence, it is likely that G6PD-patients with chemotherapy induced neutropenia are particularly vulnerable to these germs [72]. A previous study about G6PD Mediterranean variant showed that G6PD-deficient granulocytes display a reduced function in-vitro, ranging from 25% to 33% of normal activity [49, 73].

Nevertheless, in-vitro response of G6PD- granulocytes against Aspergillus and Candida strains has never been analyzed; this important aspect deserves further investigation.

Historically, the role of G6PD deficiency in determining susceptibility to infectious disease has been quite controversial, with contrasting results from several studies [41, 42, 66, 67]. In particular, to my best knowledge, there are no robust evidences supporting the involvement of G6PD Mediterranean variant in susceptibility to infections. So, the findings of my study could appear unexpected; however, my observations are contrasting with those emerging from literature only in appearance. In fact, it is possible that, in normal conditions, the large number of circulating neutrophils and the percentage of young neutrophils with more active enzyme, provide sufficient NADPH to elicit efficient oxidative killing. However, this scenario is dramatically changed in patients affected by AML with chemotherapy induced neutropenia. In these patients, circulating neutrophils are rare, and there is no efficacious generation of young neutrophils that could help providing active enzyme. It is likely, as my findings seem to suggest, that also G6PD- patients with Mediterranean variant are exposed to some kind of infectious disease in this situation.

I did not find any association between G6PD activity and infection related death, but this result could be biased by the small size of the patient cohorts. A strength in this study was the homogeneity of the two cohorts: all patients underwent the same kind of treatment and antifungal prophylaxis.

In this cohort, surveillance was performed twice weekly using the Galactomannan assay; however, this test is only useful for the detection of Invasive Aspergillosis [74]. These data suggest that G6PD-

patients are exposed to a higher risk of yeast infections. Assays such as β-D Glucan, Mannan/Antimannan Antigen Antibodies and Candida PCR seem to be more useful in the detection and diagnosis of Candida sepsis [75]. Recent guidelines indicate that the β-D Glucan test would be more useful if targeted to subgroups of patients in which the clinical course or risk factors are particularly suggestive of IC or other fungal infections. The test could identify IC cases from days to weeks before positivity of blood cultures, thus considerably reducing the median time for starting antifungal therapy [75].

The hypothesis proposed in my study will need to be confirmed in larger prospective clinical trials. In fact, one of the limitations of my research is the small number of recruited patients which may have limited my ability to detect associations reported by other groups.

Moreover, another limitation is the lack of molecular data on G6PD mutations. I defined G6PD-patients according to an enzyme activity <10%. Therefore, this cohort included only patients harboring class I or II mutations, that means that likely most patients have Mediterranean variant, considering G6PD deficiency epidemiology in Sardinia [62].

One could argue that my observations are not suitable for broad applications, because Sardinia is an area with a high prevalence of G6PD deficiency. However, G6PD deficiency is the most common enzyme defect in the world, affecting more than 400 million people and so it is not unlikely that these results may have an impact on routine practice in hematology departments [66].

Overall, this study is the first to report the association between G6PD activity and susceptibility to IFD. The results obtained suggest that AML patients with chemotherapy induced neutropenia and low levels of G6PD activity have a higher risk of developing IFD. Furthermore, I identified G6PD activity as a risk factor for Candida sepsis in patients with acute myeloid leukemia. Although studies on larger cohorts of patients are required, these findings could help clinicians to identify patients at

higher risk of Candidemia, and thus allow for timely and targeted strategies for IFD prophylaxis and surveillance.

Part 3: How to manage Invasive Fungal Disease in Acute Myeloid Leukemia Patients with Glucose 6 Dehydrogenase Deficiency

G6PD is the key enzyme in the pentose-phosphate pathway and mediates production of nicotinamide adenine dinucleotide phosphate (NADPH), protecting cells from oxidative stress and promoting neutrophil oxidative burst responses against microorganisms. About 140 mutations in the G6PD gene have been described, many of them influencing its activity. The most common G6PD variants are the African G6PD A-, frequently observed in tropical regions of Africa and North and South America, and the so called "Mediterranean" variant, widely found in Italy, Spain, Portugal and the Middle East [62]. Females who have two copies of the G6PD gene on each X chromosome can present normal gene expression, a heterozygous pattern or, in rare cases, a complete enzyme deficiency. Heterozygous females are genetic mosaics as a result of X-chromosome inactivation. Thus, clinical presentations are commonly seen in deficient male patients but are rare in heterozygous females. Traditionally the clinical picture of G6PD deficiency arises from his absence in red blood cells, with acute hemolytic anemia secondary to exogenous oxidative agents and neonatal jaundice [46]. The role of G6PD deficiency in susceptibility to infections has rarely been investigated [41,42,66,67]. Biological background of this supposed increased risk is not clear. Previous studies of the G6PD Mediterranean variant showed that G6PD deficient granulocytes display a reduced function in-vitro, ranging from 25% to 33% [49, 73]. Theoretically, G6PD deficiency provokes the lack of NADPH, essential to NADPH oxidase enzyme; therefore, patients with poor functioning of NADPH oxidase enzyme are exposed to recurrent infections by catalase-positive organisms [71], like Candida and Aspergillus. During my research, I identified G6PD deficiency as a risk factor for IFD in a large cohort of patients with AML undergoing intensive chemotherapy or allogeneic HSCT. In particular, I observed that patients with G6PD deficiency presented a significantly higher incidence of IFD than patients with wild type enzyme (35.7% vs 5%). This finding

was due to differences in frequency of mold infections (17.8% vs 5%), but mostly of Candida sepsis (17.8% vs 0).

Thus, considering this, there is a compelling need for prospective clinical trials to elaborate recommendations to guide antimicrobial surveillance, prophylaxis and treatment of G6PD- AML patients. However, while results from trials are available, clinicians need help to manage these patients. I tried to elaborate some indications to guide hematologist in the care of this complex patients, developing some algorithms that may be useful in clinical practice. I analyzed the literature and data arising from my research to elaborate a strategy to solve some classical clinical scenarios in AML patients care.

I recommend the determination of G6PD activity at AML diagnosis in all patients eligible for intensive chemotherapy. There are many available assays to evaluate G6PD activity, but I suggest to use quantitative tests to assess G6PD activity [76]. I defined patients with activity <10% as deficient, but it is possible that, if evaluated with other assays, the threshold defining high-risk population may be higher, between 20 or 30%. Females with hyperleucocytosis and enzyme activity in the range of heterozygous people (e.g. between 11 and 84% with G6PD/6PDG Automatic Analyzed (KUADRO), Nurex SRL) should be managed with close attention, because the G6PD activity may be over-estimated because of the high number of circulating white cells. In these cases, the molecular test may be indicated to confirm heterozygous status and exclude enzyme deficiency. The finding of G6PD activity in heterozygous range in a male patient should be considered as an interference due to circulating blast cells, hyperleucocytosis or recent transfusion, and this group of patients should be treated as those with complete deficiency. I showed that patients with enzyme activity below 10% are at higher risk of IFD, in particular non-Albicans species IC. This group of patients needs a more intensive strategy, with markers that allow prompt detection not only of impending infections from molds but also from yeasts. B-D Glucan assay was showed to have a high

negative predictive value [77] and to potentially detect IC cases from days to weeks before positivity of blood cultures, thus considerably reducing the median time for starting appropriate antifungal therapy [75]. So this marker could be of great impact in this kind of patients. In patients with G6PD deficiency undergoing intensive chemotherapy at our center, now we perform β-D Glucan assay 2-3 time\weekly, associated with the Galactomannan assay twice weekly. The surveillance strategy is illustrated in Figure 8.

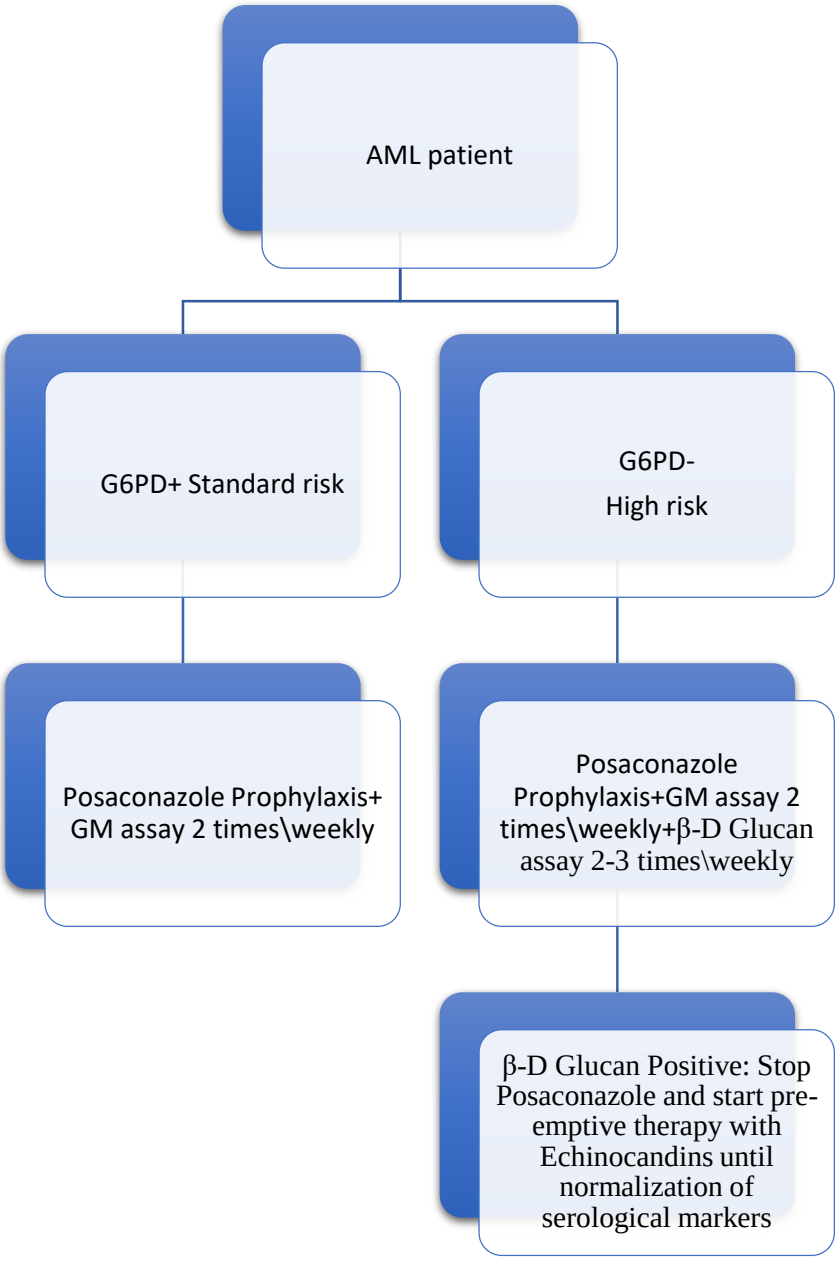

Figure 8: Proposed serological surveillance algorithm in Acute Myeloid Leukemia patients with G6PD deficiency

Some authors questioned the usefulness of IFD screening with β-D Glucan assay in patients with hematologic malignancies, because of the high incidence of false-positive results due to exposure to some drugs (e.g. Cefepime), other infections (e.g. bacteria or Pneumocystis Jirovecii) or plasma and immunoglobulin administration [78]. However, data from a large meta-analysis suggest that two consecutive positive β-D Glucan assays have a positive predictive value of 83.5% and a negative predictive value of 94.5%, and recently ECIL expert panel proposed a grade BII recommendation for the use in hemato-oncological patients [77]. During chemotherapy-induced neutropenia, G6PD- patients with two consecutive positives β-D Glucan assays may benefit from stopping prophylaxis with Posaconazole and starting preemptive therapy with Echinocandins, even in the absence of signs of infection. Prospective studies, assessing the role of β-D Glucan surveillance in combination with clinical, radiological and microbiological findings in this patient setting are lacking, and the positive predictive value of two consecutive β-D Glucan assays is only 83.5% [77], so it is possible that this approach is redundant and not cost-effective. However, I think that a 17.8% risk of Candida sepsis is too high to support a wait and watch strategy. I recommend to collect three sets of blood culture from central venous catheter, to assess for fungal colonization. Preemptive therapy should be continued until normalization of serological markers, ideally, with two consecutive negative β-D Glucan assays, that means a negative predictive value of 94.6% [77]. However, it is known that positive β-D Glucan results may persist long after blood cultures became sterile, and so also clinical variables have to be considered when deciding to stop pre-emptive therapy (e.g. neutropenia recovery, absence of fever, stable condition, negative blood cultures). I still recommend Posaconazole as drug of choice for standard antifungal prophylaxis in G6PD- patients.

For G6PD- AML patients developing febrile neutropenia, after 72 hours of appropriate antibiotic therapy, I recommend starting an empirical de-escalation therapy, stopping prophylaxis with Posaconazole and administering a broad antifungal agent such as Liposomal Amphotericin B.

Liposomal Amphotericin B has a good activity against Aspergillus, but is straightly recommended for treatment of IC [79], and so may give a good protection in this situation. This strategy is illustrated in Figure 9.

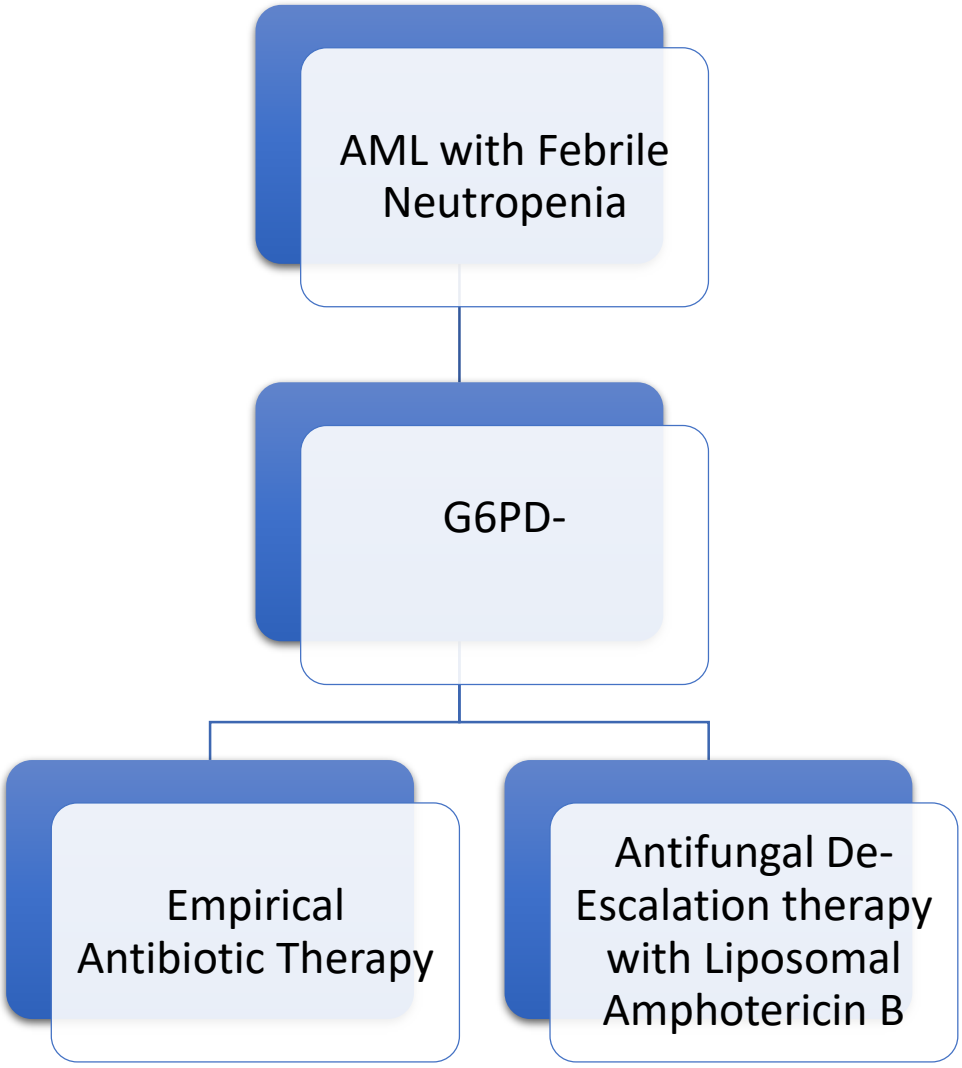

Figure 9: Proposed management algorithm of Acute Myeloid Leukemia patients with G6PD deficiency developing febrile neutropenia

Next, I recommend an aggressive diagnostic strategy, with the execution of TC scans of chest and sinuses and collection of three sets of blood culture, possibly during fever outbreak. For patients with signs or radiological evidence of lung disease, I recommend to execute bronchoalveolar lavage, with research of Galactomannan and culture and research for Aspergillus PCR and other pathogens. I suggest to pursue empirical therapy until identification of other causes of neutropenic fever, as recovery by culture of bacteria or Aspergillus. In patients with persistent fever without clinical or radiological signs of infection and with two consecutive negative β-D Glucan assays, I recommend to stop empirical therapy and resume prophylaxis. Special efforts should be made in those centers with a high prevalence of Zygomycetes, for which empirical therapy with Voriconazole should be avoided. A G6PD- patient diagnosed with IC should be aggressively managed, as illustrated in Figure 10.

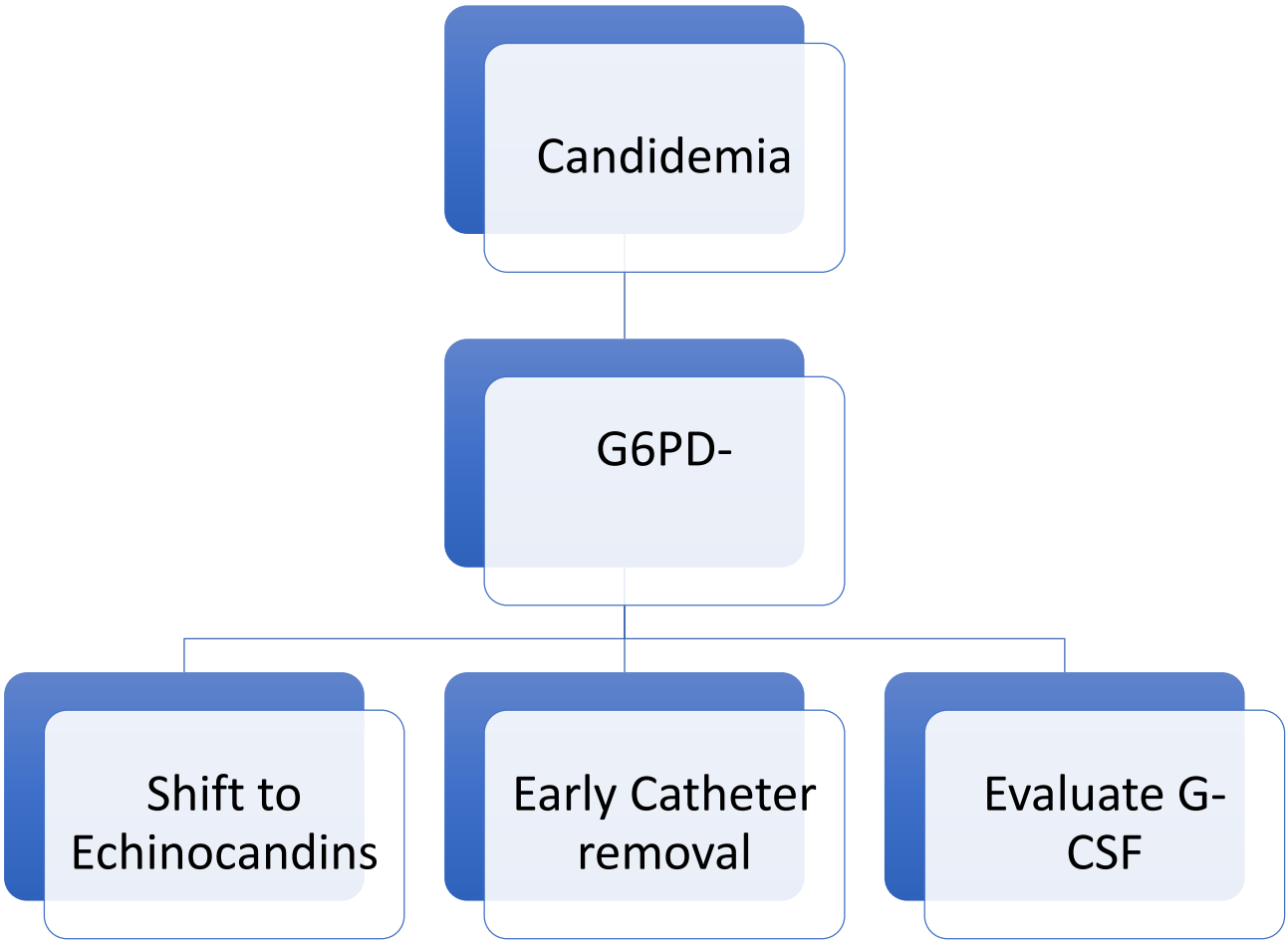

Figure 10: Proposed management algorithm of Acute Myeloid Leukemia patients with G6PD deficiency developing Candida sepsis

Recently published guidelines address management of IC in hematologic patients [79]. Echinocandins treatment with Caspofungin, Anidulafungin or Micafungin should be rapidly begun also in G6PD- patients. ECIL6 guidelines suggest with a grade II recommendation to early remove the central venous catheter, and data from recent studies suggest that early catheter removal is associated with decreased mortality [80,81]. In my experience, despite aggressive treatment with biofilm active agents as Echinocandins or Liposomal Amphotericin B, all patients who did not early remove catheter died, so I think that early catheter removal is mandatory in this subgroup of patients. The addition of G-CSF stimulation may further contribute to therapy, as I observed better outcome in patients who underwent G-CSF therapy during neutropenia. This finding deserves to be further evaluated with in-vitro studies. In conclusion, G6PD- patients with AML represent a particular subgroup at high risk of IFD, especially IC. This therapeutic algorithm may be helpful in the management of this kind of patients, while evidence from prospective clinical trials could give future evidence-based recommendations.

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