PENICILLIN G PLUS AN AMINOGLYCOSIDE IN FEBRILE NEUTROPENIA

Dag Torfoss

Faculty of Medicine
University of Oslo

Department of Clinical Oncology
Division of Cancer Medicine, Surgery and Transplantation
The Norwegian Radium Hospital
Oslo University Hospital Trust,
Oslo, Norway

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Aims

The aims of this thesis were to explore strategies to treat FN patients effectively and safely while simultaneously contributing to avoid development and spread of unnecessary antibiotic resistance.

In Norway, there has been a long medical tradition to treat severe infections, including FN, with penicillin G plus an aminoglycoside. There are reasons to assume that this regimen causes less antibiotic resistance than indiscriminate use of broad-spectrum antibiotics.

To achieve the overall objectives, we decided to focus on three main topics:

1. To summarize the evidence in the medical literature for the efficacy and safety of penicillin G plus an aminoglycoside in the initial treatment of FN.

2. To conduct a randomized, controlled trial to evaluate if tobramycin administered once daily was as effective as tobramycin administrated three times daily, both given with penicillin G, as initial empiric therapy in FN cancer patients.

3. To map the inflammatory response in FN patients and to explore if inflammatory markers of patients with newly developed FN were sensitive and specific enough to characterize and predict the severity and the clinical outcome of the FN episode as aid in deciding the antibiotic treatment.

List of papers

Paper 1

The Norwegian experience with penicillin G plus an aminoglycoside as initial empiric therapy in febrile neutropenia; a review

Torfoss D, Høiby EA, Holte H, Kvaløy S.

Acta Oncol 2012; 51: 433-40.

Paper 2

Tobramycin once versus three times daily, given with penicillin G, to febrile neutropenic cancer patients in Norway: a prospective, randomized, multicentre trial

Torfoss D, Høiby EA, Tangen JM, Holte H, Bø K, Meyer P, Grøttum K, Weyde K, Fossum Lauritzsen G, Sandstad B, Jacobsen AB, Olsen H, Kvaløy S.

J Antimicrob Chemother 2007; 59: 711-7.

Paper 3

The mild inflammatory response in febrile neutropenic lymphoma patients with low risk of complications is more pronounced in patients receiving tobramycin once daily compared with three times daily

Torfoss D, Sandstad B, Mollnes TE, Høiby EA, Holte H, Bjerner J, Bjøro T, Gaudernack G, Kvalheim G, Kvaløy S.

Scand J Immunol 2011; 74: 632-9.

1. Introduction

Febrile neutropenia (FN) has been one of the major challenges in oncology and hematology since modern anticancer chemotherapy became standard of care [Silver, *et al.,* 1958; Boggs and Frei, III, 1960]. Prior to 1961, less than 10 % of patients with serious underlying diseases survived an episode of pseudomonas septicemia [Bodey, *et al.,* 1973]. “In a classic study of Gram-negative bacillary bacteraemia, McCabe and Jackson [McCabe and Jackson, 1962] reported a 90 % mortality rate in patients with rapidly fatal underlying illnesses, most of whom had neutropenia and cancer” [Klastersky, 1993]. The oncological and hematological communities soon saw the need for systematic research to develop the medical field of FN patient management, one of the most important complications of modern cancer chemotherapy, a condition responsible for a major share of the fatalities and morbidities in cancer patients in the Western World.

In Europe, The European Organization for Research and Treatment of Cancer (EORTC) was established in Belgium in 1962, and the branch called the International Antimicrobial Therapy Cooperative Group (IATCG) was founded in 1973 ”…with a protocol designed to investigate the optimal initial antimicrobial therapy for FN patients with malignant diseases. Before this time, the outcome of sepsis in granulocytopenic cancer patients had been dismal” [Klastersky, 1993].

Major improvements in therapy of FN and clinical and epidemiological knowledge about the condition were gained through the many studies that followed. Four major randomized and controlled trials (RCT) had established a standard of care at the beginning of the 1990s, and one of the most important leaders of these trials, professor Jean Klastersky, summarized the findings in an important review from 1993 [Klastersky, 1993]. He concluded that an anti-pseudomonal β-lactam and an aminoglycoside should be started empirically as soon as possible when a new FN episode developed, “…especially in patients with severe and persistent granulocytopenia who have suspected or demonstrated Gram-negative bacillary bacteraemia.”

Further studies established monotherapy with a broad-spectrum β-lactam antibiotic as standard therapy in FN in many countries [Paul, *et al.,* 2003; Klastersky, 2009; Paul, *et al.,* 2010]. The 2010 Cochrane Collaboration metaanalysis, based on 68

RCTs, concluded that a broad-spectrum β-lactam with addition of an aminoglycoside, compared to monotherapy with a broad-spectrum β-lactam, showed a trend toward all-cause higher mortality (RR 0.87, 95 % confidence interval (CI): 0.75 to 1.02), and more adverse events "…with combination therapy, numbers needed to harm four (95 % CI: 4 to 5). Specifically, the difference with regard to nephrotoxicity was highly significant" [Paul, *et al.,* 2010]. But "nearly all trials were open-labeled", and no evaluation of antibiotic resistance development were done in these RCTs.
So, even if the arguments for broad-spectrum monotherapy may be strong, we shall see that this issue is more complicated.

Klastersky [1993] pointed out that aminoglycoside monotherapy was dismal in FN, but aminoglycosides prevented both the development of β-lactam resistance and at the same time enhanced the efficacy of the β-lactam through synergy or additive effects [Amsterdam, 1996]. The issue of aminoglycoside protection against resistance development against β-lactams is complicated and has never been fully elucidated [Gribble, *et al.*, 1983].

This thesis argues that the combination of penicillin G plus an aminoglycoside is both safe and efficient in many cases of FN, and that the addition of an aminoglycoside to a β-lactam regimen may protect the β-lactam against development of resistance [Gilbert and Leggett, 2010].

There are reasons to assume that the combination of penicillin G plus an aminoglycoside may confer important synergistic or additive effects to each other and is different from aminoglycoside monotherapy; as discussed by Klastersky [1993]. It was said that sensitivity to both antibiotics was needed, but *in vitro* studies have documented that synergy may have important implications even when the bacterial isolate was resistant toward both the aminoglycoside and the β-lactam. Already in 1969 an *in vitro* study [Sonne and Jawetz, 1969] showed synergistic effects between β-lactams and aminoglycosides in some strains of *Pseudomonas aeruginosa* that were inhibited, but not killed, by carbencillin. The Gram-negative bacilli showed up to 10-fold decrease in the minimum inhibitory concentration (MIC) when a small concentration of gentamicin (from < 0.125 to 1.0 mg/L) was present. In combination, all the bacteria were killed within 24 hours; compared to when the antibiotics were tested singly. Then the bacteria continued to grow. Similar results were found in an animal study by Lumish and Norden [Lumish and Norden, 1976].

An *in vitro* study of *P. aeruginosa* [Song, *et al.*, 2003] documented synergy in most

isolates that singly were resistant to both drugs tested when the drugs were β-lactams and aminoglycosides. Applied to clinical situations, the increased sensitivity in resistant isolates promoted by synergy or additive effects may be important [Andriole, 1971; Klastersky and Zinner, 1982].

This is important as a background for the results of our study “Tobramycin once versus three times daily, given with penicillin G, to febrile neutropenic cancer patients in Norway: a prospective, randomized, multicentre trial” for short called The Tobramycin Trial. When we take into account the fact that important Gram-negative bacilli such as wild-type *Escherichia coli* have minimal inhibitory concentration (MIC) values toward penicillin G of 34 - 64 mg/L [Kucers, *et al.,* 1997], the penicillin serum concentrations may be sufficient to exert an antibacterial effect against *E. coli* when an aminoglycoside works in combination with penicillin G.

Another example of Gram-negative bacilli with penicillin G MIC values that may be overcome by the combination of penicillin G and an aminoglycoside was a study with a wild type *E. coli* with an MIC value toward penicillin G of 32 mg/L, and a *Proteus mirabilis* with an MIC value of 16 mg/L toward penicillin G [Livermore and Williams, 1996]. Livermore and Williams did not study neutropenic patients. However, they showed that penicillin G may have antibacterial effect against some Gram-negative bacilli. Neutropenic patients need higher antibiotic concentrations and more effective antibiotic therapy than immunocompetent patients. Still, the results from The Tobramycin Trial suggested that high doses of penicillin G administered with an aminoglycoside toward which the bacteria were sensitive, might have killed these bacteria with the help of the synergy between the two antibiotics.

Penicillin G has a protein-binding of 55 % and a half-life of 30 minutes. One million units give a mean serum concentration of 9.9 mg/L (range 4.3 to 17.5) after one hour [Bamberger, *et al.,* 2005]. The concentrations vary according to the different body fluids, with highest concentration in bile (mean 45.7; range 2.2 to 189 after one hour) where it is excreted. The penicillin G dose in our study was 5 million units. Thus, penicillin G probably participated in the bacterial killing of the Gram-negative bacilli, but the importance of high aminoglycoside concentrations must be underlined for the effective bacterial killing.

Resistance to penicillin G in Gram-negative bacilli is often based on β-lactamase production and problems with penetrance and efflux [Kucers, *et al.*, 1997]. In the cell wall of the bacteria penicillin and other β-lactam antibiotics bind covalently to

transpeptidases (penicillin-binding proteins) of which there are several different kinds. The transpeptidases cross link the peptidoglycan strands which constitute an important part of the bacterial cell wall. This cell wall is the exoskeleton of the bacterium and thereby supports the high intracellular osmotic pressure necessary for the survival of the bacterium. Inhibition of peptidoglycan production effectively breaks down the cell wall in growing bacteria; and this is a main antibacterial mechanism of the β-lactam antibiotics. However, only actively growing and dividing bacteria produce peptidoglycan [Chambers, 2010]. But this is exactly the situation in bacterial sepsis with bacteria growing in the blood, the most important situation we try to fight in FN.

In Norway, the regimen of penicillin G plus an aminoglycoside has remained the standard of care as initial empiric therapy in FN as well as in community-acquired sepsis of unknown origin. The Norwegian human microbiological epidemiology, as well as the antibiotic consumption, has been monitored since year 2000 by the government financed NORM/NORM VET program [Nyquist, 2000]. NORM/NORM VET presents their national results in annual reports of antimicrobial resistance and antibiotic consumption both in human and veterinary medicine, as well as in agriculture and aquaculture. The last published report [NORM/NORM VET, 2011] presented the results from 2011. This document confirmed the general low rate of resistance and the prevailing use of narrow-spectrum antibiotics in Norway, giving an epidemiological support for continued use of penicillin G plus an aminoglycoside in FN and other septic infections.

The β-lactam coverage of penicillin G is substantially narrower than the anti-pseudomonal β-lactam recommended by Klastersky in 1993. According to the NORM/NORMVET 2011 report, analyzing isolates from blood cultures, with the addition of isolates from cerebrospinal fluids for *Streptococcus pneumonia*, penicillin G monotherapy would cover most streptococci (*S. pneumoniae* was analyzed and 96 % of the isolates were sensitive to penicillin), and 70 % of the enterococci would be covered by ampicillin among the main pathogens. Still, the key to the success of The Tobramycin Trial seems to have been the synergy and the additive effects of penicillin G plus an aminoglycoside.

If this regimen is efficient and safe in severe infections, including bacteremia with Gram-negative bacilli, the primary choice of penicillin G plus an aminoglycoside in FN

and in sepsis of unknown origin may be an important contribution to prevent the development, selection and spread of antibiotic resistance.

2. The scientific background of the Tobramycin Trial

2.1 The four major EORTC trials on febrile neutropenia

These four open-labeled RCTs aimed at defining the optimal antibiotic regimen in FN cancer patients, based on the available antibiotics at the time (1975 to 1990). A second benefit of these trials was the establishment of the clinical epidemiology of FN. The focus was on classifying the febrile episodes and defining other prognostic factors, with regard to clinical outcome and microbiological findings, especially bacteremias. The total number of FN episodes evaluated for participation in the four trials varied from 625 in the first trial to 1074 in the fourth trial. Sixty-six to 79 % of the patients had leukemia or lymphoma.

In the first trial, responses were defined as "…(1) improvement if there was a lasting return of temperature to normal or the level before infection and resolution of all signs and symptoms without addition of other antibiotics, (2) temporary improvement if there was improvement in (1) but relapse occurred within five to seven days, (3) failure if there was no or minimal response to antibiotics or if antibiotics required changes or additions, or (4) not evaluable if the patient had viral or fungal infection, a protocol violation had occurred, or the episode was classified as a doubtful infection" [Klastersky, *et al.,* 1978].

This definition of response was only modified to minor degrees in the following three trials. When "not evaluable" episodes were excluded, evaluable episodes ranged from 50 to 81 % of the total number of FN episodes in trial two, three and four (trial one did not report the number of non-evaluable episodes). The number of patients with bacteremia ranged from 13 to 29 % of the evaluable patients. The number of episodes with renal dysfunction was in general low (3 - 7 %), with no difference in toxicity between any of the antibiotic regimens; except for the cephalothin plus gentamicin arm in the first EORTC trial, where 16 % of the episodes developed renal dysfunction [Klastersky, *et al.,* 1978].

This nephrotoxic effect of cephalosporins applies only to some cephalosporins in combination with an aminoglycoside; but the specific mechanism has still to be elucidated [Luft, 1982].

The first EORTC trial [Klastersky, *et al.*, 1978] compared three antimicrobial regimens. It was based on the preceding work by Schimpff [Schimpff, *et al.*, 1971]. The antipseudomonal penicillin, carbencillin, plus gentamicin, were found to be the superior regimen, compared to carbencillin plus cephalothin and cephalothin plus gentamicin. This trial also established the basic epidemiology of FN. Approximately 40 % of the patients had a microbiological cause for their FN. Twenty-two percent of the patients (140 of 625) had bacteremia. The response to empirical antimicrobial therapy was almost similar in patients with increasing granulocytes, whether the isolate was sensitive to both (50 % of the isolates) or only to one of the antibiotics (43 % of the isolates). Improvement was registered in 58 % of the episodes with bacteremia sensitive to both antibiotics, and in 47 % of the episodes sensitive to only one of the antibiotics. Response rates were better for Gram-positive bacteremias (72 %) than for Gram-negative bacteremias (56 %). Fatality rate was higher in mixed Gram-negative bacteremias (63 %) than in single Gram-negative bacteremias (25 %). Of the total 20 candida infections, seven were candidemias. Use of corticosteroids increased the risk of infection with candida (55 %; 11 infections in 20 patients); however, the cancer diagnosis of acute lymphocytic leukemia had a five-fold increase in candida infection (12 %; 7 infections in 60 patients) compared to acute myelocytic leukemia (2 %; 6 infections in 310 patients) and other cancers (3 %; 7 infections in 255 patients).

"However, when all of the prognostic factors were considered together, change in granulocyte count during therapy became the critical factor provided that the patient had been given antibiotics to which the organism was susceptible" [Klastersky, *et al.,* 1978].

This first trial raised the question of the role of aminoglycosides in FN. Aminoglycosides were considered suboptimal as monotherapy in Gram-negative FN bacteremia "…despite their *in vitro* activity and the minimal emergence of resistance strains (sic)" [Klastersky, 1993], even though aminoglycosides might reduce resistance to β-lactams when used in combination [Gribble, *et al.*, 1983] and augment bactericidal efficacy of synergistic β-lactams [Klastersky, *et al.*, 1982].

The second EORTC trial [Zinner, *et al.*, 1983] established that double β-lactam therapy (carbencillin plus cefazolin) with aminoglycoside (amikacin) was not better than carbencillin plus amikacin alone. All the patients received the allocated antibiotic regimen "…for the first five days of therapy unless there was microbiologic

documentation of inadequate antibiotic therapy associated with poor clinical response." The addition of new antibiotics classified the treatment response to trial antibiotics as a failure. "Improvement occurred in 35 (64 %) of 55 bacteremic patients treated with two antibiotics and 39 (65 %) of 60 treated with three antibiotics." The abstract stated that "…the two antibiotic regimens were equal in efficacy and in nephrotoxicity." The increased nephrotoxicity found with cephalothin in the first EORCT trial was not found with cefazolin in this trial, even though Luft [1982] grouped both these first-generation cephalosporins as agents with the potential to increase the nephrotoxicity when given in combination with an aminoglycoside.

Another conclusion from the second EORTC trial was that oral intestinal decontamination regimens resulted in fewer episodes of bacteremia, even though the study was not designed to test this effect [Zinner, *et al.,* 1983]. Further, Klastersky, who participated in the writing committee, added a third conclusion in his 1993 summary of these four EORTC trials "…that the early empirical use of granulocyte transfusions in combination with broad-spectrum antibiotics was not useful in the management of granulocytopenic cancer patients" [Klastersky, 1993; EORTC, 1983]. With "early", Klastersky here meant prophylactic transfusions. Other studies had documented "beneficial" and even "lifesaving" effects of granulocyte transfusions from "related histocompatible donors" [EORTC, 1983] when given to patients with prolonged neutropenia and documented Gram-negative bacteremia. This did not appear in the original trial report; but the protocol paper explained that granulocyte transfusions were tested in a "poor prognosis" arm with available white blood cells for transfusion [EORTC, IATPG, 1977]. Prior to this trial, granulocyte transfusions had been considered effective in the management of pseudomonas septicemia [Frei, III, *et al.,* 1965].

The third EORTC trial [Klastersky, *et al.*, 1986] compared three different broad-spectrum β-lactams, two penicillins (ticarcillin and azlocillin) and cefotaxime, all given with amikacin. All the patients were classified according to the results of their microbiological findings, and the focus was on patients with Gram-negative bacteremias. In 83 episodes of Gram-negative bacteremias, the azlocillin-based regimen had the best result with a 66 % response rate, versus 37 to 42 % positive response rates with the two other regimens. All the Gram-negative bacilli were sensitive to amikacin. As expected from the first EORTC trial, patients with persistent, profound granulocytopenia had an even poorer response rate (37 %) than the

patients with rising granulocyte counts (73 %). “A logistic regression analysis indicated that the following factors also affected infection resolution: beta-lactam utilization in the regimen (azlocillin was better than ticarcillin or cefotaxime), resolution of profound granulocytopenia (< 100 cells per µl) during therapy, and susceptibility to the beta-lactam antibiotic” [Klastersky, *et al.,* 1986]. The importance of the aminoglycoside was probably taken for granted.

The fourth EORTC trial [EORTC, 1987] introduced for the first time the question of whether a broad-spectrum β-lactam would be efficient without the addition of an aminoglycoside. The assumption that an aminoglycoside was necessary for efficacy was so strong that even in this trial, amikacin was administered in all three arms starting the first day. However, a shorter course of amikacin was supposed to reduce the problem of nephrotoxicity. After three days, the amikacin was stopped in the arm consisting of ceftazidime plus the short course of amikacin. In the two other arms, amikacin was continued for nine days with either ceftazidime or azlocillin (the latter was the control arm based on the third EORTC trial).

After nine days, ceftazidime plus the long course (nine days) amikacin, 91 % of the patients had become afebrile which was the clinical definition of success. In the ceftazidime plus amikacin three days arm, and in the azlocillin plus amikacin nine days arm, the success rates were both 64 %. A Kaplan-Meyer plot registering the success of the patients for 14 days, showed that the proportion of success continued to drop after nine days. In the ceftazidime plus the long course amikacin arm the success rate ended at approximately 80 %. In the ceftazidime plus the short course arm the success rate ended at approximately 45 %, and in the azlocillin arm the success rate ended at approximately 20 % [Figure 1 in EORTC, 1987]. However, total deaths from the presenting infection was only 16 patients in total (< 2 %) of the 872 evaluable patients, with no difference between the three arm of the trial, so most patients with failure must have responded to modifications of the initial antibiotic regimen.

Ceftazidime was a new anti-pseudomonal cephalosporin when this trial was conducted, and resistance to ceftazidime was unusual; as it often is when a new antibiotic is introduced. This trial also found that the median time to modification was more than six days in all the three regimens that were tested. Modification during the first three days was unusual and occurred in only 10 % of the patients in all the three arms.

Table 1 Summary of the four EORTC trials

EORTC trial	Year of print	Antibiotics tested	Number with improvement	Number of Gram-positive isolates in blood	Number of Gram-negative isolates in blood	Number of deaths among evaluable patients
1	1978	**Carbencillin + gentamicin** **vs Carbencillin + cephalothin** **vs Cephalothin + gentamicin**	110/156 (71 %) 97/135 (72 %) 110/162 (68 %)			41/453 (9 %) died from infection
2	1983	**Carbencillin + amikacin** **vs Carbencillin +** **amikacin + cefazolin**	158/224 (71 %) 142/195 (73 %)			
3	1986	**Azlocillin + amikacin** **vs Cefotaxime + amikacin** **vs Ticarcillin + amikacin**	138/197 (70 %) 105/162 (65 %) 131/223 (59 %)	**10/15 (67 %)** 9/21 (43 %) 6/22 (32 %)	**21/32 (66 %)** 7/19 (37 %) 15/32 (47 %)	31 % total 17 % single Gram-negative bacteremia 4 % died from superinfection 11 % died from a non-infectious cause
4	1987	**Azlocillin +** **9 days with amikacin** **vs Ceftazidime +** **3 days with amikacin** **vs Ceftazidime +** **9 days with amikacin**		19/37 (51 %) 8/23 (35 %) 14/30 (47 %)	16/40 (40 %) 20/42 (48 %) **38/47 (81 %)**	13/298 (4 %) 13/266 (5 %) 8/308 (3 %)

In this fourth trial, there was a special focus on single-organism Gram-negative bacteremias. It occurred in 129 patients. The response rates in patients with single-organism Gram-negative bacteremia were 81 % in the ceftazidime with the long course of amikacin, 48 % in the ceftazidime with the short course of amikacin, and 40 % in the azlocillin plus nine days of amikacin arm [Table 3 in EORTC, 1987]. There may seem to be a contradiction between these results from patients with single-organism Gram-negative bacteremia and the results mentioned presented in page 13; however, the results in page 13 were based on treatment failure, previously defined as defervescence [Figure 1 in EORTC, 1987]. The difference was even more pronounced in patients with persistant < 0.1 x 10^9 granulocytes/L, with 50 % response rate in the ceftazidime with the long course of amikacin arm versus six percent response rate in the ceftazidime plus the short course of amikacin arm. The ten patients who developed a bacterial superinfection were mainly infected by Gram-positive organisms.

As initial, empiric therapy aminoglycosides were potent drugs, even when given twice daily with probably frequent suboptimal peak concentrations. Compared to tobramycin and gentamicin the potency of amikacin is generally considered to be three times lower; suggesting that a daily dose of 15 mg/kg of amikacin should compare to 5 mg/kg of tobramycin or gentamicin [Gilbert and Leggett, 2008]. Dosing amikacin twice daily was standard therapy in all the three EORTC trials that used the aminoglycoside amikacin. The peak serum concentrations, based on amikacin given twice daily, would then probably not be high enough to prevent aminoglycoside adaptive resistance or resistant small-colony variants when amikacin had been used for nine days (see page 49) [Gilbert and Leggett, 2008]. This could be the reason for some of the late failures in the ceftazidime plus the long course of amikacin arm.

Based on these four trials Klastersky concluded in 1993 that ”the combination of an anti-pseudomonal β-lactam with an aminoglycoside is recommended as the standard for empirical therapy in FN patients, especially in patients with severe and persistent granulocytopenia who have suspected or demonstrated Gram-negative bacillary bacteraemia. However, patients who are less neutropenic and/or less symptomatic may benefit from monotherapy.” [Klastersky, 1993]

In the publication “Why empirical therapy?” from 2009, Klastersky discussed why monotherapy with a broad-spectrum β-lactam has later been proven as efficient as the combination of a β-lactam with an aminoglycoside. He underlined that the

reasons for this were unclear, but pointed to several possible explanations. There might be "…improved intrinsic efficacies of antibiotics such as piperacillin-tazobactam, ceftazidime, cefepime or the carbapenems…". Further "…our practice in managing FN patients had changed so that prompt, earlier empirical therapy has become the norm." Finally "More effective cytostatic chemotherapy leading to better remission of the underlying disease and perhaps the use of agents stimulating granulopoiesis may also have contributed." He was still concerned about the 40 % case fatality rate in some groups of FN patients with Gram-negative bacteremia. Klastersky suggested that antimicrobial synergism may be important under these circumstances [Klastersky, 2009]. On one hand, these patients might have an invasive fungal infection. On the other hand, the possibility of a multiresistant superinfection might gradually become more and more important [Austin, *et al.,* 1999; Goossens, *et al.,* 2005; Haug, *et al.,* 2011]. Another important reason why monotherapy with a broad-spectrum β-lactam antibiotic might be as efficient as therapy with the combination of a β-lactam with an aminoglycoside is that the impact of different kinds of extended spectrum β-lactamases may not yet have been large enough to affect the microbiological epidemiology of FN [Bush and Jacoby, 2010]. In certain places in Asia the percentage of ESBL among the Gram-negatve bacilli is so high that it may be dangerous to treat FN with monotherapy broad-spectrum β-lactams [Peripi *et al.,* 2012; Kumarasamy *et al.,* 2010] .

These four EORTC trials all suffered from some weaknesses. At least the first and the second trials [Klastersky, *et al.*, 1978; Zinner, *et al.*, 1983], and maybe the third and the fourth [Klastersky, *et al.,* 1986; EORTC, 1987] accepted patients to be randomized several times according to the trial protocols [Gaya, *et al.*, 1975; Gaya, *et al.*, 1977].

The episodes of FN were randomized, and the studies were described as "open-labeled". Blinding this kind of trial is possible, but it requires a huge amount of double book-keeping along with a logistically complicated organization conducting the trial. Only one RCT with double-blinding, comparing tobramycin once versus three times daily with a broad-spectrum β-lactam antibiotic in FN children undergoing stem cell transplantation has been published [Sung, *et al.,* 2003]. Sung and coworkers found less nephrotoxicity and more efficacy in the once daily arm. But Sung's study was small, including only 60 episodes of FN, and it did not change the knowledge base on how to treat FN.

Finally, the definition of the primary endpoint in the four EORTC trials was not optimal. An episode of FN may last for two to three weeks or more. Considering late modifications of the antibiotic regimens as failures, made it impossible to evaluate the initial effect of the antibiotic regimen. The situation in an oncological or hematological department is different. If the patient responds to the initial empiric antibiotic regimen and is clinically stable during the first three days of FN, then the initial antibiotic regimen should be considered successful [Feld, *et al.*, 2003]. Later modification of the antibiotic regimen remains a secondary problem. New recommendations on how to conduct clinical trials in FN have adopted this way of evaluating responses [Feld, *et al.*, 2003]. "Treatment failure should not be regarded as the primary outcome in open-label trials, as it reflects mainly treatment modifications" [Paul, *et al.,* 2010].

If the Norwegian regimen of penicillin G plus an aminoglycoside as the initial, empiric therapy in FN is successful during the first three days, the total consumption of broad-spectrum β-lactams may be substantially reduced. This may be possible in many immunocompromised cancer patients, and in many patients with immunopathies, as well as in many patients who have had stem-cell or solid-organ transplantations. Initial, empiric therapy with penicillin G plus an aminoglycoside may have a substantial impact on the evolution, selection and spread of antibiotic resistance.

2.2 The origin of The Tobramycin Trial

The Tobramycin Trial is the main result of this thesis. It was a Norwegian, multicenter RCT in hemodynamically stable FN patients. The study was researcher-initiated and only sponsored by The Norwegian Radium Hospital.

At the turn of the century, most Norwegian institutions caring for FN cancer patients administered aminoglycosides three times daily, despite the fact that aminoglycoside given once daily had been introduced as standard practice in most other clinical situations (except primarily for bacterial endocarditis and pregnancy) [Cronberg, 1993; Blaser, *et al.,* 1994; Lund, *et al.,* 1997; Berild, *et al.,* 1999; Hansen, *et al.,* 2001]. The once daily dosing practice was based on the recent understanding of the aminoglycosides' concentration-dependent bacterial killing [Craig, 1998]. The idea of

a toxic upper limit for aminoglycosides had been discarded about ten years earlier [Kumana and Yuen, 1994]. The Department of Hematology, Oslo University Hospital, had, however, already introduced gentamicin 7.5 mg/kg once daily with penicillin G in FN patients. Their experience was excellent [Lorentz Brinch, personal communication, 2000].

An important question was if the aminoglycoside administered once daily had the same antibacterial efficacy as aminoglycosides given three times daily in FN patients. If the patient had a Gram-negative bacteremia, she would be without antibiotic coverage for more than 50 % of the time interval between two doses even when considering the post-antibiotic effect (PAE) of one to two hours. The PAE is longer the higher the serum concentration is, and shorter in patients with neutropenia. The PAE also depends on the bacterium, being longer for *P. aeruginosa* (1-3 hours), and not existing for *Streptococcus pneumonia*. A small inoculum, a high oxygen tension, and a high pH result in longer PAE. Imipenem, given with tobramycin or gentamicin, results in a longer PAE, while other β-lactams do not affect the PAE of the aminoglycosides [Gilbert and Leggett, 2010].

We wanted to examine if the difference between aminoglycosides given once or three times daily, with penicillin G, to cancer patients with FN had any clinical relevance. We chose to restrict the aminoglycoside to only one drug. One of the Norwegian experts in this field, Professor Johan Bruun, suggested that we used tobramycin, because, as he said: fewer clinical studies have been done with tobramycin than with gentamicin. Tobramycin was also the aminoglycoside most used at The Norwegian Radium Hospital where the trial was initiated. The trial was approved at the Institutional Review Board (IRB) at The Norwegian Radium Hospital as well as at The National Committee for Medical and Health Research Ethics (NEM) and at the other bodies that needed to approve the trial.

2.3 Would inflammatory markers add to the clinical assessment?

The biology of immunosuppression caused by anti-cancer chemotherapy has been extensively studied [Engervall, *et al.,* 1995; Buyukberber, *et al.,* 2009]. The most important phenotypic manifestation of many anti-cancer chemotherapeutic regimens

is neutrophilic granulocytopenia, affecting one of the most important protective mechanisms of the innate immune response. These regimens also affect other parts of the immune response, both the non-specific and the acquired responses, causing a wide range of immunodeficiency syndromes with variable risks of opportunistic infections [Rauch, *et al.,* 2012].

Neutropenia is characterized by increased risk of early bacterial infections, secondary both to the neutropenia itself and also to the barrier breakdown of the skin from central venous catheters and mucositis-prone chemotherapeutic agents like methotrexate [Dahl, *et al.*, 2009]. Long-lasting and deep neutropenia increases the risk for opportunistic infections. Herpes simplex reactivation is common, and prophylaxis with aciclovir is used. After three to five days of broad-spectrum antibiotic therapy in FN, systemic candidemia may occur even though the proven rate of invasive fungal infections in FN is < 5 % of all the positive blood cultures [Torfoss and Sandven, 2005]. Invasive mould infections may rarely occur, usually after more than twelve to fourteen days of neutropenia, especially if construction sites are present at or nearby the hospital with increased amounts of dust in the air [Ross, *et al.,* 2011].

The immunosuppressive condition caused by anti-cancer chemotherapy has led to research for inflammatory markers, primarily able to reflect and survey systemic bacterial infections. Developing tests that can rapidly detect induced inflammatory responses with high sensitivity and specificity that signal systemic bacterial infections and need of anti-bacterial therapy is a strong incentive for the pharmaceutical industry. From a more academic point of view, understanding the biology of neutropenia and inflammation is a driving force behind these developments.

So far, this research has not led to any laboratory test being more sensitive and specific than a detailed daily clinical examination of the FN patient. Some tests, however, have the potential to help in the diagnostic assessment, in particular procalcitonin (PCT) when used systematically and the results are immediately available to the clinician [Meisner, 2000; and Crist-Crain and Muller, 2005].

2.4 Antimicrobial resistance when The Tobramycin Trial started

When The Tobramycin Trial was initiated the greater perspective was how we could contribute to an evolution where antibiotic agents remain effective and safe in a world with increasing antimicrobial resistance [Newton, 2005; Leibovici, *et al.,* 2011]. This perspective is even more important today. In clinical situations where carbapenem- and multiresistant Gram-negative bacilli causes bacteremia and clinical sepsis [NORM/NORM VET, 2011], we have already returned to the early antibiotic era. Old, toxic antibiotics, like colistin, are being used more often, and some patients are again in need of granulocyte transfusions [Al-Tanbal, *et al.,* 2010].

The major strategies to prevent evolution and spread of antimicrobial resistance is using the antibiotic agents we have at hand in the most appropriate way, and to avoid unnecessary use of antibiotics [Jawetz, 1956; de Man, *et al.,* 2000; Austin, *et al.,* 1999; Goossens, *et al.,* 2005; Haug, *et al.,* 2011; Gammelsrud, *et al.,* unpublished]. Prevention of some infections by vaccination, and strict hospital and community hygiene practices, are the other main strategies.

Sepsis with bacteremia in FN will still continue to be a major risk factor affecting the optimal outcome in anti-cancer therapy. Documenting the efficacy and safety of the Norwegian antibiotic tradition with penicillin G plus an aminoglycoside as initial, empirical therapy in FN, is one important step in accord with these strategies.

Based on the fear of non-efficacy, the international medical community has been sceptic to a regimen based on the narrow-spectrum penicillin G in FN and other severe infections. We have, so far, not been able to give substantial theoretical proof or practical evidence for this efficacy. Thus, it has been even more important to present the Norwegian clinical experience, and the published evidence of the efficacy and safety of penicillin G plus an aminoglycoside, to the global medical community.

Only one clinical trial from The Netherlands has been published comparing penicillin G plus an aminoglycoside to broad-spectrum β-lactam antibiotics, evaluating the effect on the colonizing microbial intestinal flora according to the antibiotic regimen used [de Man, *et al.*, 2000]. Two neonatal intensive care units located on each side of a corridor were compared in a prospective cohort trial. The same number of patients was admitted to the two units (218 admissions to each unit).

There was always a shortage of unit beds and every new patient was admitted to the first unit that had a free bed. Unit A used intravenous amoxicillin plus cefotaxime and unit B used penicillin G plus tobramycin. Six months later, the units switched the antibiotic regimens (cross-over phase) to balance the effects of possible unit-related confounding variables.

Bacterial screening included cultures of respiratory aspirates and rectal swabs at admission and once a week thereafter. There were substantial differences between the two regimens with regard to the rate of colonization with Gram-negative bacilli. With the penicillin plus tobramycin regimen, *E. coli* predominated and made up 53 % of all the Gram-negative isolates. For the amoxicillin plus cefotaxime regimen, 77 % of all the Gram-negative isolates were *Enterobacter species*. The risk of colonization with resistant Gram-negative bacilli was expressed as colonizing events per 1000 patient days. The most striking difference was found in the analyses of colonizing events with strains resistant to the agents of the empirical antibiotic regimen in use. Colonizing events with such strains occurred 18 times more frequently with the amoxicillin plus cefotaxime regimen. *Enterobacter* spp. bacteremia occurred only in the amoxicillin plus cefotaxime regimen. The total rate of bacteremias did not reach the 0.05 level of significance, but there was a trend towards fewer bacteremias in the penicillin plus tobramycin regimen (27 episodes versus 37 episodes, $p = 0.22$).

The flora of the body's inner and outer surfaces forms a reservoir for opportunistic invasive infections. The 18-fold higher rate of colonization with resistant strains in the amoxicillin plus cefotaxime regimen probably put these children at a higher risk for nosocomial bacteremias with resistant bacteria. An antibiotic regimen in this population should not only treat the most common pathogens of neonatal septicemia, but it should also not contribute to selection of resistant nosocomial pathogens. With amoxicillin plus cefotaxime, they demonstrated a selective pressure toward β-lactamase producers that can degrade amoxicillin, for example *Klebsiella* spp. Further, Gram-negative bacilli such as *Enterobacter* spp. and *Serratia* spp. that can degrade cefotaxime and amoxicillin, might also have been selected [Filius, *et al.,* 2005]. The penicillin plus tobramycin regimen included a β-lactam with minor effect toward Gram-negative bacilli. The use of an aminoglycoside was not associated with any significant emergence of resistance, since tobramycin only to a minor degree penetrated the mucosal surface of the gastrointestinal tract, and anaerobic conditions

counteracted the aminoglycoside that needed aerobic energy-production to be imported into the bacterial cells [Gilbert and Leggett, 2010].

Except for the study of de Man and co-workers, all studies, to our knowledge, of penicillin G plus aminoglycoside have been conducted in Norway. The seven studies presented in Paper 1 consequently created the existing evidence for this antibiotic regimen. Three of the studies were published in Norwegian or presented at a local conference. The first study [Frøland, *et al.,* 1989] was an important RCT, but unfortunately it was never published in a peer-reviewed journal. A Norwegian publication in preparation in 2012 [Gammelsrud, *et al.,* unpublished] supports the findings of de Man and coworkers, as well as our conclusions in Paper 1 and 2.

3. Materials and methods

3.1 Material and methods of Paper 1

The Norwegian experience with penicillin G plus an aminoglycoside as initial empiric therapy in febrile neutropenia; a review

"A [non-systematic] literature search in Medline and EMBASE was unable to identify other clinical studies of penicillin G and an aminoglycoside than the seven Norwegian studies presented here. Experts in the field are not aware of any other relevant studies" [Torfoss, *et al.*, 2012]. When the studies were identified, the work that resulted in this paper mainly consisted in organizing and grouping the studies in a way that yielded meaningful results that could be compared to the international literature describing therapy based on broad-spectrum β-lactam antibiotics [Paul, *et al.,* 2002; Freifeld, *et al.,* 2011]. The seven studies were grouped and evaluated according to whether they studied the clinical response to FN therapy or microbiological sensitivities to penicillin G and to aminoglycosides.

3.2 Material and methods of Paper 2

Tobramycin once versus three times daily, given with penicillin G, to febrile neutropenic cancer patients in Norway: a prospective, randomized, multicentre trial

We decided to conduct a non-inferiority one-sided trial [Guyatt, *et al.*, 2008] documenting that once daily dosing was at least as effective as three times daily dosings. The benefits of administering the aminoglycoside once daily were sufficient enough to change our practice if the result of the study would show that once daily dosing was not inferior to three times daily dosing.

We decided to follow the same formal procedures for material and methods that were used in the four major EORTC trials. Key points in these procedures included: 1) Randomization of episodes of FN and not of patients; implying that patients could participate several times in the same trial [Gaya, *et al.,* 1975; EORTC, *et al.,* 1977].

However, when analyzing and presenting the data, it was decided that each patient could be randomized only once (see pages 40-41).
2) The primary endpoint was no modification of the initial antibiotic regimen during the full episode of FN, even if the modification occurred late in the treatment period [Gaya, *et al.,* 1975].
3) Finally, randomization took place using numbered, sealed envelopes and not by computer or by calling a study center. Figure 1 (page 28) describes the distribution of episodes and the main explanations for episodes and patients that were not included [Gaya, *et al.,* 1975; EORTC, *et al.,* 1977].

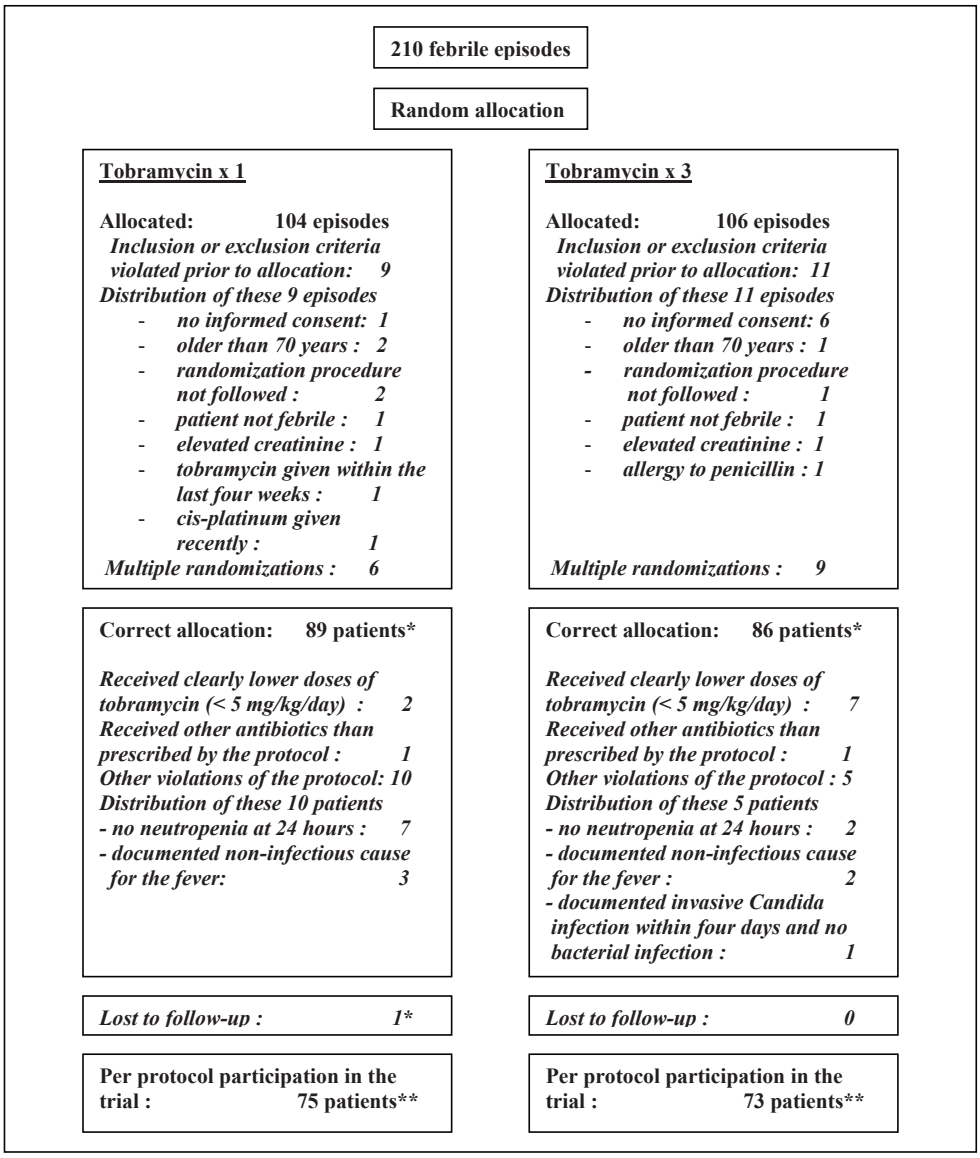
The Tobramycin Trial included patients with "…cancer and febrile neutropenia. Neutropenia was defined as a neutrophil granulocyte count ≤ 0.5 x 10^9/L, and it had to be present twenty-four hours after randomization at the latest. Fever was defined as a temperature ≥ 38.0° C. Age limits were sixteen to seventy years. A signed, informed consent was mandatory" [Torfoss, *et al.,* 2007]. For practical reasons the temperature requirement was modified to one measurement ≥ 38.5° C, or two measurements ≥ 38.0° C over a time period of two hours.

"Exclusion criteria were: allergy to penicillins or to aminoglycosides, serum creatinine level >100 µmol/L in women, or >120 µmol/L in men, massive ascites, neurogenically decreased hearing or a vestibular dysfunction, treatment with nephrotoxic medications, such as cis-platinum, treatment with an aminoglycoside within the four weeks prior to randomization, parenteral antibacterial therapy within the last four days, pregnancy or nursing, or a hemodynamically unstable condition" [Torfoss, *et al.,* 2007].

Metronidazole therapy for anaerobic infections including *Clostridium difficile* toxin associated diarrhoea, trimethoprim-sulfamethoxazole (TS) given for *Pneumocystis jiroveci* pneumonia prophylaxis, and antiviral- and antifungal therapy were allowed. For practical reasons, patients who did not tolerate TS and who took dapsone instead, were equvally accepted for inclusion.

FN cancer patients constitute a heterogeneous group of patients [Klastersky, *et al.*, 2000]. Assuming that the outcome would vary among the participating cancer patients, we decided to randomize the FN episodes within each of three strata, defined as:
1) Episodes with leukemia undergoing induction or consolidation chemotherapy. This is intensive chemotherapy with an expected long duration of neutropenia.

Figure 1 The randomization process of The Tobramycin Trial and the distribution of FN patients



** The 174 evaluable patients are the patients with correct allocations (89 + 86 = 175) minus the one patient lost to follow-up. ** Among the correctly allocated patients, several patients had different kinds of protocol violations, leading to 148 (75 + 73) patients who remained included according to all the protocol criteria.*

2) Episodes with lymphoma undergoing high dose chemotherapy with autologous stem cell support, for short called high dose chemotherapy (HDT). This is also a stratum of patients with an expected duration of neutropenia of more than a week.
3) A stratum of other episodes with cancer, including leukemia and lymphoma episodes, receiving other anti-cancer regimens than the ones described above.

To avoid logistic problems in the randomization procedure, the different centers could chose to include episodes into only one or two of the stratification groups. No center randomized FN episodes into all three stratification groups.

Before starting the trial, our statistician estimated the needed sample size based on the following assumptions:

- 15 % of the episodes were estimated to be non-evaluable.
- The probability of a beneficial outcome, defined as ”No modification”, was estimated to be 30 to 50 %. The definition of the primary end point was ”No modification” of the initial, randomized antibiotic regimen during the episode of FN. Besides no modification of the antibiotic regimen, except for the above-mentioned exceptions, “No modification” included a sterile blood culture within 24 hours of the first positive blood culture, and survival until the patient had recovered from the FN episode. We followed the patients for until 30 days after randomization. This resulted in an estimated need for 210 randomized episodes.

For logistic reasons, not all the patients with a positive blood culture had the second blood culture drawn approximately 24 hours after the first blood culture. This was accepted, and only participants with a second blood culture were evaluated with regard to a possible positive second blood culture.

The trial started at The Norwegian Radium Hospital in the fall of 2001, and it was gradually expanded to include more centers in order to recruit the necessary 210 estimated episodes of FN.

We used block-randomization to secure a balanced distribution of tobramycin once daily and three times daily in all the strata of FN episodes (diagnosis and planned therapy on the one side, and randomization center on the other side) participating in the trial [Guyatt, *et al.,* 2008]. Numbered, computer-prepared, sealed envelopes in stacks of 24 were prepared at the study secretariat for each stratum at The Department of Clinical Research Support at The Oslo University Hospital; in 2001, Kontoret for Klinisk Forskning, at The Norwegian Radium Hospital.

3.3 Material and methods of Paper 3

The mild inflammatory response in febrile neutropenic lymphoma patients with low risk of complications is more pronounced in patients receiving tobramycin once daily compared with three times daily

Patients in The Tobramycin Trial [Torfoss, *et al.,* 2007] at The Norwegian Radium Hospital gave their informed consent to collect extra blood samples in order to study inflammatory markers when the clinical trial was completed. Serum and EDTA-plasma samples from 61 patients in the HDT arm were collected and frozen at - 70°C.

Sample one was collected when the FN episode started, but before the first dose of antibiotics was given (serum and, or EDTA-plasma were collected in 53 to 56 patients; depending on the laboratory test performed, Table 2, Paper 3).

Sample two was collected one to two days later (median 24 hours; range 16 to 56 hours later), when the first tobramycin serum concentration sample was collected (serum and, or EDTA-plasma were collected in 60 to 61 patients; depending on the laboratory test) according to the protocol.

We examined the following markers:

C-reactive protein (CRP) was examined in EDTA-plasma and determined by a high-sensitive particle-enhanced immunoturbidometric assay (Roche Diagnostica, Mannheim, Germany) by technical biochemistry personnel at the Department of Medical Biochemistry at The National Hospital, Oslo University Hospital.

PCT was determined in EDTA-plasma by the BRAHMS PCT-sensitive KRYPTOR Model F Mono Cavro (Brahms Diagnostica, Hennigsdorf, Germany) at The Norwegian Radium Hospital, Oslo University Hospital, in cooperation with technical biochemistry personnel.

The C3 complement activation product C3bc and the terminal, soluble C5b-9 complex (TCC) were quantified in EDTA-plasma using enzyme-linked immunosorbent assay (ELISA) at The National Hospital, Oslo University Hospital.

Mannose-binding lectin (MBL) was quantified in EDTA-plasma by a double antibody ELISA at The National Hospital, Oslo University Hospital.

A 17-kit cytokine assay was examined in serum with Bioplex cytokine assays (Bio-Rad Laboratories, Hercules, CA, USA) at The Norwegian Radium Hospital.

Table 2 Normal values* and cytokine values (according to Biorad, CA, USA). Modified from Table 2, Paper 3

Analyses	Normal values and Cytokine values from Biorad	Samples	Sample 1	
			Median	Range
CRP	**< 10**	55	**16**	**1 - 197**
PCT	**< 0.5**	55	**0,13**	**0,05 - 0,89**
C3bc	**< 20**	53	**49**	**14 - 163**
TCC	**< 1.0**	53	**0,7**	**0 - 8,2**
MBL	**< 100 = deficient < 400 = decreased**	53	**2603**	**0 - 7772**
IL-1β	**undetectable -15**	56	**0,7**	**0 - 341**
IL-2	**< 7**	56	**0**	**0 - 10**
IL-4	**undetectable - 13.1**	56	**0**	**0 - 1,6**
IL-5	**< 5**	56	**309**	**2 - 4057**
IL-6	**undetectable - 8.5**	56	**61**	**6 - 513**
IL-7	**0.27 - 8.7**	56	**3,0**	**0 - 14,0**
IL-8	**undetectable - 47**	56	**31**	**7 - 305**
IL-10	**< 1**	56	**0,1**	**0 - 22**
IL-12	**40.4 - 150**	56	**0,3**	**0 - 16**
IL-13	**undetectable - 79**	56	**0**	**0 - 6,6**
IL-17	**< 31**	56	**0**	**0 - 0,74**
G-CSF		56	**135**	**0 - 1845**
GM-CSF	**< 3.9**	56	**0**	**0 - 40**
INF-γ	**undetectable - 5.6**	56	**9**	**0 - 119**
MPC-1	**26.9 - 375.5**	56	**316**	**83 - 2797**
MIP-1β	**24 - 211**	56	**142**	**58 - 425**
TNF-α	**undetectable - 20**	56	**8**	**0 - 86**

** Units are for CRP: mg/L, for PCT: µg/L, for C3bc and TCC, AU/mL, for MBL: µg/L, and for the different cytokines: ng/L. For other comments, see Table 2, Paper 3.*

The 17 cytokine kit contained interleukin (IL) IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, granulocyte colony-stimulating factor G-CSF), granulocyte macrophage-CSF (GM-CSF), interferon γ (INF γ), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1β (MIP-1β), and tumor necrosis factor α (TNFα).

No conventional normal values have been established for the 17 cytokines. Another Norwegian research team surprisingly found serum concentrations below the detection level in several of the cytokines they examined. They then asked the manufacturer if normal values existed. The research department at Biorad had, in fact, suggested a set of normal values, but these values had not been formally validated [personal communication, Tor Erling Lea, Norwegian University of Life Sciences, Department of Chemistry, Ås, Norway; 2011]. These manufacturer-based normal values are presented in the Table 2 page 31 above.

The set of inflammatory markers was analyzed for the whole series of FN patients, (Table 2, Paper 3), and then the results were compared between the two trial arms (Table 3, Paper 3).

4. Brief summary of the results

4.1 Paper 1

The Norwegian experience with penicillin G plus an aminoglycoside as initial empiric therapy in febrile neutropenia; a review

The occurrence of antibiotic resistance and the use of broad-spectrum antibiotics are relatively low in Norway [NORM/NORM VET, 2011; Goossens, *et al.*, 2005]. The national and local recommendations for antibiotic therapy in FN and in sepsis of unknown origin are to start with penicillin G plus an aminoglycoside [von der Lippe, 2001; NORM, 2011; Torfoss and Høiby, 2010]. There are reasons to assume that this regimen contributes to the prevention of antibacterial resistance [de Man, *et al.*, 2000; Gammelsrud, *et al.,* 2011].

In a situation with increasing world wide antimicrobial resistance and few new antibacterial drugs being developed [Newton, 2005], the initial choice of penicillin G plus an aminoglycoside may be one of the strategies to keep antibacterial drugs effective for future patients [Leibovici, *et al.,* 2011].

Seven studies were reviewed of which, unfortunately, only four have been published in international peer-reviewed journals. Clinical outcomes and, or the sensitivities of the blood culture isolates for penicillin G and for the actual aminoglycosides, were the results reported in the studies. A summary of the five studies that reported the clinical outcome (781 episodes) in episodes treated with penicillin G plus an aminoglycoside, demonstrated that 53 % of the episodes had antibiotic modifications during the episodes of infection. The overall success rates (survival) were 90 - 100 % when episodes where the antibiotic regimen had been modified were included. The total case fatality rates after the onset of infection were 5 - 10 %, and the case fatality rates related to the initial therapy with penicillin G plus an aminoglycoside were 0 - 1 %. The number of days before the antibiotic modifications took place was only reported in one trial [Torfoss, *et al.,* 2007]. In this trial, the mean time interval to modification was five days. In comparison, the fourth EORTC trial found a median time to modification of more than six days.

Four studies reported the microbiological sensitivities of Gram-positive bacteria (314 isolates) toward penicillin G. It was 49 %. Sensitivity toward aminoglycosides was 57 %. Unfortunately, no information on the combined sensitivities was reported. For the Gram-negative bacteria (308 isolates), sensitivity toward the relevant aminoglycoside (netilmicin, gentamicin or tobramycin) was 99 %.

These two qualitatively different outcomes could not be unified into one parameter, even though there might be an association between the two. Still, together they give a reasonable picture of the microbiological epidemiology, and of the clinical outcomes, when choosing penicillin G plus an aminoglycoside in patients with malignant disease and fever. The majority of the cases suffered a hematological malignancy. The number of patients who were in septic shock was only reported in one trial [Torfoss, *et al.,* 2007], which specified that hemodynamically unstable patients were excluded from participation in the trial. Medical tradition in Norway has been to provide patients in septic shock with double Gram-negative antibiotic coverage [Torfoss and Høiby, 2010]. Septic shock is relatively unusual in FN [Klastersky, *et al.,* 2000], therefore hemodynamically unstable patients were probably relatively unusual among the patients included in the seven studies in this report.

An additional comment to these results is the similar conclusions of all the seven studies*: initial therapy with penicillin G plus an aminoglycoside was efficacious and safe, provided the regimen was modified when the clinical development was not satisfactory.*

4.2 Paper 2

Tobramycin once versus three times daily, given with penicillin G, to febrile neutropenic cancer patients in Norway: a prospective, randomized, multicentre trial

Two hundred and ten episodes were included and randomized into one of the two main study arms between 2001 and 2005. During the study, our statistician and others forwarded strong arguments that patients should only be included once; as was also later recommended by Guyatt and coworkers [2008]. Accordingly, we decided to only present the results of the first randomizations. A total of 174 patients were considered evaluable.

The overall result was that 40 % of the evaluable patients in both study arms had ”No modification” of the antibiotic regimen. Furthermore, the two arms did not differ with regard to toxicity.

The mean time until treatment modification was five days; with no difference between the two arms. Modifications during the first three days occurred in 18 patients (10 %); the same as in the fourth EORTC trial [EORTC, 1987].

Thirty-one (18 %) of the 174 evaluable patients had a positive blood culture. This was slightly lower than the combined results from the four EORTC trials. This may be due to the fact that patients in suspected septic shock (hemodynamically unstable patients) were excluded from our trial. Patients in septic shock were routinely treated with a broad-spectrum β-lactam antibiotic plus an aminoglycoside (double Gram-negative coverage). Otherwise, our distribution of the classification of the febrile episodes was similar to most other similar clinical antibiotic trials in FN.

With regard to nephrotoxicity, the mean increase in serum creatinine was only seven µmol/L (95 % CI: 6 – 9 µmol/L) with no difference between the two arms. Ten patients, five in each arm, had an increase of more than 50 % from their baseline creatinine serum concentration [Mavros, *et al.*, 2011]. The highest absolute elevation in serum creatinine in any one patient was 47 µmol/L. No other serious adverse events were reported.

All our FN patients survived 30 days after the randomization; except for one patient who expired 29 days after randomization, several weeks after the HDT discharge. She died from a suspected respiratory infection (further described in the Discussion, page 47).

4.3 Paper 3

The mild inflammatory response in febrile neutropenic lymphoma patients with low risk of complications is more pronounced in patients receiving tobramycin once daily compared with three times daily

The third of our aims was to characterize the inflammatory response in FN patients and evaluate if any of the inflammatory markers examined could be sensitive and specific enough to influence the therapeutic decisions in initial FN. However, as all the participants turned out to be patients with low risk of complications, we were

unable to make any conclusions as to our hypothesis. “The results reflect only the situation in patients with a benign course of FN, and they say nothing about the inflammatory response in patients with Gram-negative sepsis or a more severe course of FN” [Torfoss, *et al.,* 2011].

Sixty-one of the HDT patients from The Norwegian Radium Hospital participating in The Tobramycin Trial had given blood samples that were analyzed. The patients constituted a clinical homogeneous group with a benign initial course. These 61 patients remained neutropenic for a median time of 10 days (range 5 - 16 days). Thirty-two patients received tobramycin once daily and 29 patients three times daily. Four patients had a positive blood culture; three grew Gram-positive viridans streptococci and one a coagulase-negative staphylococcus.

The analyses revealed a mild increase in the proinflammatory cytokines in both samples, as well as from the first to the second sample.

CRP was a non-specific parameter in the assessment of the severity of the clinical condition.

PCT results were in accord with previous research that has shown a value < 0.5 µg/L to be a strong, negative predictor for bacteremia [Christ-Crain and Muller, 2005].

The complement activation factors showed a mild increase; this supported the notion of a mild sterile inflammation.

MBL values were deficient in five patients, and decreased in five other patients. All of these patients had a clinical course similar to the other patients. However, one of the patients with a decreased MBL level was the one who died 29 days after randomization (see page 47).

The 17 cytokines showed a mild increase in the proinflammatory cytokines from the first to the second sample (primarily IL-6, IL-8, INF-γ, and TNF-α); supporting the notion of a mild sterile inflammatory response. The only cytokine with a significantly reduced concentration in the second sample compared to the first sample was IL-5. This cytokine is associated with eosinophil activation [Sanderson, 1992], allergies and a TH 2 cytokine pattern [Upadhyaya, *et al.,* 2011].

When comparing the results of the two tobramycin arms, most of these results did not differ significantly. However, there may have been a trend that the patients who had received tobramycin once daily had higher proinflammatory cytokine values compared to those who had received tobramycin three times daily. This trend may be

difficult to evaluate since the interindividual differences may be substantial [Wong, *et al.,* 2008].

We concluded that none of the inflammatory markers in this series of FN patients correlated better with the clinical outcome of the FN episode than daily and thorough clinical examinations.

5. Discussion

5.1 Evaluation of the three papers

The main strength of this thesis is the fact that we conducted a clinical RCT and found and documented that penicillin G plus tobramycin was effective and safe as initial, empiric therapy in hemodynamically stable FN patients (Paper 2) [Torfoss, *et al.,* 2007].

The focus of the trial was tobramycin once versus three times daily. As most similar trials, including the four EORTC trials, this was an open-label trial. Once the randomization envelope was opened, the treatment regimen was known to the physicians taking care of the patient, including the physician who made the decision of antibiotic modification, the critical event that could alter the outcome of the trial for that patient.

The results of the study were similar in the two randomized treatment regimens in all the different arms and subgroups of patients who took part in the trial, as documented in Tables 3 and 4, Paper 2. This supported the assumption that there was no major bias to modify the antibiotic regimen depending on the randomization arm of the patient. The conduction of the trial at The Norwegian Radium Hospital gave the principal investigator, who was also the local investigator, the impression that the decisions to modify the antibiotic regimen depended on the clinical conditions of the patients, based on the clinical traditions at the institution; and not on the fact that the patient took part in a clinical trial evaluating the initial antibiotic regimen administered to the patient. It is reasonable to assume that the situation have been similar at the other participating institutions.

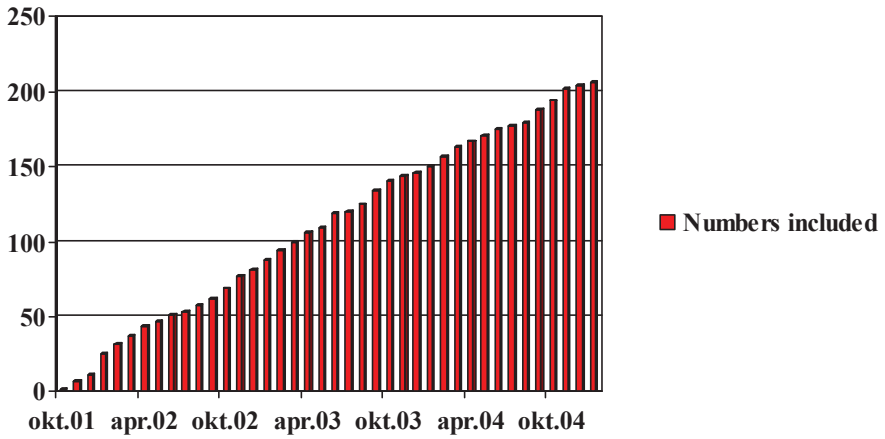
At the closure of the trial, 100 of the 210 included episodes were randomized at The Norwegian Radium Hospital. The rest of the episodes were randomized in various numbers (range 1 – 39) at the other participating institutions.

Our experience from The Norwegian Radium Hospital was that the stack of envelopes and the equipment necessary for inclusions and randomizations (informed consent-forms, new empty CRF-forms etc.) needed to be checked almost every day. A method that worked well consisted of giving the necessary information about the

study to the doctors coming on call at the daily afternoon report meeting. However, this was labor-intensive and required a persistent dedication from the local investigator.

In the end, we were able to complete the trial as planned. The measures of gradually recruiting new centers into the trial and the monthly bulletin sent to all the local coordinators made it possible to keep a quite constant randomization rate; as is shown in Figure 2 below.

Figure 2 Accumulated number of randomizations per month in The Tobramycin Trial



The four EORTC trials focused on the antibiotic response in patients with Gram-negative bacteremia. In our study, 31 patients (18 %) had bacteremias. In the once daily arm there were 13 bacteremias, and in the three times daily arm, there were 18 bacteremias. Four Gram-negative bacteremias occurred in the once daily arm, and two of those (50 %) had a successful “No modification” outcome. Seven Gram-

negative bacteremias occurred in the three times daily arm, and none of them had a "No modification" outcome. These numbers are small, but they may at least support the hypothesis of the trial. Tobramycin once daily is at least as efficient as tobramycin three times daily.

Eight of the eleven participating centers randomized ≤ 12 episodes of FN each. We therefore decided not to present results for the individual centers. The difference in successful "No modification" between the intensive leukemia stratum (in total 40 %) that was randomized at several different centers, and the HDT lymphoma stratum (in total 16 % "No modification") that was only randomized at The Norwegian Radium Hospital, can only be explained by different clinical traditions. More than 20 oncologists participated in the on-call pool at this institution; most of them worked with solid tumor patients who did not have neutropenia of long duration. Therefore, since HDT was initiated at this institution 20 years ago, there has been a consistent practice to inform the on-call team with detailed suggestions on how to handle HDT patients with probable deteriorating clinical situations. This information has been given both orally, in a clinical discussion at the afternoon report meeting, and as a written report in the patients' charts. Still, when an HDT patient had persistent fever during a night- or weekend shift of an oncologist who did not work daily with lymphoma patients, the oncologist might have had a tendency to modify the antibiotic regimen sooner than our more experienced lymphoma-oncologists. This happened in spite of repeated suggestions of starting the diagnostic work-up of fever during the on-call period, and to await antimicrobial modification until the patient's regular doctor would take care of the patient the next day.

The study of Tangen [Tangen, *et al.*, 1999] observed the same phenomenon. Similar patients with leukemia and FN receiving penicillin G plus an aminoglycoside had a "No modification" rate of 48 and 50 % at two of the three participating centers whereas the "No modification" rate was only 8 % at the third center. The reason for these differences must be other than purely medically based.

In our RCT, "The patients were stratified by study center and by three combined diagnosis and treatment groups: (i) acute-leukaemia-receiving-intensive-chemotherapy; (ii) malignant- lymphoma-undergoing-intensive-chemotherapy-with-autologous-stem-cell-support; (iii) other-cancer-patients, including leukaemia and lymphoma patients not receiving the above mentioned therapies. Block randomization was done by the computer using random block sizes of four and six

episodes and presented at the participating centres in sealed, consecutive, pre-numbered envelopes. The envelope with the lowest number was drawn when an eligible patient developed febrile neutropenia" [Torfoss, *et al.,* 2007].

The plan behind this stratification strategy was to study patients with different expected risks of complications, mainly based on different expected durations of neutropenia [Guyatt, *et al.,* 2008]. The stratum of "other" patients was planned to consist of patients with solid tumors and short periods of FN. However, most of the "other" patients (47 of 66) turned out to be leukemia and lymphoma patients who had received other anti-cancer chemotherapy regimens than the ones specified as initiation and consolidation chemotherapy in leukemia patients and HDT in lymphoma patients. Some of these anti-cancer chemotherapy regimens were followed by long periods of neutropenia.

Even though the total results would not have been affected by a different stratification strategy, we might have found a somewhat different distribution of the results if we had used two stratification groups, one with an expected long period of neutropenia and one with an expected short period. The low rate of "No modification" at The Norwegian Radium Hospital would have been discovered by combining the results from each participating institution and the reported diagnostic code.

We recorded time to defervescence in episodes with a successful "No modification" outcome. There was no difference between the two arms. A preliminary Kaplan-Meyer plot of time to defervescence in patients with a "No modification" outcome, showed two almost identical curves (results not shown). "The highest mean serum creatinine after therapy with tobramycin (n = 172) was 69 µmol/L (95% CI: 66 - 71 µmol/L) and the mean increase in serum creatinine (n = 169) was seven µmol/L (95% CI: 6 - 9 µmol/L), with no difference between the two arms. The highest absolute elevation in serum creatinine was 47 µmol/L. No ototoxicity or other side effects were reported" [Torfoss, *et al.,* 2007]. However, no audiometry analyses were performed. We tried to do audiometry at The Norwegian Radium Hospital when we started the study, but after a short time we realized that the logistics did not work in the busy pre-HDT period of these patients, so the protocol was amended and the requirement for audiometry was suspended.

A metaanalysis on nephrotoxicity in FN patients who received aminoglycoside once or multiple times daily was published in 2011. At the request of the researchers of this metaanalysis, we calculated the number of patients who had an increase in

baseline serum creatinine of more than 50 %; and we found this to be ten patients [Mavros, *et al.,* 2011]. Our contribution was acknowledged at the end of their publication.

As a conclusion to the question of how to monitor aminoglycoside serum concentrations, when aminoglycosides are administered once daily, in a dose of at least 6 mg/kg, the peak serum concentration will almost always be > 12 mg/L. For all practical purposes this is > 10-12 times the MIC of most the common bacterial isolates found in bacteremias of FN patients in Norway [Craig, 1998]. The 2011 survey of serum aminoglycoside concentrations at The Norwegian Radium Hospital found a median peak concentration of 16.0 mg/L (range: 8.1 to 32.4 mg/L). Unfortunately, lower doses than six mg/kg are still occasionally administered, probably explaining the lower end of this range interval. A similar survey at The Norwegian Radium Hospital from before The Tobramycin Trial (in 2001), when aminoglycosides were administered three times daily, showed that 25 % of the peak concentrations were < 8 mg/L. When dosing the aminoglycoside once daily, the only monitoring that usually remains important is the trough serum concentration to avoid accumulation of aminoglycosides with risk of nephrotoxicity and ototoxicity.

Other outcomes of The Tobramycin Trial were less labor-intensive treatment, administering aminoglycosides once daily compared to three times daily. During the trial a couple of experienced nurses were observed, and the time they used to prepare and administer a dose of tobramycin was found to be approximately 15 minutes. Collecting samples for trough and peak serum concentrations in addition to the administration of the aminoglycoside dose, in general required one hour.

In The Tobramycin Trial, we changed the decision of the evaluability of the patients, specifically to include only the first episode of FN in the patients who were randomized several times. This was done before the results were analyzed. Originally, patients could participate in the study several times, as had been the practice in the EORTC trials [Gaya, *et al.,* 1975; and EORTC, 1977]. Before analyzing the results we decided that only values from the first episode of FN should be included in the analyses. The reason for this is that it is known that patients who have had one episode of FN have a higher risk of FN after the next anti-cancer chemotherapy regimen [Aapro, *et al.,* 2011]. This caused 15 episodes in 11 patients to be withdrawn as non-evaluable. Altogether 17 % (36 FN episodes) ended up being non-evaluable (see Figure 1, page 28).

A total of 210 episodes of FN in 199 patients were randomized. More than one randomization occurred 15 times. The 15 “double or triple” randomizations is thus explained by seven patients randomized two times and four patients randomized three times. This comes down to seven episodes in the seven patients randomized twice and eight episodes in the four patients randomized three times. Seven plus eight makes the 15 episodes that were randomized more than once.

As Figure 1 explains, a total of 174 patients were considered evaluable. The results for these 174 patients were erroneously presented as an intention-to-treat (ITT) analysis [Torfoss, *et al.,* 2007]. The need to correct the use of the word “ITT” was first discovered when this thesis was written. We here present an analysis based on a review of the available study material. Unfortunately, some of the local investigators did not fill in the case report forms (CRF) when they realized that the patient would not be evaluable. Because of this, we are only able to present results for 196 of the 199 patients. In total, 83 of 196 patients (42 %) had a recorded “No modification” outcome. In the tobramycin once daily arm 44 of 101 patients (44 %), and in the tobramycin three times daily arm 39 of 95 patients (41%) had a successful “No modification” outcome. We conclude that this analysis shows results similar to the analysis of the 174 published “evaluable” patients (Paper 2). *Tobramycin once daily is as effective and safe as tobramycin three times daily, given with penicillin G, as initial, empiric therapy in FN cancer patients.*

Paper 1. The seven Norwegian studies consisted of two RCTs and five observational studies. The first RCT [Frøland, *et al.,* 1989] was conducted in the 1980s in a single institution. They compared episodes of FN treated with penicillin G plus netilmicin versus ceftazidime monotherapy. Their hypothesis was that ceftazidime monotherapy was as effective as penicillin G and netilmicin. They found that there was no statistical difference neither between the patients nor between the two antibiotic regimens.

In the 1980s, the question of whether broad-spectrum β-lactam monotherapy was as effective as a combination of a broad-spectrum β-lactam plus aminoglycoside was raised. This question was also the focus of the forth EORTC trial, where ceftazidime given with a long (nine days) course of amikacin had a significantly higher success rate than ceftazidime given with a short (three days) course of amikacin [EORTC, 1987]. However, the belief in the fourth EORTC trial seemed to be strong that

treatment with an aminoglycoside for at least some days was necessary. And, as we have seen, the addition of an aminoglycoside has benefits [Klastersky, 1990], and not only negative side effects.

The initiative by Frøland and coworkers to compare the Norwegian regimen with the narrow-spectrum β-lactam, penicillin G plus netilmicin was both innovative and important at the time, both with regard to the Norwegian antibiotic tradition, and as an argument for penicillin G plus an aminoglycoside in the international discussion, even though the hypothesis of their trial was to examine if ceftazidime monotherapy was a desirable antibiotic regimen in Norway. Unfortunately, since the study of Frøland and co-workers was never published in a peer-reviewed journal, it did not get international scientific attention. Today, unfortunately, it is difficult to find the document from the symposium. The complete manuscript was written in Norwegian in the Glaxo-sponsored Norwegian Ceftazidime-Symposium publication (Norsk Ceftazidim-symposium) held in 1989 [Frøland, *et al.,* 1989].

Many international studies with a broad-spectrum β-lactam with and without aminoglycosides were undertaken at about the same time. This resulted in broad-spectrum β-lactam monotherapy becoming accepted as the international standard of care. Especially monotherapy with ceftazidime became the drug of choice at many institutions, based on a long list of RCTs where ceftazidime was compared to different other broad-spectrum regimens. A Pub-Med search based on the key words “febrile neutropenia” and “randomized clinical comparison with ceftazidime” gave 18 hits. Five studies in which ceftazidime monotherapy was compared with other different antibiotic regimens, conducted before the year 2000, concluded that ceftazidime was as effective as the other regimens consisting of one of the following antibiotics: ciprofloxacin, cefepime, imipenem, or piperacillin [Bayston, *et al.,* 1989; Perkkiø, *et al.,* 1990; Dranitsaris, *et al.,* 1995; Freifeld, *et al.,* 1995; and Aparicio, *et al.,* 1996]. One trial published after the year 2000 found cefepime equivalent to ceftazidime monotherapy [Kebudi, *et al.,* 2001]. The other publications were a review [Paul, *et al.,* 2010] and trials where ceftazidime was given with an aminoglycoside. In one study, ceftazidime was compared with trimetoprim-sulfamethoxazole plus amikacin. In only one trial ceftazidime monotherapy came out slightly inferior (38 % success) to imipenem (50 % success) [Rolston, *et al.,* 1992].

In conclusion, ceftazidime was considered as effective as any other broad-spectrum β-lactam. This gave an unfortunate credit to ceftazidime as the drug of

choice for empirical therapy in FN. The problem was that ceftazidime was one of the few antibiotics available as treatment for pseudomonas infections. Ceftazidime should therefore be reserved for treatments of proven or suspected pseudomonas infections. When ceftazidime is regularly used in FN as the drug of empirical choice we risk loosing ceftazidime as a tool for dealing with often difficult to treat pseudomonas infections because of increasing resistance,

A Danish study [Hansen, *et al.,* 1988] that evaluated aminoglycoside dosing once or three times daily, added to our evidence base before we started The Tobramycin Trial. This RCT used a today relatively narrow-spectrum β-lactam, the second generation cephalosporin, cefuroxime, along with netilmicin. Sixty-four episodes of suspected septicemia in 56 immunocompromized patients (mainly hematological cancer patients), were analyzed. They found that patients given netilmicin once daily defervesced sooner than those given netilmicin three times daily. Otherwise the outcomes were similar. Cefuroxime is no longer a very effective Gram-negative antibiotic agent. In general, its MIC values for the common Gram-negative bacilli are ten to 30 times higher than the MICs of cefotaxime [Andes and Craig, 2010].

Results from another Danish study [Freundlich, *et al.,* 2007] have supported the findings of the seven Norwegian studies. It was a retrospective analysis based on patients who had received appropriate antibiotic therapy for registered bacteremias. It found that the cumulative 30 day case fatality rate was 17 % in an aminoglycoside plus a β-lactam cohort (the β-lactams most used were penicillin G, ampicillin and cefuroxime) and 18 % in a non-aminoglycoside cohort (in most episodes an aminopenicillin or cefuroxime were given as monotherapy). The editorial commentary to this study [Leibovici and Paul, 2007] concluded, ”In countries where the resistance is low enough to use ”old” β-lactams, and there is an unwillingness to use broad-spectrum β-lactams, evidence for the efficacy of combination treatment and for its role in keeping the resistance at a low level is wanting.” Northern Europe, including Scandinavia, belongs to those countries. However, on the background of changing epidemiology, such evidence is only valid for the time the study took place, unless close surveillance of clinical results and antibiotic resistance is carried out continuously. However, it was important that a famous international peer-reviewed journal like The Journal of Antimicrobial Chemotherapy for the first time in history became the international “spokesperson” for a policy that was sympathetic toward the restrictive Scandinavian antibiotic traditions.

Paper 3. The study of inflammatory markers. FN is a heterogeneous condition; but most of the patients with FN belong to the group of patients with little risk of complications [Klastersky, *et al.*, 2000]. Thus, even though the patients in the Paper 3 study are not representative for all patients with FN, they still belong to the large group of low risk lymphoma HDT patients who can be identified by a MASCC-score ≥ 21.The results from this study of low risk patients may serve as a useful comparator for similar studies in the future.

Our conclusion and suggestion in the headline of the paper, that this mild inflammatory response is more pronounced in patients receiving tobramycin once daily compared to three times daily, cannot be more than a suggestion of a possible trend.

We measured inflammatory markers, cytokines and MBL in blood samples from our patients. It is worth remembering that other possibilities of bias also may be important. The values we found in the blood samples were not necessarily the same values that might have been found at the local infection sites of infection. Still, in FN with bacterial infections, the site of infection may primarily be systemic, even though the origin of the infection will always be local. In FN, oral mucositis, local central venous catheter infections, and infections originating in the colonic part of the gastrointestinal tract are the most common local sites for the origin of systemic bacterial infections. However, it may be almost impossible to measure inflammatory markers at these sites. Blood cultures will almost always be the surrogate sample for the early site of infection, and the samples available for early, rapid analysis.

Another consideration of possible bias may be the handling of the samples before the analyses. Our experience at The Department of Medical Biochemistry at The Norwegian Radium Hospital is that this variation may be more important than previously estimated [personal communication, Trine Bjøro and Nils Bolstad, June 2012]. Different handling of the samples may affect the results of the individual samples. In our study, however, all the samples were handled simultaneously and in the same way, and all the serum and plasma samples were frozen within two hours after collection. This should suggest a reasonably similar treatment of the samples. But, in order to be able to prepare and conduct analyses in several different laboratories, the samples had to be thawed and refrozen more than once.

Finally, it may seem to be a paradox that inflammatory markers increase in neutropenic patients. With fewer leucocytes to produce the inflammatory markers, the

measured levels should be low. But the true biological reality may be different. There are many cell types not belonging to the hematological cells that produce inflammatory markers. In fact, almost all cell types in the body possess the property to produce inflammatory markers. One example are epithelial cells, especially the lining cells of the gastrointestinal tract [Sharma, *et al.,* 2010], and the endothelial cells of the vasculature. Fibroblasts [Kato-Kogoe, *et al.*, 2010], dendritic cells, Kuppfer cells in the liver, Sertoli cells of the testes, and melanocytes are other examples of cells that produce inflammatory markers [personal communication, Pål Aukrust, Oslo University Hospital, June 2012].

Sixty of the 61 patients survived through day 30 after randomization. The one patient, mentioned before, with a reduced level of MBL (326 µg/L), unfortunately died 29 days after randomization. During her HDT, all blood cultures and other microbiological tests were negative. She felt well and was in her normal state of health when she was discharged to home only eight days after randomization. Twelve days later she noticed fever and cough. The condition quickly deteriorated. Eight days later she was intubated and transferred to the regional medical center with a clinical picture of adult respiratory distress syndrome. She expired the next day. A post-mortem examination did not reveal any microbiological organisms as the cause of her fatal outcome. However, she had received broad-spectrum antibiotics. We do not know if the decreased MBL values that were found in her case contributed to the rapidly fatal course she experienced; still, this cannot be ruled out.

Several studies point at an increase in the risk of different serious infections in immunocompromised hosts with deficient or decreased MBL levels [Dommett, *et al.,* 2006; and Peterslund, *et al.,* 2001]. The role of MBL in the innate immune response still needs elucidation; however, opsonization impairment and defects in the complement response are probably among the major mechanisms involved, when reduced levels of MBL occur together with other immunodeficiencies like status after anti-cancer chemotherapy [Dommett, *et al.*, 2006].

5.2 Aminoglycosides: synergy and antimicrobial resistance

The synergy between an aminoglycoside and a cell wall active antimicrobial agent is a desirable interaction that has several implications. The mechanism of the aminoglycoside's synergistic activity with other antimicrobial agents may not be the same for all target organisms. Still, increased uptake of the aminoglycoside in the presence of a cell wall active agent seems to be important, at least for the action against enterococci, viridans streptococci, *Staphylococcus aureus*, and *P. aeruginosa* [Gilbert and Leggett, 2010].

The positively charged cationic aminoglycosides bind both to the negatively charged RNA backbone of the ribosomes, to the Gram-negative cell wall lipopolysaccarides (LPS), to the cell membrane phospholipids, and to other anionic molecules. For example, cationic aminoglycosides interact chemically with anionic β-lactam antibiotics with mutual loss of antibacterial activity. This reaction is not very rapid. It occurs molecule to molecule, and it requires several hours to be of importance. Because of the 40-fold difference in serum concentrations, there is no important reduction in the β-lactam concentration. However, there may be a 10 – 20 % reduction in the aminoglycoside serum concentration. Therefore, the recommendation in Mandell's textbook is that the aminoglycoside should always be administered first [Gilbert and Leggett, 2010].

Aminoglycosides work fast and it is the initial peak serum concentration that is important. This knowledge may not be adequately appreciated. The third EORTC trial [Klastersky, *et al.,* 1986] stated explicitly, for unknown reasons, that the β-lactam should be administered before the aminoglycoside. Neither were we aware of this phenomenon; the protocol of The Tobramycin Trial (Paper 2) did not specify the sequence of administration of the aminoglycoside and the penicillin G.

A telephone survey to 22 Norwegian departments of hematology, oncology and infectious diseases in March 2012 revealed that no department had guidelines for the sequence of administration of aminoglycosides and cell-wall active antibiotics. In general, this was left to the discretion of the nurses. Twelve departments reported that they usually gave the penicillin G first, mainly because that was easier and more convenient. One department simply stated that no guideline existed. Seven departments stated that they gave the aminoglycoside first. One reason given for this

practice was that it was more important to give the anti-Gram-negative drug as soon as possible. Two departments at regional medical centers reported that they did not use the combination of aminoglycoside plus penicillin G at all.

There may seem to be a contradiction here. On one side, the presence of the cell-wall active drug appears to improve the uptake of the aminoglycoside into the bacterial cytoplasm. On the other side, the aminoglycoside should be given first to prevent the reduction in aminoglycoside serum concentration by the mutual inactivation of the cationic aminoglycoside and the anionic β-lactam. Which is more important? Aminoglycosides do have an immediate action when it binds to all anionic molecules on and in the bacterium and in its environment, including the lipopolysaccaride (endotoxin) in the bacterial Gram-negative outer membrane, causing a rearrangement of these molecules with a subsequent “bleeding” of the outer membrane, with formation of transient holes in the cell wall that starts the killing of the bacterium and allows the aminoglycoside to enter the microbe’s inner part. One example of this is that empirical monotherapy with an aminoglycoside in urinary tract infections in most cases is the most effective therapy that may be given for this condition with urinary concentrations of the aminoglycoside being 25 to 100 times higher than the corresponding serum concentration [Gilbert and Leggett, 2010]. The β-lactam thus is not mandatory in the initial phase of the aminoglycoside attack, even though the β-lactam would help the aminoglycoside to enter the cell. But also, the high serum concentration of the aminoglycoside, when given once daily, would in most cases be high enough that eventual inactivation would be without clinical significance. Being aware of these two principles should make an individual evaluation possible when the bacterial MIC toward the aminoglycoside is known. Otherwise, the advice should be to follow the recommendation from Mandell’s textbook [Gilbert and Leggett, 2010] to give the aminoglycoside first.

Binding of aminoglycosides to prokaryotic ribosomes is a prerequisite for the drug’s antimicrobial activity. But, the exact mechanism of the cidal activity remains somewhat unclear. Recent work suggests that only bactericidal drugs stimulate hydroxyl radical formation in bacteria [Kohansky, *et al.,* 2007], whatever their antibacterial mechanism may otherwise be.

To understand how the aminoglycoside antibiotics work, and the mechanisms of resistance to aminoglycosides, some further basic in-depth presentation is

necessary. Aminoglycosides bind with high avidity to a region of highly conserved nucleotides in the mRNA decoding region of the 30S subunit of prokaryotic ribosomes. The critical translation of the mRNA codon and anticodon of the aminoacyl-transfer RNA occurs at the A (acceptance) site in the 16S reverse transfer RNA portion of the 30S ribosomal subunit, where two adenine residues are inhibited by aminoglycoside interference. This leads to misreading of the mRNA and effectively stops the polypeptide production [Gilbert and Leggett, 2010].

This mechanism is a key to understanding the differential aminoglycoside effects on prokaryotic and eukaryotic cells; and also one of the bacterial resistance mechanisms toward aminoglycosides. In eukaryotic cells, the corresponding aminoglycoside binding site carries a guanosine instead of the prokaryotic adenosine, making eukaryotic ribosomes almost completely resistant to the effect of aminoglycosides, even though interesting research may prove aminoglycosides useful in therapy of selected genetic human diseases [Gilbert and Leggett, 2010].

Methylating enzymes that modify the 16S rRNA binding site result in high-level aminoglycoside resistance; this mechanism seems to be growing in clinical isolates worldwide. However, the most common mechanism of bacterial aminoglycoside resistance consists of three different aminoglycoside-modifying enzymes that all result in poor ribosome binding, and a high level of resistance [Shaw, *et al.*, 1993]. The genes for these modifying enzymes can be spread by plasmids, sometimes in combination with other resistance genes on mobile genetic elements [Lindemann, *et al.,* 2012], but they may also be chromosomal. For unknown reasons, aminoglycoside resistance seems to be a smaller problem compared to β-lactam and fluoroquinolone resistance in Northern Europe; even though Gram-negative aminoglycoside resistance may reach 60 - 70 % of the clinical isolates in certain parts of the world [Peripi, *et al.,* 2012]. The low frequency of aminoglycoside resistance in our part of the world may be an effect of more appropriate and restricted use of aminoglycosides. However, aminoglycoside resistance also threatens to become a problem in Norway [NORM/NORMVET, 2011; Haldorsen, *et al.,* 2011; and Lindemann, *et al.,* 2012].

Are there any new aminoglycosides with reduced resistance pattern in the pipeline? Amikacin was used in the last three of the four EORTC trials because there was a problem with increasing gentamicin resistance since the first EORTC trial. Since then, amikacin has been the aminoglycoside of choice in many countries. Norway is

one of the few countries where amikacin has not been available; and we have had few problems with gentamicin and tobramycin resistance. If aminoglycoside resistance should become more prevalent, amikacin might still be an option, and it is available in Sweden (Biklin® from Bristol-Myers-Squibb; “registreringsfritak” in Norway). Moreover, the second-generation aminoglycoside plazomicin, currently being evaluated in a phase II trial, is resistant to the aminoglycoside-modifying enzymes. However, it has no clinical effect against bacteria with the 16S rRNA binding site methylating enzymes [Zhanel, *et al.,* 2012].

Low-level aminoglycoside resistance may occur secondarily to drug efflux mechanisms, causing the so-called adaptive resistance. This is a phenomenon described as a transient resistance, occurring after a treatment with an aminoglycoside, when the peak concentration only reaches a moderate value, for example secondary to dosing three times daily. Adaptive resistance follows the rapid, early concentration-dependent killing of susceptible bacteria, and lasts beyond the postantibiotic effective period into the time of regrowth. However, this effect lasts only a few hours. It may be an explanation for our results with higher concentrations of the proinflammatory markers (Paper 3) when the aminoglycoside was dosed once daily; by the hypothetical explanation, that higher number of living bacterial cells on the mucosa of the gastrointestinal tract secondary to lower serum aminoglycoside concentration with dosing three times daily, prevented the increase in the proinflammatory response. This hypothesis is based on the adaptive resistance that seems to disappear with the high serum peak concentrations of once daily dosing [Elisabeth von der Lippe, personal communication, 2011].

Aminoglycoside resistance is even more complex. Exposure of susceptible bacteria to aminoglycosides can select for two types of drug-resistant subpopulations. One is the phenomenon of adaptive resistance. The other results from small colony variants with deficient energy-dependent uptake of aminoglycosides, and may result in clinical treatment failure when the aminoglycoside has been used for a while [Gilbert and Leggett, 2010]. Initial aminoglycoside therapy is one of the most potent and rapidly working antibacterial treatments available. Prolonged therapy with aminoglycosides, on the other side, may cause clinical failures and unwanted side effects. A rule of thumb may be not to give aminoglycosides for more than eight to ten days if it is clinically possible.

5.3 Aminoglycosides and nephrotoxicity

An important question in aminoglycoside research and therapy has been whether once daily therapy versus multiple daily dosing has less renal side effects. We found only a minor increase in serum creatinine; equal in the two arms of our RCT study. Mavros and his co-workers [Mavros, *et al.,* 2011] concluded that the occurrence of nephrotoxicity was similar between two dosing regimens of aminoglycosides, once daily or multiple daily doses, based on seven RCTs with 1643 patient episodes (RR = 0.74; 95 % CI: 0.36 - 1.50), with a trend in favor of once daily dosing.

To understand the risk of severe aminoglycoside nephrotoxicity, studies from the 1970s provide one perspective on this discussion. In studies conducted before the concentration-dependent pharmacodynamic mode of action of aminoglycosides was known [Craig, 1998], gentamicin was often administered four times daily with doses of 0.75 mg/kg in every dose. They aimed at peak serum concentrations of four mg/L [Bodey, *et al.,* 1972; Bodey, *et al.,* 1973; and Schimpff, *et al.,* 1971].

In one study, gentamicin was even administered as a continuous infusion [Keating, *et al.,* 1979], and gentamicin levels were sought kept at four to five mg/L. The patients received gentamicin for a minimum of seven days, or four days after becoming afebrile. Nephrotoxicity was described as azotemia, and major azotemia was defined as an increase in serum creatinine to above 2.5 mg/dL (191 µmol/L) or as an increase in serum BUN (urea) to above 50 mg/dL (18 mmol/L). Major azotemia occurred in 19 % of the patients who had serum concentrations of gentamicin above five mg/L. Minor azotemia "…was usually transient and of no clinical significance" [Keating, *et al.,* 1979]. These results may suggest that the minor increases in serum creatinine that we found in The Tobramycin Trial were not of clinical significance. Moreover, aminoglycoside nephrotoxicity is reversible in most cases. The cells of the proximal renal tubules start regeneration already while the aminoglycoside administration is still being continued [Gilbert and Leggett, 2010].

In our regular daily work with cancer patients, after The Tobramycin Trial, the question of who may safely receive aminoglycosides and who should be deferred from such therapy is important. We have decided not to use aminoglycosides in patients with multiple myeloma, to avoid causing severe nephrotoxicity in patients with myeloma renal disease, even though this is an unusual condition [Peters, *et al.,*

2011]. We also avoid the use of aminoglycosides in patients who are planned to receive future nephrotoxic anti-cancer therapy, in particular cis-platinum. Several studies have documented a prolonged, maybe life-long, subclinical reduced renal function after therapy with cis-platinum, often suggested by a low serum magnesium level [Aass, *et al.,* 1990; Fosså, *et al.,* 2002]. In this situation, when the cancer is cured, starting therapy with penicillin G plus an aminoglycoside may be appropriate as long as their creatinine levels and other signs of nephrotoxicity are closely followed.

5.4 Important developments in the last decade

Over the years, internationally and in Norway, Gram-positive coccal bacteremias became more prevalent in FN cancer patients. This probably happened as a consequence of more intensive anti-cancer chemotherapy, causing more mucositis, rendering the patients prone to invasion primarily by α-hemolytic oral streptococci. The more widespread use of central venous accesses also rendered the patients susceptible to infections with *S. aureus* and even more to coagulase-negative staphylococci. The focus on methicillin-resistant *S. aureus* (MRSA) and other Gram-positive cocci resistant to the available Gram-positive antibacterial agents, triggered the fifth EORTC trial in the 1980s and 90s, where the question was if empirical addition of vancomycin to the established antibiotic regimen would decrease the fatality rate in FN [EORTC, 1991]. The trial concluded that vancomycin should not be added to the initial empiric regimen, unless the local epidemiology showed a high rate of Gram-positive cocci resistant to the standard antibiotic regimen.

Ten years ago, the multiresistant Gram-positive cocci seemed to represent the major threat to the global antibacterial resistance situation. In Norway, the first vancomycin-resistant *Enterococcus faecium* was found in 1998 [Torfoss, *et al.,* 1999], and MRSA continued to increase for many years. In Norway, the total MRSA rate stabilized around 1 % of the reported *S. aureus* blood culture isolates [NORM/NORMVET, 2011].

However, the most recent developments show an important increase in cases of clinical MRSA in the capital region of Norway [Jørgensen, *et al.,* 2012]. Since 2010, there has been a 34 % increase in MRSA cases. Fifteen local outbreaks in health

care institutions were reported in 2010 and 2011. In the worst case, this may be the beginning of an epidemic similar to what the United Kingdom experienced in the 1990s, leading to an epidemic with > 40 % of the *S. aureus* isolates being MRSA during the course of a couple of years [Iversen, 2004]. This alarming situation may call for all available interventions with a possible effect. Combination chemotherapy, especially the combination of an aminoglycoside with a β-lactam, may reduce the evolution of resistance [Gilbert and Leggett, 2010; Gerber, *et al.,* 1982]. Important research showing benefit of antimicrobial combinations to reduce the emergence of resistance was done in the 1980s [Michea-Hamzehpour, *et al.,* 1986; Pechere, *et al.,* 1986]. This may be another argument for the increased use of aminoglycosides in combination both with penicillin G and with other β-lactams even though we really do not know if this measure may reduce the further evolution and spread of antibiotic resistance. In addition, strict attention to, and enforcement of, hospital infection control principles remain of utter importance.

Resistant Gram-positive cocci certainly remain a continuous problem. There has been an epidemic of vancomycin-resistant enterococci (VRE) that started in Bergen in 2010, and there has been another, so far smaller, VRE epidemic at Oslo University Hospital, at the Ullevål Hospital campus in 2011. A total of 391 cases of VRE have been reported from January 2010 until May 15th 2012 to “MSIS” (Meldesystem for smittsomme sykdommer, Norway) [FHI, 2012]. Most recently, in September 2012, another report of an outbreak of VRE at the main hospital in Fredrikstad has been reported.

A more virulent *Clostridium difficile* strain (0 27) [Vaishnavi C, 2011], causing increased case fatality rate has become a global problem, but so far only few cases have been reported in Norway. Widespread use of fluoroquinolones seems to be one important cause for this development. Fluoroquinolones have been an important class of antibiotics that regrettably is gradually loosing its antibacterial power by the rapid increase of resistance world wide. It is the only class of oral antibiotics, with a broad antibacterial spectrum against Gram-negative bacilli. Unfortunately, these synthetic antimicrobials are only very slowly metabolized in the nature; and the huge consumption of fluoroquinolones both in human medicine and as veterinary (especially aquacultural) growth promoters, probably will throw a long ecological shadow of constant promotion of microbial resistance.

In the last decade, international resistance in Gram-negative bacteria has emerged as one of the major problems in antibiotic therapy in severe infections. Norway has also had many local outbreaks of strains with extended-spectrum-β-lactamases (ESBL) in Gram-negative bacilli [NORM/NORMVET, 2011; Haldorsen, 2011; and Lindemann, *et al.*, 2012]. Recently, the first *E. coli* strain containing a New Dehli Metallocarbapenemase - 1 (NDM - 1) was isolated in Norway [Haldorsen, 2011]. Similar situations are frequently reported in other countries [Kumarasamy, *et al.,* 2010; Nemec and Krizova, 2012; Livermore, 2012]. The total global antibiotic resistance situation seems to be increasing, and Gram-negative resistance may be the major threat at the moment. But as we have seen, Gram-positive resistance also seems to be increasing. In the worst case, this may take us back into the situation we had before penicillin was discovered by Alexander Fleming in 1928 and put into medical use in the 1940s.

Based on important research, the epidemiology of FN again came into focus at the turn of the century. FN patients constitute a heterogeneous group of patients. Algorithms have been developed to identify patients with low risk for complications and fatal outcomes. The trial by the Multinational Association for Supportive Care in Cancer (MASCC) has established an algorithm which makes it possible to identify patients with low risk for complications based on a few immediately available clinical parameters [Klastersky, *et al.,* 2000]. This algorithm opens for safe treatment of FN patients with low risk for complications with oral antibiotics. Unfortunately, the oral regimen has to rely on the use of fluoroquinolones. Hand in hand with this development, hospitals have built patient hotels, both improving the quality of life of FN patients, and reducing the cost of care for these patients.

Other important events relating to FN during the last decade has been the substantial increase in new antifungal drugs [Nordøy and Gaustad, 2008]. Invasive fungal infections were frequently fatal a decade ago. These new drugs have to some extent changed the poor prognosis of invasive fungal infections. However, with more intensive and aggressive cancer therapy, more invasive fungal infections are likely to occur. A challenge with the new drugs is the tendency to overuse them, resulting in development of antifungal resistance [Nordøy and Gaustad, 2008].

In Norway, we have tried to prevent unnecessary use of both antibacterial and antifungal drugs to reduce the spread of antimicrobial resistance. Antifungal prophylaxis is seldom used. *Candida albicans* is still the most common clinical candida isolate in Norwegian hospitals; and *C. albicans* has remained sensitive to fluconazole [Sandven*, et al.*, 2006; Gammelsrud, *et al.*, 2011]. In Norway, recommendations against prophylactic use of antibiotics and antifungal drugs are common [Torfoss and Høiby, 2010; Simonsen, 2010]. Reported case fatality in initial FN is very low, and systemic fungal infections are rare [Torfoss and Sandven, 2005].

Prevention of FN complications by trying to avoid neutropenia, or to abate the seriousness and duration of neutropenia, has lead to systematic use of granulocyte growth factors (G-CSF). Patients with a risk of FN > 20 % should always get G-CSF along with the cancer chemotherapy treatment, and patients with a risk of FN between 10 and 20 % should receive G-CSF if they have additional risk factors like certain comorbidities (especially pulmonary comorbidities), age > 65 years and previous FN [Aapro, *et al.,* 2011]. This is a strategy that reduces the occurrence and severity of FN; and thus reduces the consumption of antibiotics. Even though G-CSF has some unwanted side effects, the avoidance and the reduction in severity of FN are in general beneficial both to the patients and to the microbiological resistance epidemiology as well as the cost of antimicrobial chemotherapy.

The new 13-valent pneumococcal conjugate vaccine, a further evolution of the seven-valent pneumococcal conjugate vaccine, is probably one step forward regarding prophylaxis in immunocompromised patients [Vestrheim, *et al.,* 2008]. Further progress for conjugated pneumococcal vaccines is probable. This vaccine, and the annual influenza vaccine, should in general be offered to cancer patients even though the effect of vaccination may sometimes be hampered by immunosuppression caused by drugs and the diseases [Brydak and Calbecka, 1999; Nordøy, *et al.,* 2002; Yri, *et al.,* 2011; de Lavallade *et al.,* 2011]. In particular, patients with decreased or absent splenic function should be vaccinated against infections caused by bacteria with virulent capsules like pneumococci and *Haemophilus influenzae*.

In Norway, as in most developed countries, governmental bodies have established programs to fight antibiotic resistance. The first program was launched at the turn of

the century, “Regjeringens tiltaksplan mot antibiotikaresistens 2000-2004” [Nyquist, *et al.*, 2000], with follow-up programs that are currently in action, ”Nasjonal strategi for forebygging av infeksjoner i helsetjenesten og antibiotikaresistens, 2008-2012” [FHI, 2008].

The medical microbiology and the medical infectious diseases specialists’ communities in Norway unanimously participate in this program. This thesis might also be seen as part of this program.

When The Tobramycin Trial was published in 2007, a plan for a follow-up trial comparing penicillin G plus an aminoglycoside with meropenem “Penicillin G and an aminoglycoside versus meropenem as initial, empiric therapy in lymphoma and leukemia patients with febrile neutropenia of expected long duration” had already started. It had been successful in recruiting 323 patients when it was completed and closed in October 2011. Hopefully, the first publication from this trial will be submitted at the end of 2012.

Two important organizations have been established during the last decade: first, a center dedicated to facilitate trials and prudent use of antibiotics in general practice, “The Antibiotic Centre for Primary Care”. In 2011, a center focusing on improving antibiotic use in hospitals was launched: “Nasjonalt kompetansesenter for antibiotikabruk i spesialisthelsetjenesten”.

Clinical studies of the specific Norwegian antibiotic policy, often restrictive and different from international guidelines, have, unfortunately, been a neglected area of research. Thanks to a few strong opinion-leaders among the Norwegian experienced infectious diseases faculty, the Norwegian antibiotic policy has been established and to a great extent followed by medical doctors in Norway; sometimes despite lack of scientific evidence. This situation will probably not continue into the future unless clinical trials are being conducted.

We do have an advantageous situation in Norway, both with regard to the antimicrobial epidemiology and the antibiotic consumption pattern. However, parts of the general and specialist antibiotic policy need to be evaluated or reevaluated, and parts of it need to be documented. The way the infectious diseases community and the medical microbiological community have organized themselves during the last decade, and the general tasks we work to complete, along with governmental support, has improved our chances to guard and to promote the goal of preventing

overwhelming antibiotic resistance in Norway, in spite of increased traveling, trade with food etc.

6. Conclusions and future perspectives

Penicillin G plus an aminoglycoside has been the standard, initial therapy in FN and in sepsis of unknown origin in Norway for decades, as confirmed by von der Lippe in 2001 [von der Lippe, 2001]. As new broad-spectrum β-lactams and fluoroquinolones were developed, Norwegian clinicians and authorities in general chose to continue the tradition of using penicillin G plus an aminoglycoside in severe and septic conditions. This has been an important strategic decision by the infectious diseases and the medical microbiological communities since the 1970s. This decision opposed the larger international infectious diseases communities, as well as the results of multiple RCTs, in general sponsored by the pharmaceutical industry; and pressure from the pharmaceutical industry. It is therefore surprising that almost no clinical RCT was initiated at that time in order to document the Norwegian antibiotic policy. Finally, it was primarily the Norwegian hematological and oncological communities that launched the initiative to document the efficacy and the safety of penicillin G plus an aminoglycoside in severe infections.

The fact that all the seven studies resulted in the same conclusion strengthens the evidence for our antibiotic policy. They all, and especially our RCT, concluded: *penicillin G plus an aminoglycoside is effective and safe initial, empiric therapy in FN provided the regimen is modified if the clinical response is unsatisfactory.*

However, with time, the microbiological epidemiology will change. This makes surveillance of results, and preferably follow-up antibiotic trials important quality measures for our continued antibiotic policy.

The reason why we insist on choosing the “old-fashioned” regimen of pencillin G plus an aminoglycoside, in our opinion, cannot be repeated often enough. This is the only broad-spectrum antibiotic combination that is likely to confer prevention against antibiotic resistance when a broad-spectrum antibiotic regimen is needed for a severe or a septic condition.

New antibiotic classes do not seem to come forward. Culture-free techniques for diagnosis and immunological or genetic methods for therapy of serious bacterial infections most likely cannot be expected within the near future [Berman, 2010]. Accordingly, all measures that reduce the problem of antibiotic resistance must be welcomed [Walsh, 2003].

A promising event in 2011 was the article by the Editor of the Journal of Antimicrobial Chemotherapy, Dr. Leonard Leibovici with co-workers. Based on a discussion of ethical dilemmas in infectious diseases, they concluded with a strong support for an antibiotic policy based on the use of narrow-spectrum regimens in moderate to severe infections. They phrased this support as an ethical statement, that patients today must accept the possible risk of "…less than maximum empirical antibiotic treatment in order to reduce the rise in resistance", and that it must be done even "…without the consent of the patient, disregarding the patient's autonomy" [Leibovici, *et al.,* 2011]. The authors concluded that "…future patients have a right to come to no harm", and "…that the right of future patients to come to less harm outweighs the right of the present patient to share in decisions on antibiotic treatment." This article, addressed to an international audience, gave credit to the anti-resistance focus of the Norwegian antibiotic policy.

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Errata

Page 17, line 26: “the three arm of the trial” should be substituted with “the three arms of the trial”

Page 19 , line 8: “page 13” should be substituted with “page 17”

Page 20, line 18: “ Gram-negatve bacilli” should be substituted with “Gram-negative bacilli”

Page 51, line 8: “This mechanism is a key to understanding…” should be substituted with “This mechanism is a key to understand…”

When printing the thesis an extra copyright page 2 was introduced. This has been corrected in the Table of contents. However, where an explanation of how to find a statement in the text is explained with a “see page so and so”, an extra page number should be added. For example, when discussing the results of Paper 2, page 36, referring to the patient who died 29 days after randomization, this is “further described in the Discussion, page 47 + 1 = 48)”

I

II

III