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National preparedness training and exercises for Ebola cases in the United Kingdom

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Abstract

In response to the outbreak of Ebola Virus Disease in West Africa, the Emergency Response Department of Public Health England produced a series of training and exercising materials to help prepare health and partner organisations in England and other jurisdictions in the United Kingdom deal with a possible case of Ebola in the United Kingdom. They were produced with input from health (NHS England, Health Protection Scotland, Public Health Wales) and other partner organisations. The exercising materials have been used by colleagues working in national and local level organisations in the United Kingdom and other countries in the European Union. Presented here is a description of these training and exercising materials and how they were delivered to the end user.

Key words: Public health England; Exercises; Training; Ebola; Preparedness

Core tip: This review summarises the public health training and exercising materials produced by Public Health England and used by national and local health responders to prepare for a case of Ebola Virus Disease in the United Kingdom. It describes the different training and exercise materials developed, how they were used and how they were delivered to their target audiences.

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INTRODUCTION

Public Health England (PHE) is a United Kingdom public sector body that combines elements of public health including health protection, science, research, emergency response, planning and training. The Emergency Response Department (ERD) of PHE has considerable experience in the development of a wide range of training and exercises

to develop preparedness in the health community, government departments and other multi-agency partners in the United Kingdom and Europe. During the outbreak of Ebola in West Africa, PHE developed guidance to help health providers prepare for an imported case in the United Kingdom. To date there have been 3 imported cases of confirmed Ebola in the United Kingdom. The training and exercises produced by ERD were based on the PHE guidance and were developed with advice and assistance from colleagues from the Royal College of General Practitioners, the Health and Safety Executive and the NHS Ambulance Service's National Ambulance Resilience Unit.

EXERCISE PROGRAMME

An exercise is a simulation of an emergency situation used to validate plans, test well established procedures, develop staff competencies and give them practice in carrying out their roles during an emergency[1]. At the request of the Department of Health, ERD developed a series of exercises for responders working at local, regional and national levels as part of their preparedness for dealing with an imported case of Ebola.

Off-the-shelf exercise

ERD developed an "off-the-shelf" exercise package to be used by the 38 multi-agency Local Resilience Forums (LRF) in England during October and November 2014. These Forums are where local responders plan, prepare, exercise and communicate in a local environment[2]. The same exercise pack was offered to each of the LRFs[3] which allowed emergency planning staff to run their own exercise using material developed by PHE. This method of exercise delivery allows a number of exercises to be run concurrently or within a short time frame.

The "off-the-shelf" exercise was designed to be used by health professionals, port staff and multi-agency partners over a period of 4 h. The exercise package contains an explanation of how to manage, run and evaluate the exercise along with a scenario, questions for the participants and specialist information about the disease to help the exercise facilitator to guide participants. This exercise was designed to be run as a discussion-based exercise providing participants with an opportunity to develop awareness of their existing plans[4]. The exercise is divided into 3 sessions each starting with a short scenario followed by questions for the participants to discuss. The exercise asks participants to consider their actions in dealing with a suspected, then confirmed, case of Ebola at: A port of entry; the emergency department of an acute hospital Trust and then the transfer of that patient to a high level isolation unit within the United Kingdom.

All 38 Local Resilience Forums in England carried out the exercise. A report on these exercises was collated by PHE and the national Department of Communities and Local Government responsible for the LRFs. An international version of this exercise using similar scenarios and question sets has been developed and issued to colleagues from 13 European countries.

National exercises

Three exercises were developed and run for the United Kingdom health authorities between October 2014 and March 2015.

In October 2014 ERD, working with colleagues from the national Department of Health and the Civil Contingencies Committee, coordinated the delivery of an eight hour national exercise designed to test preparedness and response to Ebola cases in the United Kingdom[5]. There were two live elements of the exercise which took place in Gateshead (Northern England) and Hillingdon (part of London) which involved actors playing the part of patients with Ebola Virus Disease (EVD) being treated by paramedics and hospital staff. Information from these separate "live play" incidents was fed to the United Kingdom government's national level crisis decision making process, commonly referred to as Cabinet Office Briefing Room[6].

A second, similar exercise was held in December 2014, involving representatives from the four countries of the United Kingdom (England, Scotland, Wales and Northern Ireland). This table-top exercise explored how health authorities in those countries would work together to deal with imported Ebola cases identified in each of the 3 countries outside England. The exercise was run using teleconference facilities from each of the health authorities in their respective countries.

In March 2015 ERD ran a discussion-based exercise in London to consider surge arrangements for the nominated NHS EVD (high containment) specialist treatment centres in the United Kingdom which were set aside for accepting Ebola cases. The exercise involved participants from the Department of Health, Public Health England, NHS England, the Ambulance service and the Health and Safety Executive. The exercise was divided into two sessions. The first of these sessions examined the response to six cases, enough to overwhelm the specialist centres for the treatment of Ebola patients. The second considered the response of primary care providers where there were multiple unconfirmed cases in different communities across England (Figure 1).

International exercises

As well as these exercises run in the United Kingdom, ERD has also been working with colleagues from the Royal Institute of International Affairs, Chatham House - an independent policy institute which engages governments, the private sector, civil society and its members in open debate and confidential discussion on the most significant developments in international affairs[7] - as part of their Infectious Diseases Risk Assessment and Management initiative. This included exercises to allow those working in the extractive (mining, oil and gas)

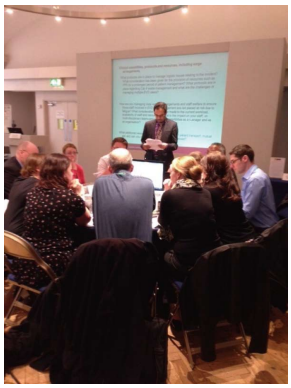

Figure 1 Participants at the surge capacity discussion based exercise in London March 2015.

Figure 2 The ambulance staff in Côte d'Ivoire undertake Personal Protective Equipment training.

industries to gain experience of the risks associated with outbreaks of infectious disease[7]. ERD's contribution has been to develop and run a series of exercises called Exercise Kulinda Afya (Swahili for "protect our health") as a method for representatives from the extractive industry, international development and governments to discuss together their response to an outbreak of Viral Haemorrhagic Fever. This discussion based exercise has been run with support from USAID with colleagues in the Democratic Republic of Congo and at extractive industry conferences in Australia and London. More are planned and this programme will continue into 2015.

In early 2015 staff from PHE and the NHS Ambulance service's National Ambulance Resilience Unit were invited by the Ministries of Health in Cameroon and Côte d'Ivoire and the United States Centers for Disease Control and Prevention to deliver Ebola preparedness workshops in these two West African countries. These week-long workshops were designed to practice participants in different aspects of national level and multi-sectorial coordination of response to EVD including: Establishment of the emergency operation centre; rapid response team's competencies and skills; outbreak control, particularly epidemiological surveillance and contact tracing; infection prevention and control and the use of personal protective equipment. A picture of staff during Personal Protective Equipment (PPE) training is at Figure 2. These exercises also included patient and sample transport and laboratory capability testing.

TRAINING PROGRAMME

PHE was requested by the national Department of Health to provide training and training materials for health workers in England to deal with suspected cases of Ebola and provide familiarisation with guidance.

NHS personal protective equipment training

ERD and the NHS Ambulance service's National Ambulance Resilience Unit delivered training to 277 NHS Emergency Department staff from 114 of the 155[8] acute Trusts in England in the first half of December 2014. This training taught the safe donning and doffing of one particular combination of PPE when dealing with a patient with suspected Ebola. The training was based on guidance produced by the United Kingdom Government Advisory Committee on Dangerous Pathogens which advises the Health and Safety Executive, and Ministers for the Department of Health and the Department for the Environment, Food and Rural Affairs and their counterparts under devolution in Scotland, Wales and Northern Ireland, as required, on all aspects of hazards and risks to workers and others from exposure to pathogens[9]. The committee's guidance on Management of Hazard Group 4 Viral Haemorrhagic fevers and similar human infectious diseases of high consequence[10] formed the basis for standard operating procedures developed by PHE staff.

The training materials included a video describing a step-by-step guide to the PPE donning and doffing procedure and the possible infection routes for Ebola to inform safe methods of working. Four A1 posters were also produced, which emergency department staff could use to help guide them through the procedures. An example of the posters showing emergency department staff the procedure for donning and doffing of PPE is shown in Figure 3. The films and posters were distributed to each person attending the training and those staff who have attended the training are able to access the materials through Public Health England's e-learning website[11]. Training and templates for the preparation of training materials have also been provided to NHS Wales.

Guidance for General Practitioners

A video based on the PHE guidance Information for Primary Care: Managing patients who require assessment for Ebola virus disease[11] and action cards produced by the Royal College of General Practitioners[11] was produced as an additional training tool for staff working in a primary care setting. The video describes the response to a suspected case of Ebola self-presenting at a doctor's surgery and describes the steps that can be taken by each member of the general practice staff to ensure that the patient is treated safely and quickly. The 20 min video takes the viewer through various scenarios, describing the different ways a patient might come into contact with primary health care providers and the actions detailed in the guidance. There are over 7900 General Practitioner

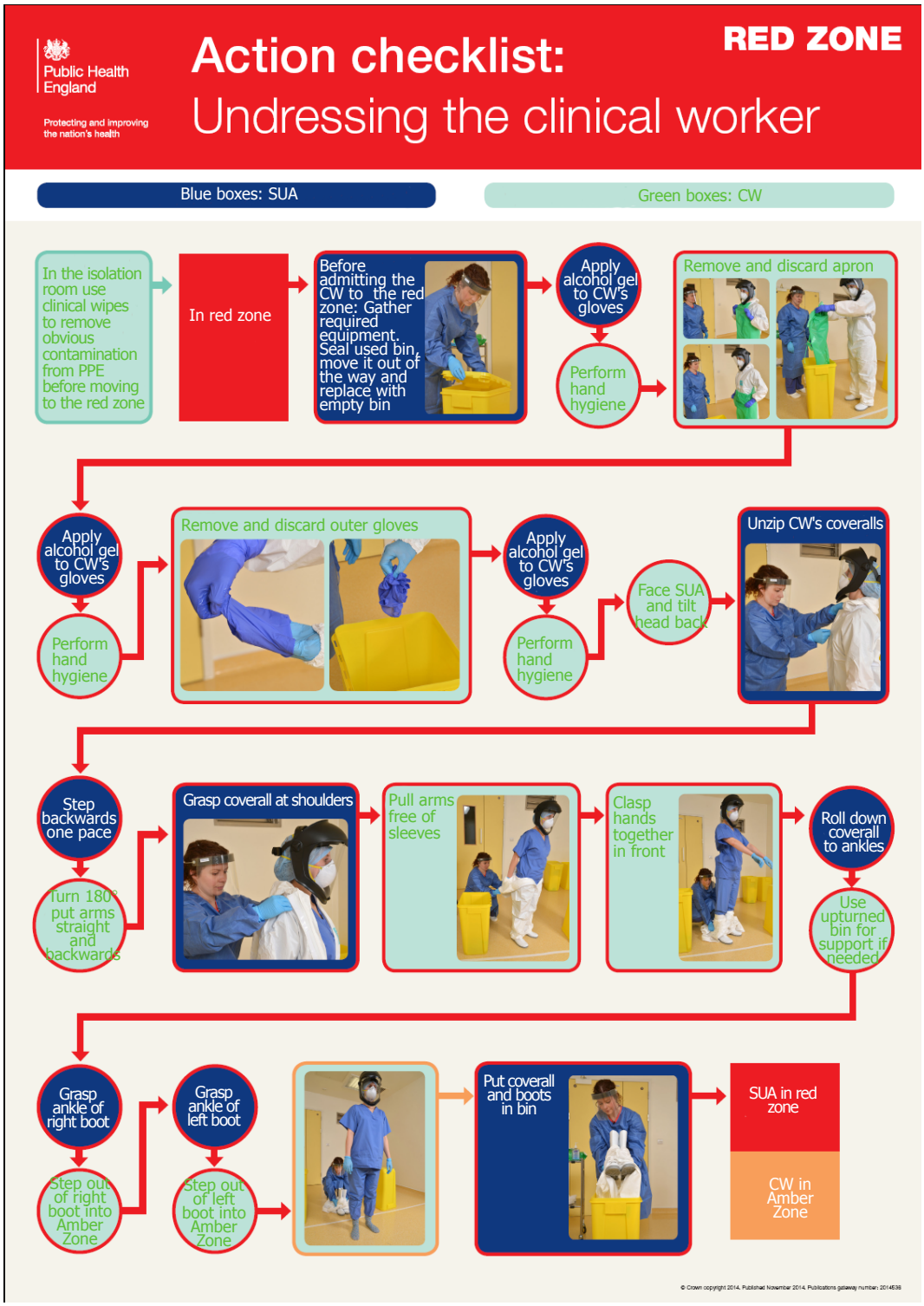

Figure 3 Poster produced by Public Health England to assist emergency department staff with doffing of Personal Protective Equipment procedures. SUA: Safe undressing assistant; CW: Clinical worker; PPE: Personal Protection Equipment.

practices in England[11] the video was posted on You Tube to make it as accessible as possible.

Port screening training

A third element of training was delivered to staff at ports of entry in England and was based on PHE internal guidance. The training describes the questionnaire and procedures to be used when screening passengers entering or returning to the United Kingdom from the Ebola affected countries in West Africa. The training was carried out with support from colleagues from local health protection teams across England.

SUMMARY

These training and exercising materials are an important element in the preparation for the health service and other national and local responders across the United

Kingdom.

Feedback confirmed that the exercises and training were valuable tools in ensuring that organisations and individual staff were familiar with the procedures to be followed in the event of a case of EVD into the United Kingdom. They helped familiarise participating organisations with each other's preparedness plans and practices, and promoted better understanding and cooperation between responding organisations. They fostered discussion, proposed realistic actions and identified important issues and areas for development. The lessons identified from all the events have been reported and allocated to the relevant organisations (a large percentage have been actioned) and in a generic form will be used to assist with future response planning for infectious diseases.

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REVIEW

Systems biology applications to study mechanisms of human immunodeficiency virus latency and reactivation

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Abstract

Eradication of human immunodeficiency virus (HIV) in infected individuals is currently not possible because of the presence of the persistent cellular reservoir of latent infection. The identification of HIV latency biomarkers and a better understanding of the molecular mechanisms contributing to regulation of HIV expression might provide essential tools to eliminate these latently infected cells. This review aims at summarizing gene expression profiling and systems biology applications to studies of HIV latency and eradication. Studies comparing gene expression in latently infected and uninfected cells identify candidate latency biomarkers and novel mechanisms of latency control. Studies that profiled gene expression changes induced by existing latency reversing agents (LRAs) highlight uniting themes driving HIV reactivation and novel mechanisms that contribute to regulation of HIV expression by different LRAs. Among the reviewed gene expression studies, the common approaches included identification of differentially expressed genes and gene functional category assessment. Integration of transcriptomic data with other biological data types is presently scarce, and the field would benefit from increased adoption of these methods in future studies. In addition, designing prospective studies that use the same methods of data acquisition and statistical analyses will facilitate a more reliable

identification of latency biomarkers using different model systems and the comparison of the effects of different LRAs on host factors with a role in HIV reactivation. The results from such studies would have the potential to significantly impact the process by which candidate drugs are selected and combined for future evaluations and advancement to clinical trials.

Key words: Gene expression; Microarrays; RNA-Seq; Systems biology; Human immunodeficiency virus; Viral latency; Disease eradication; Biomarkers; Molecular mechanisms; Latency reversing agents

Core tip: Gene expression profiling and systems biology methods are reviewed with respect to their possible application in the field of human immunodeficiency virus (HIV) research. Studies profiling gene expression in latently infected and uninfected cells are summarized to illustrate application of these methods to identification of latency biomarkers and the molecular mechanisms contributing to regulation of HIV expression. Studies that measure changes in host and HIV gene expression upon treatment with latency reversing agents (LRAs) highlight uniting themes driving HIV reactivation and identify novel mechanisms of action of LRAs. The field will further benefit from increased adoption of systems biology methods in future studies.

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INTRODUCTION

In the present era of combination anti-retroviral therapy (cART), the persistence of cellular human immunodeficiency virus (HIV) reservoir is considered to be the major barrier to a cure[1]. This cellular reservoir mainly consists of latently infected resting CD4+ T cells bearing HIV integrated provirus. It is highly stable[2-5] and inducible, necessitating life-long adherence to cART to prevent rebound of viremia. In a search for therapeutic strategies to eradicate this latent reservoir, mechanisms leading to latency have been extensively studied and include transcriptional and post-transcriptional blocks[1,6-14].

The main strategies directed toward a cure are reviewed elsewhere[6,7,9,12,15-17] and include the inactivation of replication-competent virus and the elimination of latently infected cells. An essential milestone to HIV reservoir eradication is the identification of biomarkers of latently infected cells[18,19], so that these cells can be specifically targeted by immunotoxins[20]. Currently, the foremost strategy for elimination of latently infected cells is controlled virus reactivation in the presence of continuing cART ("shock and kill")[21,22]. For this purpose, small molecule compound latency reversing agents (LRAs) are currently tested. The first LRAs used were histone deacetylase (HDAC) inhibitors (HDACi), which progressed to clinical trials[23-27] and demonstrated the ability to induce expression of HIV RNA. Unfortunately, none of the studies that followed the reservoir size post-treatment reported a significant reduction[23,25,27]. The multiplicity of molecular mechanisms involved in latency control suggests that a combination approach will likely be required to achieve the degree of reactivation necessary for the infected cell to be recognized by the immune system[28-30]. Indeed, some of the tested LRA combinations demonstrated synergy for HIV reactivation[31-35].

Gene expression profiling techniques and systems biology applications may be extremely useful in the identification of biomarkers of latency, further delineating mechanisms of regulation of HIV expression in a search for novel strategies of latency reversal, and for our understanding of the mechanisms of action of existing LRAs. Methods of analysis of gene expression data have been reviewed previously[36-40], including application of bioinformatics methods to HIV integration site analysis and the assessment of transcriptome and proteome changes induced in cells infected with HIV[41]. The present review provides a broader perspective on the use of gene expression profiling and systems biology applications in the field of HIV latency and eradication. Specifically, the objectives of the present review are: (1) to review the existing gene expression profiling and systems biology methods and their potential in the field of HIV research. We focus on the transcriptomic methods, and progress from simple approaches of differential gene expression to more complex types of analyses that integrate transcriptomic data with other biological data types, including proteomic analyses, integration site distribution, epigenetic modifications and transcription factor databases; and (2) to systematically demonstrate how methods of gene expression profiling and systems biology have been applied to answer specific questions in the fields of HIV latency and eradication. In this section we summarize specific findings that were obtained using gene expression profiling and systems biology methods, as described in existing literature.

GENE EXPRESSION PROFILING AND SYSTEMS BIOLOGY APPROACHES APPLIED IN THE FIELD OF HIV LATENCY AND ERADICATION

In this section, we describe the major methods of gene expression analysis and systems biology approaches and outline specific questions that can be addressed in

Table 1 Methods of gene expression profiling and systems biology and their applications in the field of human immunodeficiency virus latency and eradication

Method	Applications to discovery of latency biomarkers and mechanisms of regulation of HIV expression	Applications to studying the LRA mechanisms of action and evaluating combination therapies
Differential gene expression	Identification of latency biomarkers	Identification of genes responsive to LRA treatment
GO term/pathway enrichment	(1) Focusing study efforts upon gene groups of interest (*e.g.*, membrane proteins as biomarkers) (2) Identification of the mechanisms behind gene expression alterations (3) Delineating the molecular mechanisms contributing to latency control	(1) Elucidation of mechanisms of action of LRAs (2) Selection of gene targets for combination therapy based on gene function in enriched pathway
Network-based analysis	Identification of major regulators involved in HIV latency control, which may be only slightly dysregulated but significantly affect downstream molecules and pathways	(1) Elucidation of mechanisms of action of LRAs; (2) Prioritization of targets for combination therapies based upon type of connectivity (include if it regulates HIV-related processes; exclude if it regulates general intracellular processes)
Consolidating gene expression with other biological data (proteome, integration sites, chromatin features, *etc.*)	(1) Identification of latency biomarkers with transient RNA, but stable protein expression; (2) Identification of mechanisms of latency control by correlating chromatin features to gene expression	(1) Identification of post-transcriptional mechanisms of action of LRAs; (2) Assessment of chromatin features of genes and HIV integration sites responsive to LRA treatment
HIV expression and transcript type	Potential biomarker of latency	Assessment of the effectiveness of LRAs for HIV reactivation

LRA: Latency reversing agent; GO: Gene ontology; HIV: Human immunodeficiency virus.

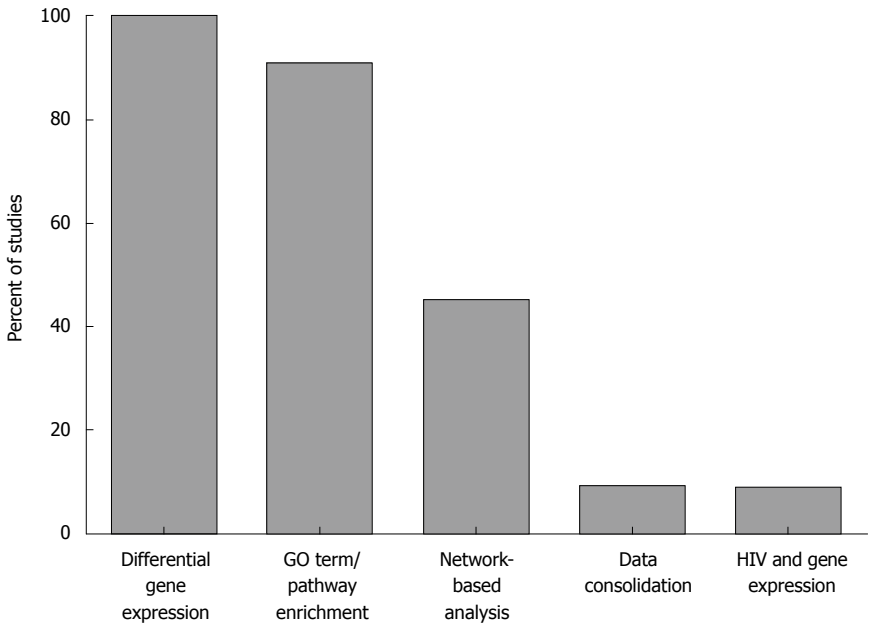

Figure 1 Summary of methods used across gene expression profiling studies in the field of human immunodeficiency virus latency and eradication. Identification of DEGs and functional analysis of GO terms and pathways enriched for DEGs are the methods that are most commonly used across studies. Network-based analyses are used in a subset of studies; while methods that consolidate host gene expression with other data types (*e.g.*, proteomics or HIV expression data) are scarce. DEGs: Differentially expressed genes; GO: Gene ontology; HIV: Human immunodeficiency virus.

the fields of HIV latency research and eradication using LRAs by each major type of application (Table 1). Where applicable, we highlight advantages and disadvantages of using individual methods over other methods for HIV latency related studies.

Differential gene expression

This basic analysis, common in all gene expression studies (Figure 1), aims at identifying genes that are expressed at different levels among the conditions tested. Gene expression can be compared in latently infected and uninfected cells to identify biomarkers of latency, and between cells treated with LRAs and untreated cells to identify genes that are responsive to LRA treatment.

To obtain gene expression data, two primary technologies are available: Microarrays and RNA-Seq. The majority of the published studies in the HIV latency field utilized microarrays, which is a well-developed technology with a fully established data analysis

pipeline. However, because microarrays use specific oligonucleotide probes, the detection is limited to only known genes. In addition, most of the microarray platforms are species-specific, which does not allow for simultaneous detection of host and pathogenic RNAs present in a sample. With advances in RNA-Seq technology and per sample cost reduction, gene expression profiling by RNA-Seq is more increasingly used. RNA-Seq allows measuring viral and cellular transcripts concomitantly in the same sample[42]. Other benefits of using RNA-Seq include increased sensitivity towards rare transcripts (as may be the case for HIV transcripts in latent state); detection of novel splice variants; and the wide dynamic range (reviewed in[43]). Numerous methods exist to analyze microarray (reviewed in[36,37,44]) and RNA-Seq datasets (reviewed in[38,39]), including methods of data processing, normalization and identification of differentially expressed genes (DEGs).

While methods of identification of DEGs are relatively straightforward, their application to mechanistic studies is limited. First, these methods usually generate far more DEGs that can be meaningfully discussed due to the lack of existing knowledge of their role in regulation of HIV expression. The second major issue in such studies is multiple comparisons. As more genes are included in either microarrays or RNA-Seq studies, the threshold for differential expression becomes much harder to reach due to the increased chance of type 1 error. Finally, a third issue arises with regards to the ranking of importance for genes which are differentially expressed. These can be ranked based upon fold change or a ranking system based upon prior knowledge of the gene. However, a gene product which is an important player of a pathway may not be well characterized, nor be heavily dysregulated, but may still cause large downstream changes.

Functional analyses to identify gene ontology terms and pathways enriched for DEGs

These frequently used methods (Figure 1) are designed to identify groups of genes sharing a common functional category or purpose that is significantly altered by gene dysregulation. Functional gene annotation may be useful for biomarker discovery to identify genes that encode membrane proteins. These proteins represent more feasible targets for antibody-bound immunotoxins as compared to intracellular proteins. Mainly, though, gene ontology (GO) term and pathway enrichment analysis is used to identify the mechanisms behind gene expression alterations in latency and during LRA treatment. Finally, specific pathways may be identified for targeting in combinatorial reactivation strategies, based on enrichment for DEGs.

There are numerous databases of annotated GO terms and pathways, and methods to analyze these functional categories, many of which are publicly available tools (reviewed in[40]). Gene set enrichment analysis (GSEA) approaches are the most commonly used method to identify GO terms and pathways that are enriched for DEGs[45-47]. Among these, ToppGene[47] has several advantages, including a user-friendly interface, allowing multiple input codes for genes, and performing both GO term and pathway enrichment analyses. Many similar functions are available in the DAVID Bioinformatics Resources tool[46]. GoSeq tool was developed specifically for RNA-Seq data and quantifies gene length bias present in the data[48]. In cases when an intervention significantly alters the expression of an extremely large number of genes, as may be the case for some LRAs, GSEA approaches may not work as most categories are enriched. An alternative method, Functional Analysis of Individual Microarray Expression[49] utilizes an exponentially decreasing weighted expression to generate a score for each GO category or pathway in both experimental and control conditions. A *t*-test, or other statistical test can be then performed to determine if the scores are significantly different. One drawback of this method is the importance placed upon highly expressed genes. However, lowly expressed genes may play other roles through post-translational modifications or hub roles which are not detected by this method or differential expression methods in general. To address these issues, network analysis techniques are extremely useful.

Network-based gene expression analyses

These tools, used in about half of the studies in the field of HIV latency (Figure 1), are designed to identify key functional regulators among DEGs, and to evaluate gene network differences among experimental conditions. In the network-based analyses, the function of a single gene may be elucidated through a "guilt by association" approach. High connectivity between a known and unknown gene may shed light upon their function. Additionally, a group of highly connected genes may indicate that a biologically relevant pathway is at work in the altered state. These pathways or networks of genes can be tested for differential expression without the high type 1 error rate, which is common when testing many thousands of individual genes. Heavily connected genes whose importance may have been missed in a standard differential expression test would show up in a network method as a hub (highly connected) gene. In this way, additional genes with a role in latency control or reactivation may be identified, which would be missed in other types of analysis. Finally, genes may be selected as therapeutic targets based on the network analysis, if they are connected to other factors with roles in HIV latency control. Conversely, if a gene is connected to genes that encode proteins with broad cellular functions, it may be selected against as side effects from a therapeutic intervention would be expected.

One well-developed network analysis tool is Weighted Gene Co-expression Network Analysis (WGCNA)[50]. In this method, the connectivity between genes is determined by correlating the expression of these genes

across samples, independent of known protein-protein and protein-DNA interactions. First, an adjacency matrix is constructed based on correlations between each gene pair, followed by creating a topological overlay map (TOM) that utilize information not only from the direct interaction between two genes, but also their neighboring nodes. Once this TOM is created, genes may be subdivided into highly connected groups or modules. The eigengene of this module represents the mathematically optimal summary of the expression profiles of all genes within the module as determined by their expression variation across samples. This eigengene may then be correlated to any trait of interest, such as the expression of specific HIV transcripts, or the degree of HIV reactivation upon treatment with LRAs. Genes with unknown function may be explored through both the behavior of the module as a whole and within the module itself (peripheral gene or a primary hub gene). Highly connected genes often represent key players in pathways and shed light upon the mechanistic differences between the two conditions being compared, such as uninfected CD4+ T-cells *vs* HIV-infected CD4+ T cells. Another network-based method, the "Active modules" algorithm[51], utilizes a different approach to network analysis by determining which portions of the network contain an unexpectedly high occurrence of genes with significant changes in expression. In contrast with WGCNA, the "active modules" algorithm utilizes protein interaction data from available databases, which allows incorporating information about the host and HIV interactions[51]. Available software packages for network analysis usually use literature curated protein-protein and protein-DNA interactions databases, but do not take into account enrichment of specific clusters for DEGs (*e.g.,* Metacore, Ingenuity, iRefWeb). A major advantage of utilizing known interactions is independence from differential expression (*i.e.,* all known protein-protein and protein-DNA interactions will be displayed for each DEG). A drawback of literature-based networks is the dependency on the accuracy of annotated sources and the robustness of the algorithms for network generation.

Integrating gene expression with other types of biological data

Methods of transcriptomics are well-developed and capture the majority of annotated genes. However, previous studies have shown that the transcriptome only partially correlates with the proteome[52-54]; therefore, assessment of gene expression at the functional (protein) level may be necessary to validate the role of specific genes in HIV latency control and reactivation. In addition, proteomics methods identify the effects that are not reflected or captured at the RNA level; for example, due to an increase of translation from existing messenger RNA[55], or because of the transient RNA expression. Thus, proteome profiling may be used to identify latency biomarkers that are stably expressed at the protein level. In addition, profiling of post-transcriptional effects of LRAs is beneficial to capture those effects that would be missed if only the transcriptome profiling were performed. Analysis of the proteome may thus shed light on the mechanisms by which LRAs regulate gene expression[56], including, possibly, transcriptional activation of HIV.

Other biological data types may be integrated with gene expression profiling data to further understand the mechanisms of HIV latency and reactivation. The activity of the HIV promoter may depend on the characteristics of the site of proviral integration[57]. Chromatin features surrounding an integration site may contribute to the levels of HIV transcription, including histone acetylation and methylation, and DNA methylation. For example, latent inducible proviruses have a tendency to be integrated into highly expressed genes, gene deserts, or alphoid repeats[58]. The transcription level of nearby genes as well as viral genome orientation may influence transcription of viral genes by RNA interference mechanisms[59-61]. However, to date, no clear feature of integration sites could be identified when comparing 5 different models of HIV latency[62]. Integration of HIV into specific genes, such as genes associated with cell cycle, may provide advantage to the maintenance of the latent reservoir through clonal expansion[63].

Depending on the type of data, different modeling methods may be used. The study described below was done with cancer cell lines; however, their method of integrating datasets would be applicable for many types of HIV latency related data. The aims of the study were to determine how DNA methylation in different genomic regions contribute to gene expression in cancer cell lines, and whether methylation of transcription factor binding sites impact transcription factor recruitment and therefore gene expression[64]. Gene expression was measured by Affymetrix microarrays, and DNA methylation by methyl-CpG binding domain-based capture (MbDCap)-Seq[65]. Pearson correlation analysis and decision tree learning were used to determine the effect of methylation in various genomic regions (promoters, first and second exons, and first introns) on the breast cancer subtype differential gene expression. To determine the role of methylation in transcription factor binding, cell line-specific consensus sequences were generated by assembling reads that mapped to the significantly hypermethylated regions and then matching these sequences to candidate transcription factors using the TRANSFAC package[66]. Similar approaches can be used to determine the role of chromatin features such as DNA methylation, as well as histone acetylation and methylation, in regulation of the expression levels of genes that control HIV latency, in the latent state and during reactivation using LRAs.

Evaluating the levels of HIV RNA using RNA-Seq datasets

HIV full length unspliced (US) genomic RNA can be spliced into different mRNA species, 47 identified in an early study[67], and 78 more recently[68]. The major classes of transcripts constitute multiply spliced (MS)

transcripts that encode regulatory and accessory proteins Tat, Rev, and Nef; and singly spliced (SS) transcripts that encode one-exon Tat, Vpr, Vif, Vpu, and Env. The US transcripts encode Gag and Gag-Pol polyproteins. In cell line models of latency (ACH-2 and U1), MS and SS transcripts were detected at early stages of replication cycle, when little or no genomic (US) RNA was produced[69]. Both MS and US transcripts were detected at low levels in resting CD4+ T cells from the HIV-infected individuals, while the majority of detected transcripts represented abortive HIV transcripts lacking polyA tail[70]. As was suggested previously[71], HIV RNA itself may represent a biomarker of latency. While multiple assays have been developed to detect HIV RNA using PCR-based methods[72,73], they require design of specific primers to detect various forms of HIV RNA, and may be plagued by inability to detect HIV RNA in a subset of patients due to virus mutations. RNA-Seq technology allows for concomitant detection and quantification of various HIV RNA species from the same samples as host transcripts, regardless of the viral sequence. Total HIV transcripts, including the abortive transcripts, can be measured by RNA-Seq using total RNA (ribo-depleted) libraries that capture non-polyadenylated RNAs.

RNA-Seq can also be used to evaluate induction of HIV expression using LRAs. In this case, libraries enriched for polyA (polyadenylated) RNAs would be a more appropriate choice, since induction of abortive transcripts or read-through transcripts from the neighboring genes is not relevant to the success of the "shock and kill" strategy, as no viral proteins will be produced. Specifically, induction of polyA US transcripts would need to be monitored, as it is indicative of productive infection (that will result in production of virions). Unfortunately, none of the existing RNA-Seq data analysis packages have reliable tools for precise splice variant measurement from standard RNA-Seq datasets (50-100 base pair reads), in particular, complex overlapping sequences as in the case of HIV[67]. Precise measurement of splice variants require longer read capacity (10 kb)[74]; otherwise, expression of the major splice variants, MS and SS, and the US genomic RNA can be only estimated. Mohammadi *et al*[42] developed a method that allows the approximation of the proportions of different HIV transcripts in the RNA-Seq data. The method is based on determining the number of reads that pass through the splice junctions D1 [directly after the long terminal repeat (LTR) region] and D4 (splice junction between Tat-Rev and Vpu) that define MS, SS, and US transcripts. If a read passes through the junction D1, then it belongs to the US transcript. Reads which align to the left of the D1 junction but are broken at D1 and align to another segment of the HIV genome correspond to reads from either SS or MS transcripts (SS + MS). Reads overlapping the D4 junction correspond to reads from either US transcripts or SS transcripts (US + SS). Finally, reads which are broken at the D4 junction correspond to reads from MS transcripts. The SS read percentage is then estimated by subtracting the US and MS percentages from 100.

USING TRANSCRIPTOME PROFILING TO IDENTIFY BIOMARKERS OF HIV LATENCY

A recent study[20] provided a proof of principle that immunotoxins can be used to target cells expressing a specific surface molecule; however, the choice of CCR5 co-receptor resulted in killing of both HIV-infected and uninfected CCR5-expressing cells. This choice of target would not be optimal for therapeutic applications, since CD4+ T cells are usually already compromised in HIV-infected individuals. Therefore, identification of a unique biomarker signature of latently infected cells is warranted to target these cells for eradication with high specificity. These biomarkers may have additional applications; for example, reliable quantification of latently infected cells *in vivo* to follow the size of the latent reservoir in patients post-treatment, and enrichment for latently infected cells for further studies.

The proof of principle that latently infected cells may have a distinct gene expression signature was provided in an early study comparing gene expression in resting CD4+ T cells from aviremic HIV-infected individuals and HIV seronegative donors as controls using microarrays[75]. Whilst less than 0.1% of cells from aviremic patients were latently HIV-infected (as determined by presence of HIV-1 proviral DNA), 165 genes showed differential expression between CD4+ T cells from aviremic patients as compared to HIV-seronegative donors. The limitations of this study were the low prevalence of latently infected cells and the confounding effect of antiretroviral therapy on gene expression. Later studies aimed at characterizing the gene expression profile of latently HIV-infected cells using chronically HIV-infected cell lines or *in vitro* infected primary resting CD4+ T cells and reporter viruses, allowing for strategies to enrich or select for latently HIV-infected cells.

Table 2 summarizes the four studies comparing gene expression in latently infected cells *vs* their uninfected counterparts. To estimate the proportions of latently infected cells present in each model, provirus expression is reactivated following establishment of latency, using strong agents that induce T cell activation, such as phorbol myristate acetate[18], anti-CD3/anti-CD28 + IL-2[42], or phytohemagglutinin and feeder peripheral blood mononuclear cells[76]. The percentage of uninfected cells may be estimated by subtracting the percentage of latently infected cells from the total (100%), assuming that all latent proviruses were induced. The percentage of cells expressing HIV Gag protein (p24+) or GFP reporter is also measured before the stimulation, to determine whether there is background expression of HIV in each latency model. These p24+ or GFP+ cells may represent productively infected cells present due

Table 2 Features of gene expression studies comparing latently infected *vs* uninfected cells

Study characteristics	Krishnan and Zeichner[18]	Iglesias-Ussel *et al*[19]	Mohammadi *et al*[42]	Evans *et al*[76]
Cells used	Cell lines ACH-2, A3.01, J1.1	Primary CD4+ T cells	Primary CD4+ T cells co-cultured with feeder H80 human brain tumor cell line	Primary resting CD4+ T cells co-cultured with dendritic cells
Virus used	CXCR4 tropic HIV-1 LAV strain	CXCR4 tropic GFP reporter virus (GFP inserted in place of Nef)	CXCR4 tropic GFP reporter virus with mutations in Gag, Vif, Vpr, Vpu, Env and Nef	CCR5 tropic GFP reporter virus (GFP inserted into the Nef open reading frame)
Proportion of uninfected cells	⩽ 1.1%	0%	8%-18%	99.7%
Proportion of GFP+ or p24+ cells	8.20%	8.15%	Approximately 16%	0% (removed by sorting)
Proportion of latently infected cells	98.9%	100%	Approximately 82%-92%	Approximately 0.3%
Time of culture	N/A (chronically infected)	20-22 d	13 wk	5 d
Experiment replicates	8	4	Not reported	4
Gene expression profiling platform	Microarrays (Hs. UniGem2)	Microarrays (Agilent-012391 Whole Human Genome Oligo Microarray G4112A)	RNA-Seq (polyA RNA library; Illumina HiSeq2000)	Microarrays (Illumina Human-Ref8)
Method to identify DEGs	Parametric one-sample random variance *t*-test (BRB-Array Tools, $P < 0.001$)	Linear modeling and using an empirical Bayes method with FDR correction (limma)	Generalized linear modeling (DESeq, FDR < 0.05)	Linear modeling and using an empirical Bayes method (limma, FDR < 0.05)
Databases used for functional analyses	NIH mAdb	GO consortium; MsigDb; KEGG pathways	Reactome pathways Ver.40; MsigDb	IPA
Total number of DEGs	32	875	227	Not reported

CXCR4: Chemokine (C-X-C motif) receptor 4; LAV: Lymphadenopathy-associated virus; CCR5: Chemokine (C-C motif) receptor 5 (gene/pseudogene); GFP: Green fluorescent protein; polyA: Polyadenylated; DEGs: Differentially expressed genes; BRB: Biometric Research Branch; FDR: False discovery rate; NIH: National Institutes of Health; mAdb: Mad Bee; GO: Gene ontology; MsigDb: Molecular Signature database; KEGG: Kyoto Encyclopedia of Genes and Genomes; IPA: Ingenuity Pathway Analysis; N/A: Not applicable.

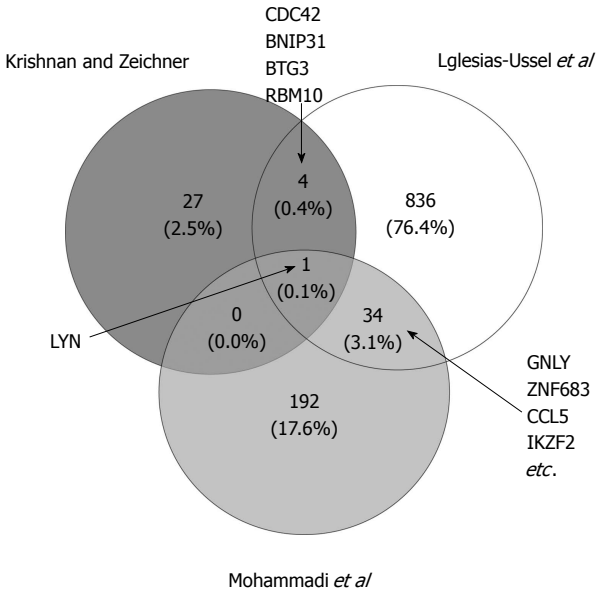

Figure 2 Venn diagram depicting differentially expressed genes across three latency models. The overlapping genes were identified using the online tool Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Shown are the total number of differentially expressed genes and percent of total identified across all models[18,19,42]. For each overlap, gene symbols are listed. For the overlap between Iglesias-Ussel *et al*[19] and Mohammadi *et al*[42] studies, the four genes with the highest average absolute fold change are listed.

the leakiness of a model, or be reflective of the viral entry in the absence of *de novo* viral production. Of note, Krishnan and Zeichner[18] provided these estimates only for one of the cell lines studied, ACH-2. The proportions of each cell type need to be taken into account when evaluating the results from differential expression analysis.

Table 2 presents additional characteristics that differed among the studies, including cells that were used (proliferating cell lines, resting CD4+ T cells or total CD4+ T cells), the duration of time in culture and viruses used to infect the cells. Finally, gene expression profiling platforms and statistical approaches to analyze the data were also different.

In order to assess whether biomarkers of latency can be reliably identified using gene expression profiling, we compared the DEG lists, where available (all studies except for Evans *et al*[76]). Krishnan and Zeichner[18] reported 32 genes that were consistently changed in latency in all three cell lines that were tested, and this list of DEGs was used. The number of DEGs from each study that participated in this analysis is indicated in Table 2 (bottom row). If consistent changes across model systems could be detected, these genes would represent strong latency biomarker candidates.

Figure 2 depicts the result of comparison of DEGs between latently infected and uninfected cells available from three published studies[18,19,42]. A total of 1094 DEGs were identified. Only one gene, LYN proto-oncogene, Src family tyrosine kinase (*LYN*), was dysregulated in latency in all three models. Not surprisingly, there were

Table 3 Limitations of the present studies that identify differentially expressed genes between latently infected and uninfected cells and possible solutions that may enable identification of solid candidate biomarkers of latency

Limitations	Solutions
Small percentage of latently infected cells	Isolate latently infected cells using reporter system OR perform gene expression profiling on a single-cell level
Effect from the exposure to the virus without infection	Use aldrithiol-2 inactivated virus[123] instead of mock-infection to compare to latently infected cell model
Identified differentially expressed genes are ubiquitously expressed on all CD4+ T cells	Identify a panel of biomarkers that best differentiates between latently infected and uninfected cells
Different models represent different aspects of latency establishment	Include additional models into analysis; use same statistical approaches to ensure differences in biomarkers are biological, not technical differences
Gene expression profiling can only identify candidate biomarkers	Perform experimental validation that latently infected cells can be detected using these biomarkers

fewer similarities between the cell lines and each of the primary cell models. In addition to *LYN*, only four genes were in common between Krishnan and Zeichner[18] and Iglesias-Ussel *et al*[19] studies. More similarities were found when comparing the two studies that performed gene expression profiling using primary CD4+ T cells (Iglesias-Ussel *et al*[19] and Mohammadi *et al*[42]): 34 genes were found in common, with the majority (29 of 34) consistently up- or down- regulated in latency in both models. The remaining genes were unique for any given study (27 of 32, or 84% for Krishnan and Zeichner[18], 836 of 875, or 96% for Iglesias-Ussel *et al*[19], and 192 of 227, or 85% for Mohammadi *et al*[42]).

This comparison indicated that despite the small proportion of overlapping genes between models, genes whose products may be able to differentiate between latently infected and uninfected cells can be identified using gene expression profiling, especially when comparing models established in primary cells. However, these studies have several limitations that presently preclude from achieving a consensus on what genes may represent suitable biomarkers of latency. These limitations and potential solutions that may advance this field are summarized in Table 3.

TRANSCRIPTOME PROFILING AND SYSTEMS BIOLOGY APPROACHES TO IDENTIFY MOLECULAR MECHANISMS OF REGULATION OF HIV EXPRESSION

Understanding the mechanisms of establishment and maintenance of HIV latency has greatly contributed to the development of strategies for eradication. It has become apparent that multiple cellular processes and pathways contribute to the control of HIV latency at both the transcriptional and post-transcriptional levels[1], suggesting that combination strategies will likely be needed to achieve eradication of the latent reservoir[28]. Block of viral transcription from the LTR is the most studied mechanism, which occurs through several proposed routes: Inhibition of transcription though histone and DNA modifications[77-79]; absence of necessary transcriptional activators and presence of transcriptional repressors in resting CD4+ T cells[80,81]; integration into inactive transcription sites[57]; or premature termination of viral transcripts in the absence of Tat and Tat-associated host factors[82]. Another mechanism suggests that latency may be maintained due to post-transcriptional blocks. HIV could be transcribed, but could fail to export MS HIV transcripts, contributing to non-productive infection in resting CD4+ T cells[83]. Finally, discoveries in the field of inhibitory micro RNAs (miRNAs) suggest a possibility of transcriptional inhibition of HIV by miRNAs encoded in HIV genome[84] and translational inhibition by host miRNAs[85].

Gene expression profiling data can be used to identify gene categories that describe cellular processes and pathways, as well as key regulatory factors with a role in HIV latency control, thus contributing to our understanding of the mechanisms that regulate HIV expression. The same studies described in Table 2 performed functional category analysis by identifying pathways and GO terms enriched for DEGs. Though these four studies utilized different cell types and viruses (Table 2), some uniting themes were observed in the mechanisms contributing to HIV latency control. We utilized the lists of GO terms and pathways that were reported in each of the four studies, to compare the gene categories dysregulated in different latency models. The reported terms were assigned to two major categories: Transcriptional regulation, including signaling pathways that regulate activity and localization of transcription factors, and functional categories related to RNA synthesis; and post-transcriptional regulation, both at the RNA and protein levels (Figure 3); terms that could not be assigned to these categories are not shown. Not surprisingly, the specific GO terms and pathways in each category were different between the studies, which was at least in part attributable to the usage of different annotated databases to obtain these terms (Table 2). However, terms associated with both transcriptional and post-transcriptional control of HIV latency were reported in more than one study. These GO terms and pathways comprise both well-established (*e.g.,* NFκB signaling and transcriptional regulation[86,87]) and novel mechanisms of regulation of HIV expression (*e.g.,* proteasome[18]).

Network-based approaches can also be utilized to identify genes that may have a role in regulation of HIV

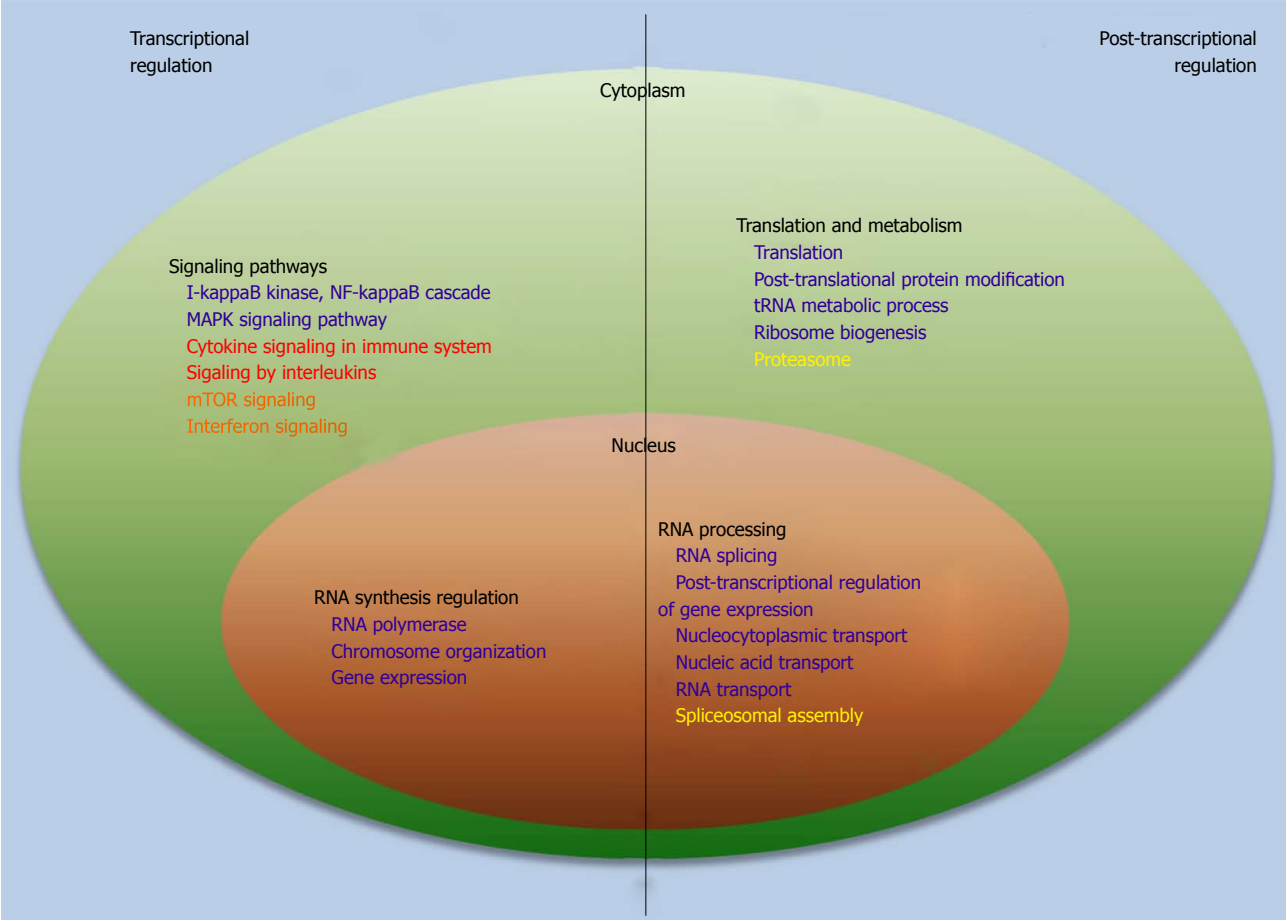

Figure 3 Transcriptional and post-transcriptional mechanisms of regulation of human immunodeficiency virus expression. Pathway and GO term categories related to transcriptional and post-transcriptional regulation of HIV expression, identified in gene expression studies that compared latently infected and uninfected cells, are shown. Dark blue, Iglesias-Ussel *et al*[19]; Red, Mohammadi *et al*[42]; Brown, Evans *et al*[76]; Yellow, Krishnan and Zeichner[18]. GO: Gene ontology; HIV: Human immunodeficiency virus; mTOR: Mammalian target of rapamycin.

expression, despite not being detected as differentially expressed in latency. For example, tubulin alpha 3 (*TUBA3)* was a well-connected gene in a network constructed by Bandyopadhyay *et al*[51] who utilized the Krishnan and Zeichner dataset[18]. *TUBA3* was connected to both Tat and Rev in the network, suggesting a possible yet unknown post-transcriptional role for this gene in regulation of HIV expression, one which would not have been detected in non-network-based approaches.

Taken together, functional studies using systems biology approaches to analyze host gene expression in the *in vitro* models of HIV latency suggest that maintenance of HIV quiescence in T cells involves basic cellular mechanisms beyond those traditionally implicated in transcriptional repression of the HIV-1 provirus.

TRANSCRIPTOME PROFILING AND SYSTEMS BIOLOGY APPROACHES TO IDENTIFY MOLECULAR MECHANISMS OF HIV REACTIVATION USING LRAS

HDACis have been the most studied LRAs, with a number of these compounds progressing to clinical trials[23-27]. The primary mechanism of action proposed for HIV reactivation using HDACis was histone acetylation and chromatin decondensation, which provide a transcriptionally favorable environment[88]. However, the results from gene expression profiling studies following the discovery of anti-cancer properties of HDACis (reviewed in[89]) strongly suggest the existence of secondary mechanisms of action of HDACis beyond chromatin remodeling. In particular, despite chromatin decondensation, as many genes were downregulated by HDACis as were upregulated. Over the years, studies using HDACis demonstrated that transformed cells responded to treatment differently as compared to primary cells[90-93]. Therefore, gene expression profiling of HDACis using primary CD4+ T cells is more relevant for delineating the mechanisms driving HIV reactivation. Most of the gene expression studies using HDACis in primary cells up-to-date have utilized the HDACi vorinostat/suberoylanilide hydroxamic acid (SAHA), which was the first of the FDA-approved HDACis for treatment of cutaneous T cell lymphoma[94]. These studies are summarized in Table 4. In addition to SAHA, the effects on gene expression were profiled for another HDACi, valproic acid (VPA) in primary CD4+ T cells

Table 4 Features of gene expression studies comparing suberoylanilide hydroxamic acid -treated and untreated primary cells

Study characteristics	Beliakova-Bethell *et al*[96]	Reardon *et al*[100]	White *et al*[99]	Mohammadi *et al*[42]	Elliott *et al*[25]
Cells used	Primary CD4+ T cells	Primary CD4+ T cells	Primary CD4+ T cells	*In vitro* primary CD4+ T cell latency model	Total blood from HIV-infected individuals on cART
Concentration or dose of SAHA	0.34 μmol/L	0.34, 1, 3, 10 μmol/L	1 μmol/L	0.5 μmol/L	400 mg orally once daily
Time of treatment	24 h	24 h	24 h	8 h and 24 h	14 d (samples analyzed at 2, 8 h; 1, 14 and 84 d)
Experiment replicates	9	6	6	Not reported	9
Gene expression profiling platform	Microarrays (Illumina HT12 Beadchips version 3)	Microarrays (Illumina HT12 Beadchips version 3)	Microarrays (Illumina HT12 Beadchips version 3)	RNA-Seq (polyA RNA library; Illumina HiSeq2000)	Microarrays (Illumina Human HT12 version 4)
Methods to identify DEGs	Multivariate permutation test (BRB-Array tools)	Dose-response analysis using likelihood ratio test (Isogene) with Bonferroni correction ($P < 0.05$)	Linear modeling (limma, FDR $P < 0.05$)	Generalized linear modeling (DESeq, FDR < 0.05)	Linear modeling (limma, $P < 0.05$)
Databases used for functional analyses	GO consortium, KEGG and Biocarta pathways (BRB-Array Tools), MetaCore networks	GO consortium, KEGG and Biocarta pathways (BRB-Array Tools), MetaCore networks	GO consortium, KEGG pathways (FAIME), MetaCore networks	Reactome pathways Ver.40; MsigDb	IPA, MsigDb
Total number of DEGs	1847	3477	2982	1289	Not reported

cART: Combination antiretroviral therapy; polyA: Polyadenylated; DEGs: Differentially expressed genes; BRB: Biometric Research Branch; FDR: False discovery rate; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MsigDb: Molecular Signature database; FAIME: Functional Analysis of Individual Microarray Expression; IPA: Ingenuity Pathway Analysis; HIV: Human immunodeficiency virus.

Table 5 Features of gene expression studies comparing cells treated with latency reversing agents of different functional classes and untreated cells

Study characteristics	Jiang *et al*[95]	Mohammadi *et al*[42]	Sung and Rice[97]	Banerjee *et al*[98]
Cells used	Primary cells from HIV-infected individuals on cART	*In vitro* primary CD4+ T cell latency model	Primary resting CD4+ T cells	J-Lat 10.6 T cell line
LRA (functional class)	Valproic acid (HDACi)	Disulfiram (alcohol dehydrogenase inhibitor)	Prostratin (PKC agonist)	JQ1 (bromodomain inhibitor)
Concentration	1 mmol/L (+20 U/mL IL-2)	0.5 μmol/L	250 ng/mL	0.1 μmol/L, 1 μmol/L
Time of treatment	6 h	8 and 24 h	48 h	24 h
Experiment replicates	4	Not reported	3	Not reported
Gene expression profiling platform	Microarrays (Agilent)	RNA-Seq (polyA RNA library; Illumina HiSeq2000)	Microarrays (Affymetrix Human Genome U133 Plus 2.0)	Microarrays (Affymetrix ST 1.0)
Methods to identify DEGs	Rosetta Resolver system ($P < 0.01$)	Generalized linear modeling (DESeq, FDR < 0.05)	*t*-test with FDR correction	ANOVA ($P < 1E-5$)
Databases used for functional analyses	Not used	Reactome pathways Ver.40; MsigDb	GO consortium, KEGG pathways	GO consortium
Total number of DEGs	199 (fold change > 3)	189	2514 (fold change > 1.5)	Not reported

cART: Combination antiretroviral therapy; LRA: Latency reversing agent; HDACi: Histone deacetylase inhibitor; PCK: Protein kinase C; polyA: Polyadenylated; DEGs: Differentially expressed genes; FDR: False discovery rate; ANOVA: Analysis of variance; MsigDb: Molecular Signature database; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; LRAs: Latency reversing agents.

from HIV-infected individuals. Treatment with either SAHA or VPA resulted in downregulation of V-Myc avian myelocytomatosis viral oncogene homolog (*MYC*)[95,96]. Among other LRA classes, the effects of alcohol dehydrogenase inhibitor Disulfiram and protein kinase C (PKC) agonist Prostratin on host gene expression were assessed using primary CD4+ T cells[42,97], while the effects of a bromodomain inhibitor, JQ1, on gene expression were assessed in a cell line model of HIV latency (J-Lat 10.6 T cell line)[98] (see Table 5 for the summary of the studies).

For all classes of compounds tested, Disulfiram appeared to induce minimal changes to host gene expression[42], while SAHA and Prostratin modulated thousands of genes[42,96,97,99,100]. Gene expression studies were able to identify novel mechanisms contributing to HIV reactivation out of latency by LRAs, besides their primary mechanisms of action. For example, in addition to chromatin decondensation, SAHA upregulated specific HIV transcriptional activators [*e.g.,* immunity-related GTPase family, M (*IRGM*)[101], heat shock protein 70 (HSP70, gene symbol *HSPA2*)[102,103] and lysine (K)-

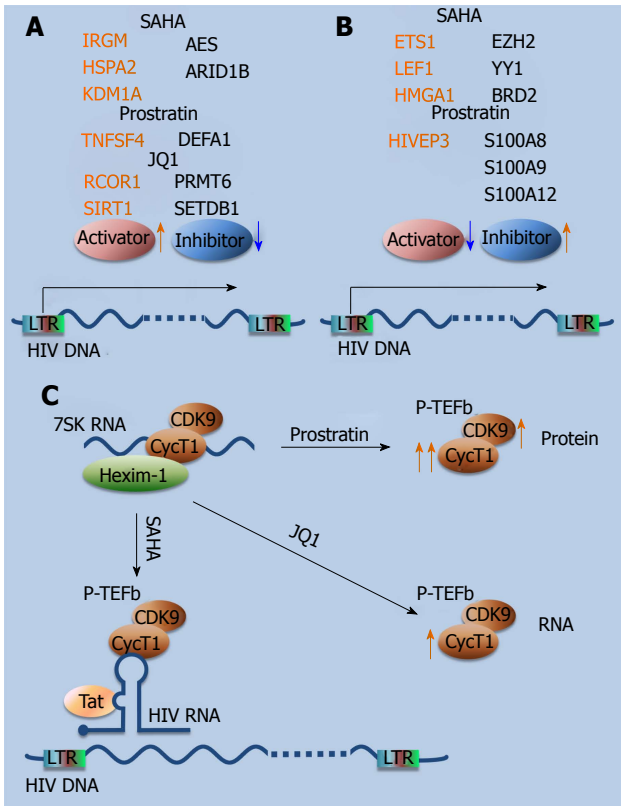

Figure 4 Main findings from gene expression studies using Latency reversing agents. A: Novel mechanisms of HIV reactivation besides primary mechanisms of action of LRAs. These include upregulation (red arrow) of HIV activators (red oval) and downregulation (blue arrow) of repressors (blue oval). Examples for LRAs from 3 functional classes (HDACi, SAHA; PKC agonist, Prostratin; and bromodomain inhibitor, JQ1) are listed; B: Effects of LRAs on host genes that are inhibitory for HIV reactivation. These include upregulation (red arrow) of HIV repressors (blue oval) and downregulation (blue arrow) of activators (red oval). Examples for LRAs from 2 functional classes (HDACi, SAHA; and PKC agonist, Prostratin) are shown; C: LRAs of different classes act on components of p-TEFb complex *via* different mechanisms, contributing to HIV reactivation. SAHA induced dissociation of p-TEFb from the inactive 7SK RNA complex and facilitated its recruitment to the HIV LTR. Prostrain and JQ1 upregulated components of p-TEFb complex at the protein and RNA level, respectively (red arrows indicate upregulation). LRA: Latency reversing agent; HDACi: Histone deacetylase inhibitor; PKC: Protein kinase C; SAHA: Suberoylanilide hydroxamic acid; IGRM: Immunity-related GTPase family, M; HSPA2: Heat shock 70 kDA protein 2; KDM1A: Lysine (K)-specific demethylase; TNFSF4: Tumor necrosis factor (ligand) superfamily, member 4; RCOR1: REST coreceptor 1; SIRT1: Sirtuin 1; AES: Amino-terminal enhancer of split; ARID1B: AT rich interactive domain 1B, SWI1-like; DEFA1: Defensin alpha 1; PRMT6: Protein arginine methyltransferase 6; SETDB1: SET domain, bifurcated 1; ETS1: V-Ets avian erythroblastosis virus E26 oncogene homolog 1; LEF1: Lymphoid enhancer-binding factor 1; HMGA1: High mobility group AT-hook 1; HIVEP3: HIV type I enhancer binding protein 3; EZH2: Enhancer of zeste 2 polycomb repressive complex 2 subunit; YY1: YY1 transcription factor; BRD2: Bromodomain protein containing 2; S100A8: S100 Calcium Binding Protein A8; S100A9: S100 Calcium Binding Protein A9; S100A12: S100 Calcium Binding Protein A12; CDK9: Cyclin-dependent kinase 9; P-TEFb: Positive transcription elongation factor; CycT1: Cyclin T1; Hexim-1: Hexamethylene Bis-Acetamide Inducible 1; LTR: Long terminal repeat; Tat: Transactivator of transcription.

specific demethylase (*KDM1A*)[104]], and downregulated repressors [amino-terminal enhancer of split[105] and AT rich interactive domain 1B, SWI1-like (*ARID1B*, or BAF250)[106]][25,99,100] (Figure 4A). Sung and Rice[97] found that Prostratin upregulated HIV activator, tumor necrosis factor (ligand) superfamily, member 4 (*TNFSF4*)[107], and downregulated defensin alpha 1, which interferes with PKC signaling[108]. Among genes with a role in regulation of HIV expression that were modulated by JQ1, Banerjee *et al*[98] noted upregulation of activators REST coreceptor 1 (*RCOR1*)[104] and the class Ⅲ deacetylase sirtuin 1 (*SIRT1*)[109], and downregulation of repressor methyltransferases, protein arginine methyltransferase 6 (*PRMT6*) and SET domain, bifurcated 1 (*SETDB1*)[110,111].

In addition to the effects of LRAs on gene expression that may promote HIV reactivation, possible inhibitory effects were also observed in gene expression studies that used SAHA and Prostratin-treated primary cells (Figure 4B). Genes encoding factors that activate HIV transcription, V-Ets avian erythroblastosis virus E26 oncogene homolog 1(*ETS1*), CCAAT/enhancer binding protein, Beta (*CEBPB*), and lymphoid enhancer-binding factor 1 (*LEF1*)[112-114], were downregulated by SAHA in primary CD4+ T cells[100]. Enhancer of zeste 2 polycomb repressive complex 2 subunit (*EZH2*), a methyltransferase implicated in HIV LTR silencing[115], was upregulated[100]. Genes encoding HIV transcriptional repressors YY1[116] and bromodomain protein containing 2 (*BRD2*)[117] were upregulated by SAHA in blood cells from HIV-infected individuals on cART[25]. Downregulation of *ETS1* and *LEF1* and upregulation of *BRD2* were confirmed at the protein level in primary CD4+ T cells[99]. In addition, a network-based approach integrating transcriptomics and proteomics datasets highlighted upregulation of high mobility group AT-hook 1[99], which represses HIV transcription by competing with Tat for TAR binding[118] and by recruiting inactive positive transcription elongation factor (p-TEFb) to the HIV LTR[119]. Possible inhibitory effects of Prostratin with respect to HIV reactivation identified by Sung and Rice[97] were upregulation of a repressor, HIV type Ⅰ enhancer binding protein 3[120], and downregulation of the three genes encoding S100 calcium-binding proteins (*S100A8, S100A9,* and *S100A12*), shown to enhance HIV-1 transcription in a NFκB-dependent manner[121].

Finally, gene expression profiling studies using LRAs of different functional classes highlighted uniting themes driving HIV reactivation, such as importance of the components of p-TEFb complex (Figure 4C). Cyclin T1 (CycT1) was upregulated at the RNA level by JQ1[98]; both CycT1 and cyclin-dependent kinase 9 were upregulated at the protein level by Prostratin[97], while SAHA induced dissociation of p-TEFb from the inactive 7SK RNA complex and facilitated its recruitment to the HIV LTR[122]. Though through different mechanisms, p-TEFb function appears to be enhanced *via* action of several classes of LRAs.

CONCLUSION AND PERSPECTIVES

This review discusses how methods of gene expression profiling and systems biology can be applied to address specific questions in the field of HIV latency and eradication. It presents a systematic analysis of the

application of these methods to discover biomarkers of latency, identify molecular mechanisms of latency control and reactivation using LRAs. Identification of DEGs and functional category assessment are the most common methods currently used in the field (Figure 1). Network-based approaches are utilized in a subset of more recent studies. Advances in RNA-Seq technologies allow for integration of HIV expression analysis with the changes in expression of host genes in a single experiment. Integration of transcriptomic data with other biological data types in the field of HIV latency is presently scarce; and the field would benefit from increased adoption of these methods in future studies.

Gene expression analysis of latently infected and uninfected cells has been used to identify candidate biomarkers of latency and to delineate the molecular mechanisms that contribute to regulation of HIV expression. Studies comparing gene expression in HIV latency models to uninfected cells have several limitations that presently preclude from achieving a consensus on what genes may represent suitable biomarkers (Table 3). Improved bioinformatics approaches (*e.g.,* using the same methods of data acquisition and statistical analyses across models) and experimental validation of candidate biomarkers would be extremely useful in future studies to more reliably identify biomarkers of latency. Studies profiling gene expression changes induced by LRAs identified novel mechanisms of action of the LRAs and their inhibitory effects with respect to HIV reactivation out of latency, as well as highlighted uniting themes driving HIV reactivation. Using similar statistical approaches in prospective studies using LRAs would facilitate prediction of whether the inhibitory effects of different LRAs on HIV reactivation could be cancelled out in a combination strategy. The results from such studies would have the potential to significantly impact the process by which candidate drugs are selected and combined for future evaluations and advancement to clinical trials.

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MINIREVIEWS

Osmolyte transport in *Staphylococcus aureus* and the role in pathogenesis

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Abstract

Osmolyte transport is a pivotal part of bacterial life, particularly in high salt environments. Several low and high affinity osmolyte transport systems have been identified in various bacterial species. A lot of research has centered on characterizing the osmolyte transport systems of Gram-negative bacteria, but less has been done to characterize the same transport systems in Gram-positive bacteria. This review will focus on the previous work that has been done to understand the osmolyte transport systems in the species *Staphylococcus aureus* and how these transporters may serve dual functions in allowing the bacteria to survive and grow in a variety of environments, including on the surface or within humans or other animals.

Key words: *PutP*; *OpuD*; *Staphylococcus aureus*; Proline transport; Osmolyte

Core tip: *Staphylococcus aureus* (*S. aureus*) is the number one cause of skin and soft tissue infections. In the United States, *S. aureus* is usually the number one hospital-acquired pathogen. The skin and urinary tract organs are high osmotic stress environments. Osmolyte transport is essential for *S. aureus* survival in different environmental niches, such as within human skin abscesses or the human urinary tract.

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INTRODUCTION

A well conserved, evolutionary strategy used by many organisms to adapt to high osmotic conditions is the transport of organic compounds, called compatible solutes[1]. These compatible solutes serve as cytoplasmic solutes that balance water relations, without interfering with normal cytoplasmic activities, within cells grown in high salt environments. Examination of the transport

systems in *Staphylococcus aureus (S. aureus)* may provide insight into how proline and glycine betaine may be transported into Gram-positive bacteria.

GENERAL OSMOLYTE TRANSPORT FEATURES IN *S. AUREUS*

Although osmolyte transport is best described in *E. coli*[1-3], there are also compatible solute transport systems in *S. aureus* to adapt to high salt environments[4]. Studies have shown that *S. aureus* cells grown in very high salt environments had increased intracellular levels of proline and glycine betaine[5-11]. Other intracellular molecules that also increased in high NaCl environments were choline, proline betaine, taurine, and glutamic acid[6,7,12]. Of these accumulated solutes, proline and glycine betaine were the most effective osmoprotectants of *S. aureus*, since *S. aureus* growth was observed when these solutes were excluded from defined high osmotic media[6,8,12].

Identification of genes that encode transport proteins and their importance for the survival of *S. aureus* coincides with previous observations that *S. aureus* requires several amino acids as a source of carbon and nitrogen[4]. Of these essential amino acids, proline and other amino acids are not synthesized by *S. aureus*[4,13,14]. The accumulation of most of the proline in *S. aureus* occurs because of proline transport proteins.

Although prior research performed using other Gram-positive bacteria may not have specifically addressed proline transport, it does help in uncovering commonly conserved mechanisms of compatible solute transport in *S. aureus*. Several studies that have examined compatible solutes accumulation in *S. aureus* grown at high osmotic environments showed increased intracellular levels of proline, aminobutyric acid, glutamic acid, choline, taurine, and glycine betaine[5-7,15,16]. Of these compatible solutes, only glutamic acid is synthesized by *S. aureus*, whereas the other compatible solutes have to be imported from the external environment[5,7,8,17-19]. To substantiate the osmoprotective importance of these transported compatible solutes, the growth rates of *S. aureus* grown in defined high osmotic media was observed to increase when supplemented with either proline or glycine betaine[8]. Although *S. aureus* normally possess relatively large concentrations of glycine betaine and potassium ions, compatible solute transport is believed to aid in creating high intracellular pressure that enables *S. aureus* to survive in high osmotic environments[15].

SPECIFIC PROLINE TRANSPORT SYSTEMS IN *S. AUREUS*

Initial proline uptake research using whole cell assays on *S. aureus* has shown the presence of at least two proline transport systems[10,17,20]: Both a low- and high-affinity system. These systems may be similar to the OpuE and OpuD transport systems found in *B. subtilis*[21,22] and they share properties with the PutP and ProP systems of *E. coli*[1]. They are both sodium-dependent transporters, since gramicidin D and monensin, which collapse Na^+ gradients, inhibit proline transport in both systems[10]. Proline transport in either system showed low susceptibility to inhibition by glycolysis and ATP formation by a combination of NaF and sodium iodoacetate or sodium arsenate, respectively. Lastly, alterations of pH from 5.5 to 8.5 had little effect on the transport rates of proline[10].

In *S. aureus*, proline transport kinetics is hard to interpret because of strain differences and the calculation setups used to determine the K_m and V_{max} values reported, one based on per mg protein and the other per mg dry weight. Reports have shown that the high-affinity proline transport system in *S. aureus* had a K_m ranging from 1.7 to 7.0 mol/L, with a V_{max} ranging from 1.1 nmol/min per milligram dry weight to 10 nmol/min per milligram protein[10,17]. Though these numbers are not directly comparative, they do give us a relative range of activity for this system, which correlates to a previously observed K_m value of 3.5 mol/L for proline uptake with vesicles prepared from *S. aureus* grown in a low-osmolarity medium[23] and K_m values of the PutP system in *E. coli*[1,17,24-26]. Moreover, like the PutP system of *E. coli*[1], the high-affinity proline transport system in *S. aureus* is specific for the transport of proline and it's activity increases when proline deprivation is encountered, suggesting that this system may also be involved in scavenging low concentrations of proline from the environment[10]. Further proof of the relatedness of these systems can be seen from the complementation of a genetic defect in proline transport within *E. coli* by the high-affinity proline transport system of *S. aureus*[27]. At the structural level, the PutP homolog of *S. aureus* shows a sodium-binding motif, the same ten conserved amino acids found in all other members of the sodium/solute symporters[28], and the predicted PutP protein of *S. aureus*[29] shares considerable similarity with the PutP protein of *E. coli*[1]. Although many similarities exist between the high-affinity proline transport systems in *S. aureus* and *E. coli*, major differences between these systems include: The concentration of NaCl appears to have no effect on proline transport in *S. aureus*[8,17]; the *S. aureus putP* gene is activated by high concentrations of osmolytes in the environment[30], whereas the *E. coli putP* gene is not[1,25,29]; and the *S. aureus putP* gene is regulated by SigB[30], which is similar to the regulation shown for *opuE* in *B. subtilis*[21]. Although PutP has a sodium binding motif and has homology with sodium/solute symporters, the concentration of NaCl does not affect proline transport[7,17], It is possible that when *S. aureus* is grown in an environment with a low sodium concentration that PutP behaves like other bacterial high affinity proline transporters that are driven by a sodium motive force. On the other hand, *S. aureus* grown in a high sodium environment may cause the PutP protein to use a proton motive force instead of a sodium motive

Table 1 Distribution of proline and glycine betaine transport genes in some sequenced

	S. aureus strains			
Gene	N315	MW2	COL	Mu50
putP	SA1718	MW1843	SACOL1963	SAV1902
putP	SA0531	MW0528	SACOL0620	SAV0573
opuD	SA1183	MW1236	Yes (2)[2]	SAV1349[4]
opuD1	-[1]	-[1]	SACOL1384	ND[3]
opuD2	-[1]	-[1]	SACOL2176	ND[3]
opuCA	SA2237	MW2372	ND[3]	SAV2448
opuCB	SA2236	MW2371	ND[3]	SAV2447
opuCC	SA2235	MW2370	ND[3]	SAV2446
opuCD	SA2234	MW2369	ND[3]	SAV2445

[1]Does not possess; [2]Multiple *opuD* genes in this species; [3]Not determined; [4]The gene appears to be fragmented into two pieces.

force to bring proline into the cell.

The low-affinity proline transport system of *S. aureus* also has similarities to the low-affinity proline transport system (ProP) of *E. coli*. For proline transport, the K_m value of *S. aureus* ATCC 12600 (K_m of 420 mol/L and V_{max} of 110 nmol/min per milligram protein) is similar to the K_m value of ProP in *E. coli* (approximately 300 mol/L)[17]. For *S. aureus* (K_m of 132 mol/L and V_{max} of 22 nmol/min per milligram dry weight), a greater difference in the K_m values for the low-affinity proline transport system can be seen between strains as compared to the difference in K_m values for the high-affinity system. Again, the K_m and V_{max} values from the ProP system of *E. coli* fit within the overall range found for *S. aureus*[1,31-33], but strain variation along with calculation setup differences may again be the cause of these divergent numbers. Excluding the differences of the K_m and V_{max} values between strains, the low-affinity proline transport systems of different *S. aureus* strains possess identical characteristics[10,17]. Many of these characteristics are similar to the regulatory and functional properties of the ProP system of *E. coli*[34] (*i.e.,* both of these systems transport proline and are stimulated by increasing osmolarity produced by either ionic or nonionic solutes)[17].

DIFFERENCES IN THE *S. AUREUS* OSMOLYTE TRANSPORT SYSTEMS COMPARED TO OTHER BACTERIA

Though these systems are similar, there are some major differences between the Gram-negative and Gram-positive low-affinity proline transport systems. One major difference is that the low-affinity proline transport systems in *S. aureus* are optimally activated at NaCl concentrations ranging from 0.75 to 1.0 mol/L[17,35], whereas the low-affinity proline transport systems in *E. coli* are inhibited by NaCl concentrations greater than 0.2 to 0.3 mol/L[29,36]. Other major differences include glycine betaine transport activity by the low-affinity proline transport system has not been conclusively established and there conflicting opinions and data presented for the glycine betaine transport activity for the low-affinity system[9,17,18,20,37]. In part, the previous lack of any low-affinity system mutants in those studies complicated the examination of glycine betaine transport activities. Since glycine betaine accumulation has been linked to proline transporters in Gram-negative bacteria[1] and *S. aureus* has been shown to transport glycine betaine from the external environment[38], this suggests that an additional glycine betaine transporter that is osmotically stimulated may be present in *S. aureus*. Moreover, *S. aureus* cells shocked with 0.5 mol/L NaCl in the presence and absence of chloramphenicol (100 g/mL) showed identical levels of transported proline, suggesting that new protein synthesis is not necessary for rapid proline uptake and that osmotic shock activates a pre-existing proline transport system[10].

BIOINFORMATIC TOOLS TO IDENTIFY OSMOLYTE TRANSPORT SYSTEMS IN *S. AUREUS*

Sequencing of several *S. aureus* genomes has provided a wealth of information on the existence of several putative osmolyte transport systems in *S. aureus*[14,39,40]. All of the strains appear to have a conserved *putP* gene for high affinity transport of proline, although there appears to be homologs for both a *proP* gene[1] and *opuD* gene[21,35] (Table 1). Additional analyses have shown that the *opuD* gene (encoding a low affinity proline transporter) is activated under osmotic stress conditions and OpuD transports proline under low affinity growth conditions[35]. Furthermore, a mutation in the *S. aureus proP* gene also causes lower proline transport in media with high concentrations of proline (Schwan WR unpublished data).

This is the first instance of both the ProP and OpuD low affinity proline/glycine betaine transport homologs being identified in one species and suggests the importance that proline transport must have in the survival of *S. aureus* cells in a variety of environments. Furthermore, the *opuC* system, which putatively transports glycine betaine/carnitine/choline, has also been observed. Together, the bioinformatic comparisons have uncovered some very interesting genomic features in *S. aureus* centered on osmolyte transport. A summary of the four osmolyte transport systems in *S. aureus* tied to proline transport and other known solutes is noted in Figure 1.

OSMOLYTE TRANSPORT TIED TO *S. AUREUS* SURVIVAL IN HUMANS AND MICE

The rationale of investigating proline and glycine betaine transport in *S. aureus* is not purely academic. In planktonic *S. aureus*, the glycine betaine level is high,

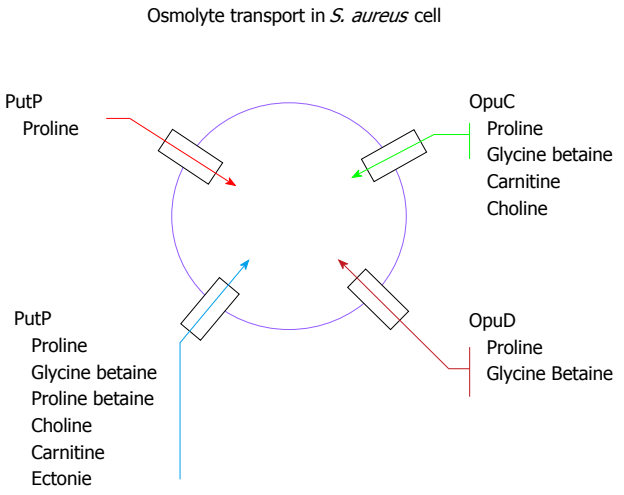

Figure 1 The four prominent osmolyte transport systems in *Staphylococcus aureus* tied to proline transport as well as other solutes.

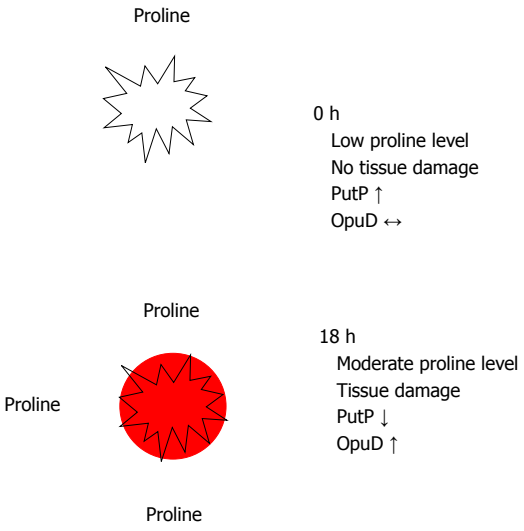

Figure 2 Model for the roles of proline transporters in *Staphylococcus aureus* pathogenesis within a murine abscess.

but lower in *S. aureus* found in biofilms[41]. Glycine betaine is the most effective osmoprotectant. To achieve the high glycine betaine level, an active glycine betaine transporter would need to be functioning in the planktonic *S. aureus* cells that are immersed in an environment of high osmotic stress, like the human skin.

Indirect effects on *S. aureus* survival have been tied to osmolyte transport systems. Defects in the cell wall caused by a *femAB* mutation caused an upregulation of *opuC* (glycine betaine/carnitine/choline transporter) and downregulation of *opuD* to compensate for the defect[42]. YhcSR encodes a two-component signal transduction system that is required for *S. aureus* survival. This two-component regulatory system regulates transcription of the *opuCABCD* operons affecting proline and glycine betaine levels in *S. aureus*[43]. One study examining daptomycin resistance revealed an accumulation of glycine betaine within *S. aureus* cells that was coupled with upregulation of the *cudT* (choline transporter) gene, a beta choline dehydrogenase gene, a *gbsA* gene (glycine betaine aldehyde dehydrogenase), an *opuD2* gene, and the *proP* gene[44]. Uptake of choline is needed to produce glycine betaine internally, the best osmoprotectant[19].

More directly, a transposon mutation in the gene for the high affinity (PutP) proline transport system of *S. aureus* rendered the bacteria less able to survive in several animal infection models[45-47]. Within cardiac vegetations, the viable *S. aureus* count was 1-3 logs lower than the wild-type parent strain[45]. Transcription of *putP* was shown to increase 105-fold shortly after *S. aureus* infection of murine kidneys[30]. In *S. aureus* infected murine bladders, spleen and livers, *putP* transcription was also elevated very quickly and then dropped markedly as the infection progressed. Proline levels in livers and spleens are very low[47] and the levels are likely low in the other organs (*e.g.*, bladder and kidney), but through tissue damage by staphylococcal toxins, the concentration of proline may increase substantially and in turn shut off transcription of the high affinity proline transport gene.

Conversely, transcription of the low affinity proline transport gene *opuD* was shown to be the highest after 4 h post-infection in murine bladders and 18 h post-infection in murine thigh abscesses[35]. Within murine bladders and kidneys, high osmotic conditions prevail. Initial observations demonstrated that at least one of the low-affinity proline transport systems of *S. aureus* was activated under moderate to high osmotic conditions[17], which has been subsequently confirmed[35].

Our model is that PutP is important in the early stages of an infection when proline concentrations are low, but OpuD expression is not as important (Figure 2). As the infection proceeds, tissue damage occurs, which releases free proline. By 18 h post-infection, the level of free proline is higher and OpuD becomes important at this stage of the infection.

These studies suggest that osmolyte transport systems may play essential roles in survival of *S. aureus* within humans or mice. Characterization of the proline and glycine betaine transport systems will provide us with experimental proof of the importance of these systems during growth in high osmotic conditions, how these systems are regulated, and will further our understanding of the significance of the proline/glycine betaine transport to the survival of *S. aureus in vivo*.

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REVIEW

Challenges in management of recurrent and refractory *Clostridium difficile* infection

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Abstract

Clostridium difficile infection (CDI) is the most common nosocomial infection in the United States and is associated with a high mortality. One quarter of patients treated for CDI have at least one recurrence. Spore persistence, impaired host immune response and alteration in the gastrointestinal microbiome due to antibiotic use are factors in recurrent disease. We review the etiology of recurrent CDI and best approaches to management including fecal microbiota transplantation.

Key words: *Clostridium difficile* infection; Epidemiology; Outcomes; Treatment; Fecal microbiota transplantation

Core tip: Recurrent *Clostridium difficile* infection (RCDI) is common and can be difficult to treat. Clostridia spores transmit disease. They are ubiquitous and hard to eradicate. The composition of the gut microbiome plays an essential yet poorly understood role in maintaining overall health, and in protecting against *Clostridium difficile* (*C. difficile*) infection. Antibiotic induced dysbiosis of the microbiome is a key contributor to RCDI. Here we review how *C. difficile* spores and alterations in the microbiome contribute to RCDI.

Meehan AM, Tariq R, Khanna S. Challenges in management of recurrent and refractory *Clostridium difficile* infection. *World J Clin Infect Dis* 2016; 6(3): 28-36 Available from: URL: http://www.wjgnet.com/2220-3176/full/v6/i3/28.htm DOI: http://dx.doi.org/10.5495/wjcid.v6.i3.28

INTRODUCTION

Clostridium difficile (*C. difficile*) is a gram positive, anaerobic, spore forming bacteria first associated with antibiotic-associated and pseudomembranous colitis in 1978[1,2]. Originally isolated from meconium and feces of newborn infants in 1935, it was dubbed "*Bacillus difficilis*" due to its poor culture growth characteristics[3]. Although

C. difficile culture is achievable now using Cycloserine Cefoxitin Fructose Agar media[4], the moniker remains apt, albeit for different reasons. A diagnosis of *Clostridium difficile* infection (CDI) adds considerably to healthcare cost, length of stay, complications and mortality[5,6].

CDI diagnosis is based on symptoms and toxin detection, and initial treatment involves oral metronidazole for mild-moderate cases or oral vancomycin if severe[7]. Both metronidazole and vancomycin lead to intestinal dysbiosis and impair "resistance to colonization" actually facilitating recurrence[8].

Recurrent CDI (RCDI) is defined as recurrence of clinical symptoms with a positive *C. difficile* stool test within 8 wk of symptom resolution[9]. Twenty to twenty five percent of CDI patients will have at least one recurrence[10] and subsequent risk can be as high as 40%-65%[11]. Reinfection *vs* relapse are indistinguishable clinically, however based on serotyping and PCR ribotyping up to 50% of patients recur with a strain that is different to the original one[12]. RCDI relates to spore production and persistence, the host immune response (or lack of it) to toxins, and alterations in the gut microbiome.

C. DIFFICILE SPORES: RESISTANCE AND PERSISTENCE

C. difficile spores are the agents of disease transmission[13]. They are ubiquitous and may survive on contaminated surfaces for months, possibly years[14-16]. *C. difficile* pathophysiology relates to spore exposure and ingestion, spore vegetation and toxin production in the setting of an altered host gut microbiome[17]. A healthy gut flora is protective against colonization and infection from *C. difficile*[18]. Asymptomatic colonization with toxin negative and positive strains has been described[8,19].

Anaerobic bacteria form spores when conditions are not conducive to growth (*i.e.*, starvation), specifically when deprived of carbon or nitrogen[16]. Clostridial spores are metabolically inactive (dormant) and impervious to most environmental assaults (except bleach)[7]. Anaerobic spore DNA is protected from damage by several mechanisms that have been established in related clostridial and bacteroides species and extrapolated to *C. difficile*. These include the fact that the spore core is anhydrous (water content 25%) and acidic (pH 6.5), which inhibits enzymatic activity and immobilizes most proteins[16]. There are high levels of ionic calcium-dipicolinic acid in the spore core, which forms a 1:1 complex with DNA. Deletion experiments suggest that saturation of DNA with α/β small acid soluble spore proteins (SASPs) is the dominant protective mechanism[20]. Mutants spores that lack α/β SASPs and calcium-dipicolinic acid lose viability rapidly during sporulation due to DNA damage[16].

Spores are the main vehicle of disease transmission, persistence and recurrence in CDI[14]. The environmental spore load necessary to infect 50% of mice after 1 h in one series of experiments was 5-10 spores/cm^2[21,22]. Spores shed through stool contaminate skin, bed clothes and even air, reaching 53-426 colony forming units/m^3 of air[15]. Mutants unable to produce Spo0A (a transcription regulatory protein essential for sporulation) do not persist or transmit disease in mice[23]. Thus elimination of spores can interrupt disease transmission. Presently this is most often pursued in the health care setting in the context of a known case (we don't as yet target spores in the community)[7]. Sodium hypochlorite (*i.e.*, bleach) is the most commonly used agent, with far UV light and vapor hydrogen peroxide also effective[14].

There are several additional issues of note. In murine gut *C. difficile* sporulates at a rapid rate - 56% relative to vegetative cells at 14 h post infection[24]. The murine colonic environment supports sporulation by phosphorylation of the master regulator Spo0A[14,23,25]. Presumably similar unknown triggers are present in the human gut.

Recent whole genome sequencing of CDI isolates in > 1200 patients with disease showed only 35% of cases were related to known cases, which suggests alternate routes of exposure (animals/food), outside of health care settings[26] (Presumably patients got the disease from spores in the community). The prevalence of asymptomatic carriage in hospital admission ranges from 7%-18%[27].

This has great clinical implications. Widespread community colonization with toxigenic *C. difficile* suggests that attempts to restrict spore spread only in the context of known exposure in healthcare settings may be insufficient. For meaningful interruption, universal modified contact precautions for all admissions may be necessary. Measures to prevent spore formation may alter the transmission cycle. Further study of the mechanism of spore formation may identify new targets. Thus far only fidaxomicin has been shown to decrease spore formation most likely by inhibiting transcription of sporulation genes[28]. Its high cost however precludes widespread use, as discussed below.

VEGETATIVE FORMS: TOXIN PRODUCTION AND CDI

Germination of spores to toxin producing vegetative forms can occur within minutes of exposure to specific triggers deemed germinants (*i.e.*, taurocholate)[14,16]. Taurocholate (a primary bile acid) is both necessary and sufficient to trigger *C. difficile* germination. L-glycine acts as a cogerminant[29]. In contrast, certain secondary bile acids, *i.e.*, deoxycholate can inhibit vegetative growth[30]. Secondary bile acids are derived by the action of endogenous flora on primary bile acids[31] and the relative ratio of each in the colon may determine spore/vegetative balance.

Toxins A (TcdA) and B (TcdB) and binary toxin (CDT) are the major virulence factors that contribute to pathogenesis[32]. Toxins A and B are multi-domain proteins that share a high degree of homology and comprise an N terminal catalytic domain with glucosyltransferase activity, a middle translocation domain and a C-terminal host cell binding region[33]. The toxin receptor remains unknown.

Both A and B are proinflammatory and cytotoxic and it is not clear if both are needed for pathogenesis[34]. Both alter the actin cytoskeleton, disrupt the epithelial barrier and cause apoptosis by glucosylation and inactivation of GTPases-Rac, Rho and Cdc42[35]. This induces mucosal damage and inflammation. Toxin expression derives from a 19.6 kb pathogenicity chromosomal locus (PaLoc) that encodes *TcdA* and *TcdB* in addition to *TcdR* (RNA polymerase sigma factor that positively regulates toxin expression), *TcdC* (putative negative regulator-deletion in 027 ribotype may increase toxin production), and *TcdE* (related to bacteriophage holins)[32,35]. The role of the toxins in the bacterial life cycle is unclear. Different *PaLoc* variants are called toxinotypes: 34 are described[36]. *PaLoc* has features of both stable integration and a mobile genetic element[37]. The CDT-binary toxin expressed in 027 ribotype ADP ribosylates G actin in target cells leading to protrusion bodies of microtubules that contact *C. difficile* and possibly increase colonization efficiency[38].

Toxigenic *C. difficile* causes disease: However colonization with toxigenic *C. difficile* can be asymptomatic[27]. After successful treatment many patients will continue to shed spores without manifesting disease. Colonization is a critical step in the pathogenic process and depends on adherence to gut epithelial cells by adhesion and flagellin proteins[39-41].

Colonization with non-toxin forming *C. difficile* may out-compete toxin forming *C. difficile*[27]. In one recent study, administration of nontoxigenic *C. difficile* spores (NCTD-M3) to patients after treatment of either first CDI episode or first recurrence, showed a 3-fold reduction (from 30% to 11%) in recurrent disease compared to placebo[42]. Patients given 10^7 spores/day for 7 d had the lowest recurrence rate (5%)[42]. The study does raise some concerns, primarily the possible acquisition of toxin containing PaLoc sequences by toxin negative strains, an event that has been shown to occur *in vitro*[43].

In theory, non-antibiotic toxin binders could ameliorate disease without disrupting intestinal flora. Cholestyramine, which binds toxin has been tried[44]. One difficulty is that it also binds vancomycin (as does colestipol and other anion exchange resins), complicating its use[45]. It can also bind bile salts and potentially stimulate *C. difficile* growth[46]. Given lack of efficacy data and possible harmful interactions use of cholestyramine or colestipol is not recommended.

Tolevamer, a polymer of styrene-sulfate that binds *C. difficile* toxin *in vitro*, was inferior to both metronidazole and vancomycin in 2 phase Ⅲ trials[47]. Only 44% of patients who took tolevamer had resolution of diarrhea or abdominal pain compared to 73% for metronidazole and 81% for vancomycin[47].

IMMUNE RESPONSE TO TOXINS AND CDI

Only half of hospitalized patients colonized with *C. difficile* develop CDI, and initial disease is associated with lack of anti-toxin A IgG[48]. The host immune response also plays a part in recurrent disease- patients with antibodies to toxin are less likely to relapse than those with undetectable toxin antibody[49,50]. Passive immunization by administration of intravenous immunoglobulin may have a role in patients with hypogammaglobulinemia[51,52], or in patients with severe disease[53].

Specific anti-toxin antibodies prevent mortality independent of antibiotic treatment. In one study a 3-fold reduction in relapse (25% to 7%) was seen when anti-toxin antibodies were used[54]. Data in animal models supports the efficacy of toxin-targeted vaccines[55]. Formalin inactivated toxin A/B (toxoid) protected hamsters from lethal *C. difficile* challenge[56]. Currently there are 2 vaccines in human trials. Sanofi Pasteur formalin inactivated toxins A/B vaccine was safe, well tolerated and immunogenic (generated antibodies to toxin)[57]. It is now in phase Ⅲ trial for primary prevention (https://clinicaltrials.gov/ct2/show/NCT01887912). An alternate approach involves a recombinant fusion protein of toxins A/B. A phase 1 trial of escalating doses of this recombinant is completed and results are pending (https://clinicaltrials.gov/ct2/show/NCT01296386).

There is some evidence of efficacy of vaccines in secondary prevention of RCDI[58], but more data is needed.

STANDARD ANTIMICROBIAL TREATMENT OF RCDI

Antimicrobial stewardship remains a key element of any RCDI management strategy. The reader is directed to other reviews for further discussion[59-61]. This review will focus on RCDI specific treatment.

Standard antimicrobial therapy targets the vegetative forms of *C. difficile*[7,52]. Spore vegetation and recurrent CDI are intricately linked. Favoring germination (by altering the germinant/sporulation ratio towards vegetation) would in theory allow eradication with antibiotics. Depending on antibiotic used however, this can also alter the microbiome and could increase the likelihood of relapse. Alternatively inhibiting germination, *i.e.*, by altering the gut flora towards secondary bile acids that inhibit vegetative forms[46] might also be a therapeutic option.

The use of vancomycin to treat CDI predates recognition of *C. difficile* as the causative agent of antibiotic associated colitis. First recurrence of CDI is treated with the same agent used for the initial episode. If clinically severe then vancomycin is used[7,52]. For second recurrence, pulsed and/or tapered vancomycin is recommended. Metronidazole is not used beyond the first recurrence due to possible cumulative neuropathy[62] (Table 1 is a summary of general clinical approach to RCDI).

Data supporting these recommendations is recognized as weak and poor quality with no corroborative randomized controlled trials.

Tedesco *et al*[63] reported on 22 patients treated for 21 d with a vancomycin taper and pulse and noted

Table 1 Management outline for recurrent *Clostridium difficile* infection[7]

General
Stop/minimize antibiotics (if possible, to allow gut flora to repopulate)
Rule out other causes of diarrhea, *i.e.*, post-infectious IBS
(check stool for C diff only in context of symptoms, not as test of cure)
Antibiotic treatment
Use the same antibiotic as initial regimen (depending on disease severity and response to initial treatment)[7,52]
Consider Vancomycin taper ± pulse[11]
Vancomycin followed by rifaximin chaser[67]
Fidaxomicin[80]
Probiotics
Probiotics with antibiotics may help[99]. Consider adding to last 2 wk of vancomycin pulse/taper and continue for 4 wk after
(caution in immunocompromised patients- may cause fungemia. Don't use in isolation. Not standardized, doses/active agents may vary)
Immunotherapy
Monoclonal antibody (neutralize toxin)[54]
IVIG[51]
Toxoid vaccine[58]
Non toxigenic strains[42]
Bacteriotherapy
Fecal microbiota transplant[111,114]

IBS: Irritable bowel syndrome; IVIG: Intravenous immunoglobulin.

no relapses (average follow-up 2-12 mo). In McFarland *et al*[11], 83 patients treated with 10-14 d course of vancomycin had an average relapse rate of 55% (range 42%-71%, depending on vancomycin dosing). Twenty-nine patients were treated with a vancomycin taper over an average of 21 d and 31% relapsed. If vancomycin taper was followed by vancomycin pulse (drug dosed every 48 or 72 h) then relapse decreased to 20% (10 patients). Lastly, 7 patients treated only with vancomycin pulse had 14% relapse[11]. The theory behind pulsed doses is to target vegetative forms of *C. difficile* but still allow restitution of the gut flora[11]. These numbers are small and the approach is not standardized. Oral vancomycin is also expensive: A 6 wk tapered course can cost hundreds of dollars[64].

Management of those who fail pulsed/tapered vancomycin is challenging.

ALTERNATIVE AGENTS FOR RCDI

Rifaximin is a synthetic rifamycin derivative that inhibits transcription[65]. It has little (< 0.4%) systemic absorption[65]. It is not used as monotherapy due to rapid emergence of resistance[66,67]. It has been used as an adjunct to vancomycin after 2 wk of standard treatment or taper[67]. Dosed at 400 mg BID for 2 wk after vancomycin taper, cure was described in 17/20 patients in 3 reports[67-69]. Recurrence rate was similar (15%) in a small (68 patients) RCT[70].

Fidaxomicin is the first macrolide antibiotic with an 18 membered macrocyclic lactone ring[71]. It is bactericidal and acts at an early step of RNA synthesis (it stops DNA strand separation)[72]. The *C. difficile* minimum inhibitory concentration is lower than that for vancomycin or metronidazolel[73]. A prolonged post antibiotic effect of at least 10 h allows twice daily dosing[74]. It is not absorbed systemically and has minimal effect on the gut microbiome. The effect on transcription inhibits both sporulation and toxin production[28,75]. The effect on sporulation may impact recurrences.

In vitro then and based on mechanism of action fidaxomicin should be an attractive option for RCDI. Indeed, in a phase 3 trial fidaxomicin was non inferior to vancomycin in terms of clinical cure[76]. Moreover, in the same study it strikingly decreased recurrence rates from 24%-25% to 13%-15%. Adverse event profiles were similar.

Subset analysis looking specifically at RCDI confirmed both the efficacy of fidaxomicin and decreased recurrence[77]. The stumbling block with fidaxomicin is the prohibitive cost ($140 per pill, 2800 for ten day course)[52].

Cadazolid, a novel hybrid antibiotic with a quinolone pharmacophore incorporated in an oxazolidinone ring has potent anti *C. difficile* activity and decreased propensity to induce antibiotic resistance[78,79]. It has a dual mechanism of action, both inhibiting translation and DNA synthesis[78,80]. Phase 1 studies with doses up to 3000 mg indicated the drug to be generally well tolerated with headache and diarrhea being most common SE.

A phase Ⅱ multi-center, double-blind, randomized study was conducted in 84 CDI patients. Cadazolid was dosed at 250, 500, or 1000 mg and deemed comparable or superior to vancomycin with respect to clinical and sustained cure rates[79,81]. Lower recurrence rates (18%-25% *vs* 50%) were noted for all doses[82]. Although there is no data as yet in RCDI, given decreased recurrence rate, and reported impact on spore production efficacy in RCDI is of significant interest.

GASTROINTESTINAL MICROBIOME: ROLE IN CDI

The adult gastrointestinal tract has 10^{14} bacterial cells from > 1000 different bacterial species[83,84], which comprise the microbiome, or gut flora. Composition varies depending on diet, age and health[85]. A "healthy" microbiome has a large number of different species of microorganisms with more of certain phyla, *i.e.*, *Firmicutes* and *Bacteroides* and less of others, *i.e.*, *Proteobacteria*[86]. Gut bacteria play critical roles in immunity, epithelial barrier function (resist pathogens) and nutrient absorption[87]. Any imbalance (in number, species, or composition) can distort this symbiosis leading to the converse, known as dysbiosis[88,89]. The microbiome varies between individuals but is generally stable over time[90].

C. difficile can be part of the normal microbiome[88], but is generally contained by other more dominant anaerobes. A healthy microbiome may protect against CDI in different ways. One may simply be due to numbers and competition for nutrients and mucosal niches[30].

Alternatively, the microbiome may elicit substances, *i.e.*, short chain fatty acids that actively inhibit *C. difficile*[91]. Normal intestinal flora primes a Myd88 TLR-5 dependent innate immune response which protects against CDI[92]. More recent data shows that certain bacteria (*i.e.*, *Clostridium schindens*) change the primary and secondary bile acids ratio[46].

The most common cause of alteration in the microbiome is antibiotic use, which can affect "mutualistic" interactions[93]. The gut microbiome in patients with *C. difficile* is indeed dysbiotic[94,95]. Probiotics have been used in an attempt to redress this.

Probiotics are preparations containing live microbial agents that may be beneficial to the host when ingested. They range from yoghurt to specific microbial extractions (*i.e.*, *lactobacillus*, *Saccharomyces boulardii*). Efficacy in RCDI may be multifactorial and comprise restitution of gut flora[96], specific anti *C. difficile* effect (*i.e.*, *S. boulardii* protease cleaves Toxin A)[96] and/or immune modulation[97].

At present preparations are not standardized or regulated, and may have no live organisms or organisms not listed on label[52]. There is risk of fungemia or bacteremia- even in immunocompetent hosts[98].

Staggered and tapered vancomycin with daily kefir (yoghurt) led to resolution of symptoms in 21/25 patients with RCDI[99]. This was a retrospective study and remains to be confirmed.

FECAL MICROBIOTA TRANSPLANTATION

Administration of exogenous fecal material *via* fecal microbiota transplant (FMT) to correct intestinal dysbiosis has been used successfully to treat CDI. FMT for pseudomembranous colitis was performed in 1950s by Eiseman *et al*[100] using fecal enemas. Successful use of FMT to treat CDI was reported in 1983[101]. A proof of principle study reported by Silverman *et al*[102] in 2010 described 7 patients with RCDI who self-administered fecal enemas at home. At an average of 14 mo follow-up there were no recurrences[102]. Brandt *et al*[103] reported long term follow-up of 77/94 patients administered colonoscopic FMT for RCDI with primary cure rate of 91% (resolution of symptoms without recurrence). Since then multiple case reports and small series have been published showing efficacy in CDI[102,104]. An open label randomized clinical trial comparing fecal transplant to vancomycin was stopped early when interim analysis showed that 94% patients in the transplant group had improvement of diarrhea compared to 31% in the vancomycin alone group[105]. FMT has been reported for more than 1000 cases worldwide with > 90% efficacy[106], including patients with severe CDI[107]. Current guidelines recommend FMT for 3rd recurrence (*i.e.*, after vancomycin taper)[7,52].

Also deemed "bacteriotherapy", FMT restores both the microbiome and favorable bile acid composition[31,108].

Barriers to mainstream use of fecal transplants have included general aversion to knowing ingestion of feces, technical issues with standardization of material (route of administration, donor, volume, preparation) and concern for transmission of disease/infection[109]. Donors are screened and stool tested for transmissible pathogens[110].

An attempt to standardize FMT involving frozen oral FMT capsules led to 90% clearance of diarrhea[111]. A recent trial from Canada directly compared efficacy of frozen- thawed *vs* fresh FMT administered *via* enema and showed equivalent outcomes (70%-75% overall cure)[112]. An alternative approach involved SER-109, a novel *Firmicutes* spore containing oral agent derived from healthy stool[113]. Thirty patients with RCDI received SER-109 after standard CDI antibiotic treatment. At 8 wk 29/30 patients showed clinical resolution and diversification of gut flora[113].

If borne out, these approaches would negate concerns for procedural risk, donor variability and disease transmission and allow standardization of transplanted material.

Many questions remain with respect to the microbiome and its role in RCDI. If indeed the main protective effect relates to bile acid composition then perhaps administration of favorable agents, *i.e.*, deoxycholate may suffice. Defined microbial systems (*i.e.*, a mixture of known specified microbes) have been used to treat CDI also[114]. The optimal composition remains to be defined. Current use of FMT is for those who have failed standard RCDI therapy. Use as first line therapy or indeed as prophylaxis in patients receiving antibiotics is possible. The role of microbiome modulation with FMT in other disease states ranging from obesity to multiple sclerosis[106] is being explored.

CONCLUSION

Recurrent/relapsing *C. difficile* remains a therapeutic challenge. *C. difficile* spores are the agents of persistence and disease and additional efforts to minimize spread are warranted. Further research on factors that affect sporulation and vegetation may yield additional therapeutic targets. The role of the gut microbiome remains mysterious; however it is clearly of great importance not only in RCDI, but in myriad disease states. FMT is an effective therapeutic modality, but long term follow-up is needed.

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REVIEW

Clinical research in febrile neutropenia in cancer patients: Past achievements and perspectives for the future

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Author contributions: Klastersky J contributed to historical background and introduction; Paesmans M contributed to risk prediction for complications and death; Klastersky J contributed to prevention according to risk; Aoun M contributed to empiric therapy according to risk; Georgala A contributed to emergence of resistant strains; Loizidou A contributed to persisting febrile neutropenia; Lalami Y contributed to cost issues; Dal Lago L contributed to febrile neutropenia at the extreme of age; Klastersky J and Aoun M contributed to conclusion.

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Abstract

Febrile neutropenia (FN) is responsible for significant morbidity and mortality. It can also be the reason for delaying or changing potentially effective treatments and generates substantial costs. It has been recognized for more than 50 years that empirical administration of broad spectrum antibiotics to patients with FN was associated with much improved outcomes; that has become a paradigm of management. Increase in the incidence of microorganisms resistant to many antibiotics represents a challenge for the empirical antimicrobial treatment and is a reason why antibiotics should not be used for the prevention of neutropenia. Prevention of neutropenia is best performed with the use of granulocyte colony-stimulating factors (G-CSFs). Prophylactic administration of G-CSFs significantly reduces the risk of developing FN and consequently the complications linked to that condition; moreover, the administration of G-CSF is associated with few complications, most of which are not severe. The most common reason for not using G-CSF as a prophylaxis of FN is the relatively high cost. If FN occurs, in spite of prophylaxis, empirical therapy with broad spectrum antibiotics is mandatory. However it should be adjusted to the risk of complications as established by reliable predictive instruments such as the Multinational Association for Supportive Care in Cancer. Patients predicted at a low level of risk of serious complications, can generally be treated with orally administered antibiotics and as out-patients. Patients with a high risk of complications should be hospitalized and treated intravenously. A short period of time between the onset of FN and beginning of empirical therapy is crucial in those patients. Persisting fever in spite of antimicrobial therapy in neutropenic patients requires a special diagnostic attention, since invasive fungal infection is a possible cause for it and might require the use of empirical antifungal therapy.

Key words: Fever; Neutropenia; Prophylaxis; Algorithm; Cancer

Core tip: The overall presentation of febrile neutropenia has considerably changed over the last 50 years. Prevention is now feasible with the use of granulocyte colony stimulating factors. If fever appears in a neutropenic patient, empirical therapy with broad spectrum antibiotics is mandatory; it should be adapted to the risk of severe complications that can be now predicted in individual patients using a reliable scoring system. Special situations such as persisting fever in neutropenic patients, the risk of invasive fungal infection and the management of older patients are crucial questions that are discussed as well as the issues linked to the high cost of prophylaxis and therapy.

Klastersky J, Paesmans M, Aoun M, Georgala A, Loizidou A, Lalami Y, Dal Lago L. Clinical research in febrile neutropenia in cancer patients: Past achievements and perspectives for the future. *World J Clin Infect Dis* 2016; 6(3): 37-60 Available from: URL: http://www.wjgnet.com/2220-3176/full/v6/i3/37.htm DOI: http://dx.doi.org/10.5495/wjcid.v6.i3.37

HISTORICAL BACKGROUND AND INTRODUCTION

In 1966, Bennett *et al*[1] showed convincingly that severe and/or protected neutropenia, in cancer patients, was associated with increased risks of severe infection. At that time, patients receiving chemotherapy (CT) were almost exclusively those with acute leukemia, a condition associated with severe bone marrow dysfunction. As a result of severe neutropenia, overwhelming infection-mainly caused by Gram-negative sepsis - was responsible for a mortality in the range of 90%, often precluding the completion of successful anti-leukemic therapy[2]. It was also observed at that time that mortality resulting from sepsis, in those severely neutropenic patients, was early after the onset of fever and that fever was often the only manifestation of the infection; this led to the concept of febrile neutropenia (FN), which was widely accepted as a significant clinical syndrome.

Today, the syndrome has become more heterogeneous; most patients with FN are receiving relatively less myelotoxic CT for solid tumors; as a consequence, the overall incidence of FN in CT-treated patients has dropped to 10% and the overall mortality, in cases of Gram-negative bacteremia, is about 20%[3]. At the same time, there has been a significant shift in the microbiological etiology of FN in neutropenic patients; gradually Gram-positive infections became more prevalent and, actually, Gram-positive and Gram-negative microorganisms are involved, as a cause of bacteremia in patients with FN, in 50% of the cases, respectively[3].

A major advance in the approach of FN has been the introduction of empirical broad spectrum antimicrobial therapy as soon as fever appeared in a neutropenic patient[4]. That concept that has never been challenged in a comparative trial, was then against the dogma of treating infection; however, it proved to be obviously so effective that it is still accepted as a paradigm for the management of FN today[5].

However, with the changing epidemiology of FN, it became obvious that all patients with FN probably had no longer the same risk of complications and death; this observation led to the search for prognostic factors of these complications and, consequently, with the possibility of prediction of that risk, to adjustments of empirical therapy. These aspects will be dealt with in details later in this paper. Finally, a major issue in CT treated cancer patients is the prevention of FN; these aspects will also be discussed in detail later.

NATURAL HISTORY OF FN

The severity of neutropenia - which directly influences the frequency of FN - is clearly related to the intensity of CT (number of agents and respective doses, as well as the myelotoxic potential of each component). However, the relationship between the type of CT and the risk of FN is far from being perfect. There are models that classify the common CT regimens according to the risk of FN as being low (< 10%), intermediate (10%-20%) or high (> 20%)[6,7] but their predictive values are far from being optimal because they do not take into account the factors linked to the patients and to the underlying disease(s) (cancer and co-morbidities) which can increase the risk of developing FN and result in different frequencies of FN with the use of the same type of CT. These factors, which also increase the risk of complications and death during an episode of FN, will be discussed later.

It has been shown, in patients with many different tumors (lymphoma, breast, colon, lung, ovary and others) that the risk of developing FN is maximal during the first cycle of CT and diminished afterwards[8]. While the precise reason for that is not known, the clinical implication is very clear: If a prophylaxis of FN exists (this will be discussed later), it should be applied from the first cycle of CT.

As shown in Table 1, FN is associated with a significant frequency of severe complications and deaths. These data are derived from a study of 2142 patients with FN registered in two observational studies conducted in different institutions and different countries[3]. It is shown that the type of underlying neoplasia, be it hematological malignancy or solid tumor, does not influence significantly the incidence of complications or deaths during episodes of FN; on the other hand, the presence of bacteremia significantly increases both morbidity and mortality. Unfortunately, bacteremia is not easy to predict on a clinical basis at the time of onset fever, although manifestations such as high fever, hypotension and thrombocytopenia are possible clues for it. It is also important to stress that the presence of a focal infection (*e.g.*, pneumonia or cellulitis) increases the

Table 1 Complications and death rates in patients with febrile neutropenia

	Complications (%)		Mortality (%)	
	Hemopathies	Solid tumors	Hemopathies	Solid tumors
No bacteremia	17	11	4	3
Bacteremia	30	35	9	13

Adapted from Klastersky *et al*[10].

risk of dying during an episode of FN; these focal infections are probably a surrogate for bacteremia but they also can lead to specific local complications by themselves[9]. Besides the severity of neutropenia (which is mainly influenced by the type of CT administrated) and the presence of bacteremia (which is difficult to predict) other factors influence significantly the risk of complications and death during an episode of FN. Among these factors, age (> 65 years) plays a critical role[10]. As shown recently, adverse events (including neutropenia) were more frequent in elderly patients[11]; the importance of prevention of severe neutropenia in elderly patients cannot be overemphasized.

Besides age and the other predisposing factors to complications and death, various comorbidities such as the stage of the neoplastic disease, poor nutrition, diabetes, chronic pulmonary disease, renal function impairment, and many others increase the morbidity and mortality of FN. Although the precise evaluation of the risk of FN associated with these various comorbidities, is not always easy to define, it is clear that it significantly increases with the number of comorbidities that are present in a patient[8,12].

Before finishing this introductory review of the past and present of FN, it is important to stress two important consequences of the development of FN in a patient. The first is the possible impact of FN on the following courses of CT as in some patients the dose of CT may be reduced or its timing modified, with possible reduction of the dose intensity, jeopardizing the efficacy of anticancer treatment; this might be particularly detrimental for patients treated with curative intent or in the adjuvant or neoadjuvant setting.

The second aspect to be stressed is that the cost of FN is substantial; it is estimated to be in the range of $16000 for each episode, with those episodes associated with complications or death being the most expensive[13]. Although these cost figures vary from country to country and from institution to institution, it is generally considered that they are underestimated, especially if all the expenses, including namely the social costs, are taken into account.

RISK PREDICTION FOR COMPLICATIONS AND DEATH

Past achievements

FN is a limiting factor for CT administration and requires prompt initiation of antimicrobial treatment. It is a possibly lethal complication with a mortality rate as high as 10% and associated costs are important especially if patients need to be hospitalized[14]. On the other hand, FN has long been recognized as a heterogeneous syndrome in terms of type and site of infection, further neutropenia duration, *etc*. Some patients at high risk may therefore be undertreated at the time of initiation of empiric treatment and some patients may be overtreated. Risk prediction is therefore an important issue with therapeutic implications: If correctly identified, low-risk patients may benefit from simplified therapy (oral therapy, outpatient treatment) and high-risk patients might benefit from more aggressive initial antimicrobial therapy and/or from early intensive care.

At least, two approaches can be considered to predict risk: One is to make use of clinical criteria defined alone without assessment of the possible interactions between them, the other is to integrate independent risk factors to produce a model predicting risk. Risk models have the following advantages: They only make use of the non-redundant information, they should produce objective and reproducible prediction, they have known characteristics. They however have drawbacks: They need to be validated, updated and tested in different settings. Nevertheless, we will focus our report on risk models only and for populations of adult patients.

When risk models are to be developed, an outcome has first to be defined: It might be development of bacteremia, development of invasive bacterial infection, response to empiric treatment, serious medical complication, death or death due to infection. This last endpoint is likely the most relevant one but due to its low frequency, developing a model for its occurrence is highly challenging due to sample size issues. The validated models have made use of a composite endpoint: Occurrence of a serious medical complication and/or death. Secondly, the clinical use for the model needs to be defined in order to optimize the model for the chosen goal.

Models developed to predict low-risk of serious medical complications and/or death

There are essentially two models that have been validated.

Talcott's model: The first one was developed and validated by Talcott *et al*[15]: It was derived, using clinical judgment, on a series of 261 febrile neutropenic episodes and firstly validated on a series of 444 episodes. Unfortunately, that model, although being reliable for predicting FN patients at low risk of complications (with an excellent positive predictive value but lacking from sensitivity), was not effective[16], as 9 patients out of 30 (30%) needed readmission. After that pilot study, a randomized clinical trial was initiated comparing management of patients with FN in-hospital or with early discharge. Planned sample size was 448 patients for showing an increase from 4% to 10% of the complication rate although an equivalence design (or a non-inferiority of the experi-

Table 2 Multinational Association for Supportive Care in Cancer scoring system

Characteristic	Weight
Burden of illness: No or mild symptoms	5
No hypotension	5
No chronic obstructive pulmonary disease	4
Solid tumor or no previous fungal infection	4
No dehydration	3
Burden of illness: Moderate symptoms	3
Outpatient status	3
Age < 60 yr	2

Points attributed to the variable "burden of illness" are not cumulative. The maximum theoretical score is therefore 26.

mental arm) would have been more convincing. The trial was closed for poor accrual after recruitment of 113 patients (66 in the in-hospital arm and 47 in the arm with early discharge). Complication rates were 9% *vs* 8%. Surprisingly, there was no evidence for improvement of patients' quality of life (QoL) in the experimental arm but costs were reduced with the home arm[17].

Multinational Association for Supportive Care in Cancer model: The Multinational Association for Supportive Care in Cancer (MASCC) risk-index score has been developed (Table 2) and its clinical prediction rule for identification of low-risk patients was first validated in the primary publication[18]. The event "occurrence of a serious medical complication" was precisely defined in the study protocol and can be found in[18]. The MASCC score has been, since 2002, accepted as a standard technique to predict low-risk of complications in patients with FN by the European Society of Medical Oncology[19] and by Infectious Diseases Society of America (IDSA)[20,21]. Indeed, several validation studies[22-28] were published and already tabulated in a review published in supportive care in cancer (Table 3)[29]. From this review, it should be stressed that the performance of the MASCC model decreases when haematological patients are present in the patients populations. The positive predictive value is > 90% when the score is used for patients with solid tumor but may decrease to 83% when haematological patients are eligible.

The MASCC model represents an improvement over the Talcott's classification[18]. The selected factors appear to be more specifically associated with the clinical severity of the FN episode rather than with the underlying cancer. A weakness of the model is that it includes a subjective assessment, burden of illness but all the attempts to substitute it with more objective factors failed. Hematological malignancy was not included in the final model. Neutropenia duration certainly plays a role too but cannot be reliably assessed at the onset of the febrile episode. The MASCC score is however not perfect, especially in patients with hematological patients. However, up to now, attempts to improve it did not lead to the development of validated models ready to use in clinical practice[30-32].

The use of the MASCC model to guide the management of a febrile neutropenic episode has been studied and includes the choice of the empiric regimen (intravenous, oral, monotherapy or combination) or the setting of treatment (in-hospital, in-hospital with early discharge or ambulatory) according to risk[33]. For instance, oral therapy has been shown to be safe in patients predicted at low-risk by the MASCC score[24,25,34-37] as well as a management including early discharge, expected to improve patients QoL, to reduce risk of nosocomial infections and costs, individual[24,38] studies as well as in meta-analyses[39,40]. Even, in hematological patients, outpatient treatment seems to be possible in patients who are clinically stable and defervesced[23]. It should be stressed however that low-risk prediction is not the only criterion for suitability for oral and/or ambulatory therapy as other factors need to be considered (like social factors and acceptance of home therapy by patients and their physicians).

Models developed to predict low-risk of serious medical complications and/or death

MASCC model: The MASCC model was developed to predict a low risk of serious complications and the threshold of 21 was chosen to optimize sensitivity for a targeted positive predictive value. However, the value of the score estimates the probability of complications and other thresholds could be considered when prediction of high-risk is the goal as the threshold of 21 is clearly associated to a too low sensitivity. Combining the data from 2 observational studies[41], overall complications rate was 79% and mortality rate was 36% in patients with a score < 15. However, no clinical prediction rule for predicting high-risk was proposed. Blot and Nitenberg[42] suggested to improve the performance of the MASCC score for high-risk prediction by repeating calculation of the severity score and by including number of organ dysfunction but they didn't propose any practical model. Some laboratory parameters have been suggested to be associated with poor outcome in patients with FN as thrombocytopenia and increased CRP[43], serum lactate[44,45], electrolytes abnormalities[46].

CISNE score: A Spanish team worked on the prediction of serious complications for patients with FN. In a first study, designed as a case-control study[28], they reviewed retrospectively 861 episodes of FN and matched patients who developed complications to patients who did not (3 controls for 1 case): They suggested that ECOG performance status $\geqslant$ 2, chronic obstructive pulmonary disease, chronic heart failure, stomatitis grade $\geqslant$ 2, monocyte count and stress hyperglycemia are factors associated to complications. From a subsequent data set of 1133 patients with FN and clinically stable 3 h after FN diagnosis, they derived, using logistic regression analysis, and validated a score predicting complications, ranging from 0 to 8 (Table 4)[47]. They defined low (score of 0) and intermediate risk (score of 1 or 2) *vs* high-risk (score > 2). The characteristics of CISNE score and MASCC

Table 3 Validation studies of Multinational Association for Supportive Care in Cancer score for predicting low-risk

Ref.	N episodes	Patients with hema-tological malignancy (%)	Predicted at low-risk (%)	Se (%)	Sp (%)	PPV (%)	NPV (%)
Klastersky *et al*[24], 2006	1003	55	72	79	56	88	40
Stratum of hematological tumors	549	100	70	77	51	84	40
Stratum of solid tumor patients	454	0	74	81	64	93	38
Uys *et al*[22], 2004	80	30	73	95	95	98	86
Cherif *et al*[23], 2006	279	100	38	59	87	85	64
Klastersky *et al*[24], 2006	611	43	72	78	54	88	36
Innes *et al*[25], 2008	100	6	90	92	40	97	20
Baskaran *et al*[26], 2008	116	100	71	93	67	83	85
Hui *et al*[27], 2011	227	20	70	81	60	86	52
Carmona-Bayonas *et al*[28], 2011[1]	169	0	?	94	36	NA	NA

[1]Selected patients population ("apparently" stable patients). The characteristics were calculated for a test aiming to identify low-risk patients and may then differ from the original publications. Due to the case-control design of the study, the rate of patients predicted at low risk as well as the negative and positive predictive values are meaningless. Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

Table 4 CISNE score

Characteristic	Weight
ECOG performance status ⩾ 2	2
Stress induced hyperglycemia	2
Chronic obstructive pulmonary disease	1
Chronic cardiovascular disease	1
Mucositis NCI grade ⩾ 2	1
Monocytes < 200/μL	1

ECOG: Electrocorticogram; NCI: National cancer institute.

score (at the threshold of 21 chosen however to predict low-risk) for predicting high-risk are shown in Table 5. Although the overall misclassification rate is lower for MASCC than for CISNE, sensitivity for predicting high-risk is much better for CISNE score as well as negative predictive value. Positive predictive value is poor for both systems. The authors acknowledged the fact that a threshold of 21 for MASCC was not intended to predict high-risk but stated that CISNE score remains more performant at other thresholds than the MASCC score.

Perspectives

Many achievements were reached for predicting low-risk for FN and allowed to successfully adapt therapeutic strategy. There is however place for improvement, especially for increasing the positive predictive value overall and certainly for patients with hematological malignancies. Further research may include further investigation of laboratory parameters, investigation genetic predisposition for infection development or monitoring of intermediate-risk patients with early repeated measurements of risk scores of whom we don't know the value. The situation is more challenging for identifying patients at high-risk. The CISNE score was only very recently proposed and its usefulness for improving patients outcome remains to be demonstrated. Clinical trials should be conducted to assess the value of "aggressive" empiric therapy or the use of early intensive care. Due to the relative low frequency of complications, further achievements in this area will be possible only thanks to large international collaboration studies that should be strongly encouraged.

Table 5 Characteristics of CISNE score and Multinational Association for Supportive Care in Cancer score for predicting high-risk

	CISNE	MASCC
Predicting high risk, complications	118	53
Predicting low risk, no complications	747	853
Predicting high risk, no complications	234	128
Predicting low risk, complications	34	99
	1133	1133
Se	0.78	0.35
Sp	0.76	0.87
PPV	0.34	0.29
NPV	0.96	0.90
Miscl rate	0.24	0.20

High-risk of prediction: CISNE > 2, MASCC < 21. Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; MASCC: Multinational Association for Supportive Care in Cancer.

PREVENTION OF FN

As has been stated in the introduction, FN is associated with serious medical complications; moreover, it can jeopardize the effectiveness of CT and represents significant extra-cost. Although, the incidence of FN and the frequency of associated complications have decreased significantly over the last 50 years, FN remains a major medical problem in patients receiving CT, especially in view of the large numbers of patients receiving CT today all over the world. It is estimated that 10% of these patients will develop FN and that 10% of them will die as a result of it; which means that eventually 1% of the patients receiving CT die as a consequence of neutropenia, a figure which is appalling for patients treated with a curative intent or in the adjuvant or neoadjuvant setting[1].

The first attempts to prevent FN in CT-treated patients has been done with antimicrobials (first non-absorbable antibiotics and later, co-trimoxazole) with some success, but also with the observation of the emer-

gence of resistant strains that limited soon or later the efficacy of that approach[2,3].

Recently, fluoroquinolones have been broadly used for that prophylaxis. Once again, most studies showed that fluoroquinolones reduced the incidence of infection and the infection-related mortality in neutropenic patients but at the expense of emergence of quinolone-resistant strains[4]. This should at the end make the prophylaxis useless; moreover, these strains jeopardize the use of fluoroquinolones as a therapy of FN, in low risk patients, as will be discussed elsewhere. For all those reasons, the use of antimicrobials, including fluoroquinolones, should be discouraged. Guidelines from American Society for Clinical Oncology limit the use of antibacterial prophylaxis to patients at high risk for FN; others recommend avoidance of such practices for the prevention of FN[5].

The use of granulocyte-colony stimulating factors (G-CSF)[1]; this approach is highly effective, without virtually any short-term side effects; on the other hand, more problematic is the cost of such a prophylaxis and this is clearly a limiting factor for a large scale use today. Two pivotal studies have established the effectiveness of primary prophylaxis with either filgrastim[6] or pegfilgrastim[1]. Pegfilgrastim differs from filgrastim by its prolonged time of action, as the polyethylene glycol tail added to the filgrastim molecule, prevents it from being excreted through the kidneys; the elimination of pegfilgrastim depends only on its inactivation by the rising numbers of neutrophils. Therefore, pegfilgrastim can be administered as a single injection after CT, whereas filgrastim requires daily injections and periodic granulocyte level monitoring until neutrophil recovery (usually 7 to 10 doses). This makes pegfilgrastim use easier for the patient and the physician, but an injection of pegfilgrastim costs at least twice as much as a full course (10 administrations) of filgrastim.

Several meta-analyses have confirmed the efficacy of G-CSF for the prevention of FN in CT-treated patients, and have shown that mortality associated with FN could be reduced[8,9].

Is pegfilgrastim more effective than filgrastim in preventing FN? A recent meta-analysis suggests that it might be the case[10]. However, outside clinical trials, it appears that in the community oncology practice, despite that filgrastim is often given later and for shorter times than officially recommended, no major differences are seen between the efficacy of pegfilgrastim and filgrastim[11,12].

The current recommendations, namely those proposed by European Organization for Research and Therapy of Cancer (EORTC)[13] state that patients with a > 20% risk of developing FN should receive G-CSF primary prophylaxis and those with a risk < 10% should not. Patients with an intermediary risk (10%-20%) should be evaluated for further risk factors, such as age > 65 years, advanced disease and various comorbidities (as discussed previously in the introductory section); if present, those factors should lead to a more liberal use of G-CSF in that group of patients. The general use of algorithm in the use of G-CSF in neutropenic patients for primary prophylaxis of FN is indicated in Figure 1.

The official recommendation to pay attention to age and other comorbidities for deciding to use G-CSF a risk of FN < 20% is an important step towards a better protection of more patients against the adverse consequences of FN. Actually, most of the patients receiving CT today have a < 20% risk of developing FN, as indicated in Figure 2; applying strictly the initial rule allowing primary prophylaxis with G-CSF only in patients with a risk > 20%, would have without protection a substantial number of patients[48]. The introduction of criteria such as age and comorbidities in patients with an intermediary risk, allows to extend the potential benefit of primary prophylaxis to more patients.

A further issue might be the optimal management of patients with a risk < 10%. It has been shown that the efficacy of primary prophylaxis is actually better in patients with a lower risk of developing FN when compared to those with a higher risk[8]. In that context, and in a retrospective analysis, it has been found that a reduced dose of filgrastim (300 μg on day 8 and 12), after a CT carrying a 7% risk of FN in patients with breast cancer, was similarly effective as a full course of filgrastim[49]. Of course, these stimulating observations need confirmatory prospective trials, to see whether it might be appropriate to propose primary prophylaxis with reduced doses, especially if there are other risk factors (*e.g.*, age and comorbidities) or if CT is given with a curative intent or in an adjuvant or neo-adjuvant context[50]. In that context, it should be stressed that, under "real life" conditions, there is wide variation in the patterns of G-CSF utilization by practicing oncologists. A recent study indicates that despite guidelines, the use of G-CSF has not been consistent. Wide variations in overuse, underuse and misuse are very common, which means possibly that physicians might perceive the usefulness of administering G-CSF, even if the guidelines are not strictly followed; alternatively, it might mean that present guidelines do not always fit clinical practice[51].

Cost is the main problem for a possible extension of the use G-CSF for primary prophylaxis of FN[51]; it is difficult to accept, on ethical grounds, that the administration of a potentially life-saving procedure is based merely on economic conditions. Moreover, the trade-off used in these early - but influential studies - is controversial, as it was based mainly on the cost for hospitalization for FN, which is definitely not the only aspect of the cost of an episode of FN. For all those reasons, the balance between the cost and the benefits of primary prophylaxis with G-CSF of FN needs to be reevaluated[50,52].

A potential solution to the limiting effect of cost on the more liberal use of G-CSF might come from the introduction of biosimilars to filgrastim or pegfilgrastim[53]. Several of such preparations have been approved in Europe and are proposed at lower prices than the original products. Thus, a combination of modified schedule of administration, tailoring the dose to the clinical needs, and a price reduction might make G-CSF prophylaxis for

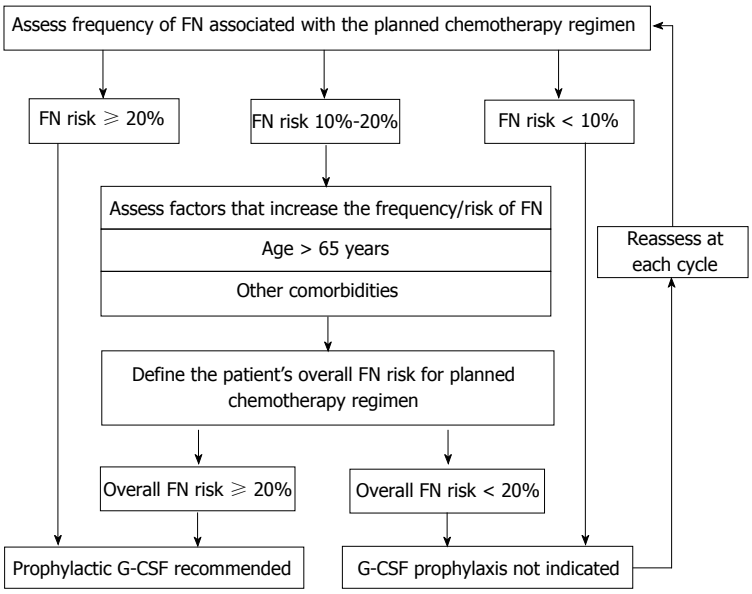

Figure 1 Algorithm to decide primary prophylactic granulocyte colony-stimulating factor usage. Adapted from European Organization for Research and Treatment of Cancer Guidelines. Data taken from[13]. FN: Febrile neutropenia; G-CSF: Granulocyte colony-stimulating factor.

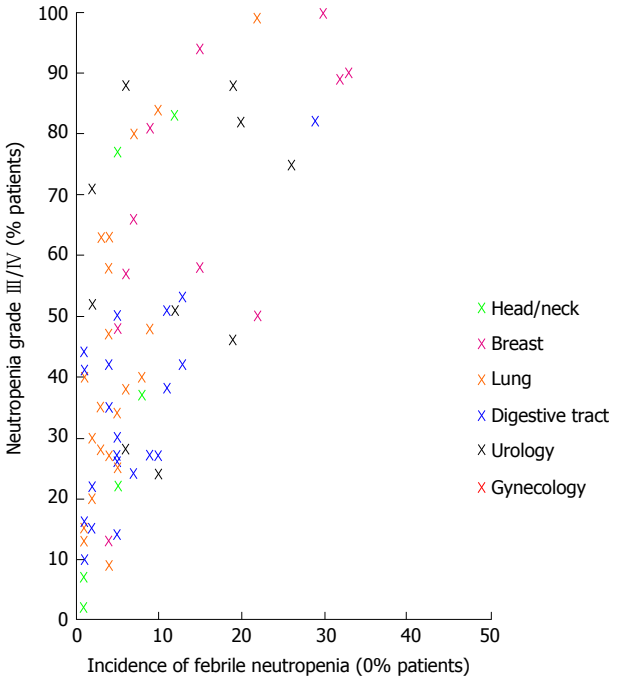

Figure 2 Relationship between the occurrence of febrile neutropenia and the severity of granulocytopenia.

FN available to more patients. Once again, it should be emphasized that new paradigms need to be based on adequately conducted clinical trials.

EMPIRIC THERAPY ACCORDING TO RISK

The elements of the management of FN have been a matter of intense research, improvement and refinement over the years (Table 6).

In the late 80's, there was a general perception that all neutropenic patients do not have the same risk of developing life-threatening complications. Not all neutropenic patients need hospitalization and intravenous antibiotics until resolution. Talcott *et al*[54] reported the first work that tried to assess the risk of adverse outcome during a neutropenia. However, the Talcott's criteria lack sensitivity (30%) and in the early 2000's, the MASCC developed an index scoring system that allows the selection of low-risk patients with good sensitivity (80%) and specificity (71%)[18]. The MASCC index has been tested in several independent trials[22,23] and is the most widely used in adult population. Thus progressively, a risk-adapted strategy for the management of FN was implemented.

Empiric treatment of low-risk patients

The major objective of identifying low-risk patients is to develop a strategy of management that decreases the costs, improves the QoL while maintaining safety. Over the time, there was an evolution in the different strategies used as well as in the selection criteria of low-risk patients. One of the first strategies consisted in early discharge to continue intravenous antibiotics on an outpatient basis and was tested successfully in two pilot trials[16,55] and in a randomized multicenter study including 80 adults[56]. In the second one, ambulatory intravenous antibiotics were given from the onset of FN. Once-daily dosing regimens such as ceftriaxone alone or combined with aminoglycoside are the most practical. Using such a strategy, a German multicenter study reported a hospital readmission rate of 24% for persisting fever or clinical deterioration[57].

The third one, a step-down strategy from inpatient intravenous antibiotics to oral antibiotics with early discharge has the advantage of allowing a period of observation and assessment of microbiology results which is critical for safety. The oral antibiotic therapy selected was

Table 6 Major elements of the management of febrile neutropenia over time

60's	High mortality (> 90%) in FN with gram-negative bacilli bacteremia
	Establishing the concept of empiric antibiotic therapy
70's	Anti-pseudomonal penicillins plus aminoglycoside combination as empiric therapy of choice
	Oral non resorbable antimicrobials (aminoglycosides, glycopeptides, polymyxines, colimycin, in different combinations with nystatin), for intestinal flora suppression
80's	Establishing empirical antifungal therapy
	Oral trimethoprim-sulfamethoxazole (or nalidixic acid and fluoroquinoles for prophylaxis in HM
	Assessment of risk factors predicting complications: Talcott's criteria
90's	Monotherapy supplanted combination
	Ambulatory management first with IV antibiotics (ceftriaxone + aminoglycoside) and then with oral fluoroquinolones
2000's	Refinement of risk assessment: MASCC score
	Risk-adapted therapy

FN: Febrile neutropenia; HM: Hematologic malignancies; MASCC: Multinational Association for Supportive Care in Cancer.

a combination of ciprofloxacin and amoxicillin/clavulanate and was used successfully in two non-randomized trials including low risk patients with hematological malignancies[23,58]. Finally, giving oral antibiotics from the onset of FN to low-risk patients, with early discharge, is probably the strategy that best meets the objectives of reducing costs and improving QoL[59]. Because of their high oral bioavailability, good tolerance and bactericidal activity particularly against GNB[60], fluoroquinolones either alone or in combination with anti-Gram-positive agents such as clindamycin[61] or amoxicillin/clavulanate[62], have been the mainstay oral therapy. A first step was to establish the safety of an oral regimen given from onset of FN. This has been accomplished through the achievement of two randomized trials comparing ciprofloxacin plus amoxycillin/clavulanate with either ceftazidime[63] or ceftriaxone plus amikacin[64], in an inpatient setting. More recently, once daily oral moxifloxacin 400 mg monotherapy has been shown to be equivalent to the standard[38]. Concern has been raised about the limited activity of moxifloxacin against Pseudomonas aeruginosa (*P. aeruginosa*). However, the frequency of this organism in the population of solid tumors or lymphoma at low risk FN is very uncommon and should be assessed locally. In this trial XV of the EORTC, 59% of patients could be discharged early with only 5% readmission rate for clinical deterioration and other medical complications.

Several studies have assessed the role of oral antibiotics given from onset of FN with immediate discharge without hospitalization for observation[60,65-68]. All excluded patients with acute leukemia and hematopoietic stem cell transplantation. Patients should be able to ingest and tolerate oral antibiotics with the first dose being tested at the emergency room. A close follow-up is undertaken with phone calls and a visit every other day until resolution. Figure 3 summarizes some of the elements that may help in the management of patients with FN at low risk.

Despite the increasing resistance of Gram-negative bacteria to fluoroquinolones over time, their efficacy in empiric oral therapy for low-risk patients does not seem to be affected. On one hand, the rate of failure because of fluoroquinolone resistance is not higher in the recent trials as compared to older ones and on the other hand, the incidence of GNB bacteremia is low. However, epidemiological variations between institutions may exist and a careful monitoring is recommended.

Empiric treatment of high-risk patients with FN

Inpatient management with parenteral broad-spectrum antibiotics is the standard care of FN patients at high-risk. A β-lactam agent active against GNB including *P. aeruginosa* remains the central core of empiric therapy. However, the increasing resistance of GNB over the years has made the β-lactam choice much more challenging[69]. There are many geographical differences in the epidemiology of microbial resistance and it is more likely that the local epidemiology than any global data, for the selection of initial for empiric therapy[70]. Until the 90's, this choice was mainly influenced by one risk which was *P. aeruginosa* resistance to the different β-lactams.

Nowadays, this choice depends on too many risks. The risk of ESBL producing GNB especially *K. pneumoniae* and *E. coli*, risk of a MDR non-fermenter such as *P. aeruginosa*, *Acinetobacter baumanii* or *S. maltophilia*, risk of carbapenemase producing pathogen in addition to the risk of MRSA, VRE and anaerobes (see epidemiological section). Any delay in the early adequate therapy is associated with an increased mortality[71,72]. Therefore, defining risk factors for MDR pathogens, in neutropenic patients, is determinant for empiric antibiotic selection and outcome. The risk factors for MDR pathogens identified include prior exposure to broad-spectrum antibiotics, the severity of underlying disease such as in acute myelocytic leukemia, and the presence of medical comorbidities, as well as the presence of urinary catheter[73]. However, these are quite common to allow a specific selection of the patients who ultimately develop an infection due to MDR pathogens. ESBL-GNB or VRE stool colonization was associated with subsequent bacteremia due to the same pathogen in a prospective study[74] in hematological malignancy patients, with a RR of 4.5 for ESBL-GNB (95%CI: 2.89-7.04) and a RR of 10.2 for VRE (95%CI: 7.87-13.32).

Thus, surveillance cultures should be reassessed and validated prospectively for both infection control purposes and selection of β-lactam empiric therapy. Patients who are not at risk of ESBL-GNB infection will receive therapy with piperacillin/tazobactam or cefepime or ceftazidime, while patients at risk of ESBL-GNB, will receive upfront a carbapenem[74]. Anti-anaerobic coverage is indicated for necrotizing gingivitis, typhlitis and peri-anal abscess[19,75]; piperacillin/tazobactam and carbapenems are, however, active against the majority of anaerobe[76]. In case of allergy to penicillin, aztreonam combined with a glycopeptide is an acceptable alternative.

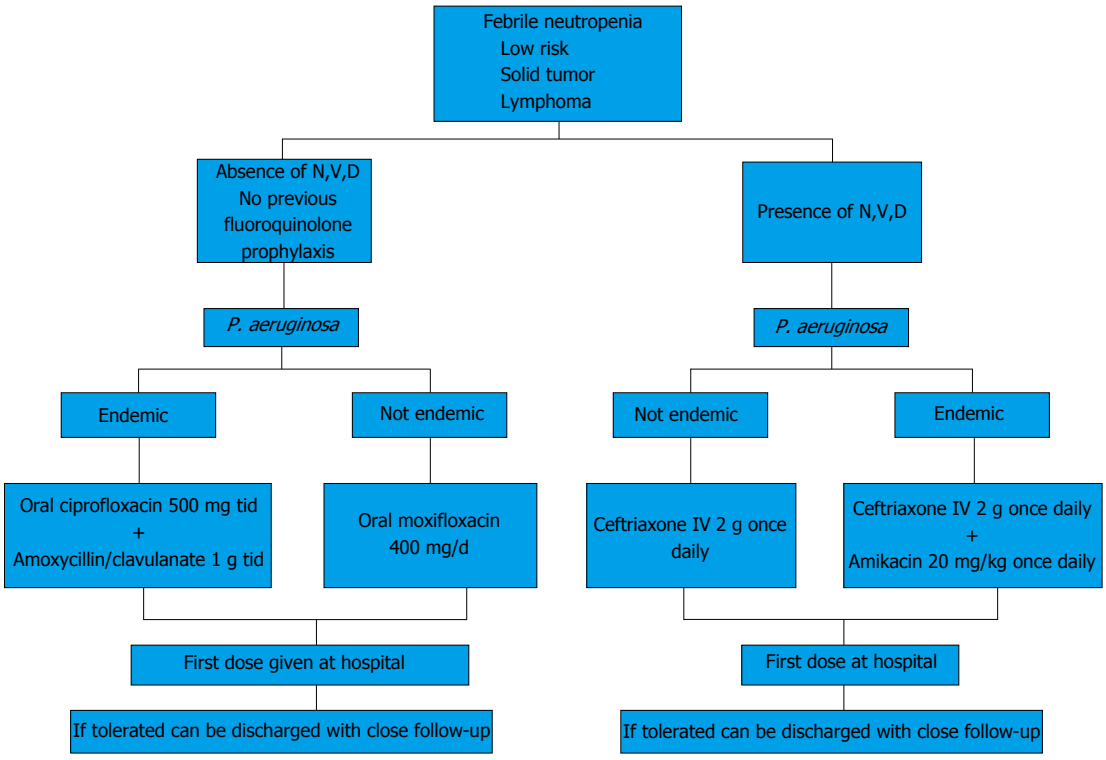

Figure 3 Decision tree for the administration of antibiotic therapy to low-risk patients with febrile nerutropenia. N: Nausea; V: Vomiting; D: Diarrhea; *P. aeruginosa*: Pseudomonas aeruginosa.

A combination therapy with an aminoglycoside has no advantage and is more toxic than monotherapy[77,78]. However, for the subgroup of patients with signs of sepsis or septic shock, the mortality is unacceptably high, especially when empiric therapy proves to be inadequate[79]. In such conditions, a combination with an aminoglycoside for a limited duration up to 3 d, seems reasonable[80,81].

In institutions where MDR non-fermenters such as *P. aeruginosa* or *Acinetobacter baumanii* or *carbapenemase*-producers enterobacteriae are endemic, combination with colistin has been advocated[82]. Empiric addition of a glycopeptide didn't show benefit in reducing treatment failure, in gram-positive infections[83]. However, addition of empiric glycopeptide under certain circumstances, is indicated such as in patients already colonized by MRSA, if MRSA is endemic in the institution, in the presence of folliculitis, furonculosis or catheter-related cellulitis and if viridans group *Streptococci* penicillin-resistance is prevalent[75].

In allogeneic hematopoietic stem cell transplant patients (HSCT) colonization by vancomycin-resistant enterococci (VRE) and T-cell depletion are important risk factors for VRE bacteremia[84]. In such patients, early empiric combination with linezolid or high-dose daptomycin (> 6 mg/kg per day) is justified[85,86]. Figure 4 provides indications for the selection of empiric therapy in high-risk patients with GN.

EMERGENCE OF RESISTANT STRAINS

The discovery and clinical use of antibiotics was officially initiated in 1936 with sulfonamides and followed in the 1940s with penicillin and streptomycin; a whole new era of anti-infective drugs was inaugurated with successful treatment of previous lethal diseases. The dream started fraying when the first resistant strains against sulfonamides[87], penicillin[88-90] and streptomycin appeared[90].

The exhilaration accompanying the modern antibiotics was over by the early 2000s; antimicrobial resistance emerged as part of the adaptive mechanisms deployed by micro-organisms (bacteria, fungi, viruses and parasites) in order to survive in a stressful environment (inside and outside the hospital). Bacteria developed successful resistance strategies through the last 6 decades. On the other hand, microbiologists and clinicians faced the ESKAPE concept: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumanii, Pseudomonas aeruginosa, Enterobacteriaceae*[91] and new comers such as *Mycobacterium tuberculosis*, HIV, *Aspergillus sp*. and malaria; very few antimicrobials were active against these bugs and the new drugs were even less designed, developed or available for human use.

In the narrow field of FN, complicating aggressive CT regimens, prophylaxis by oral antibiotics[92], broad-spectrum early antibiotherapy[75] and optimal supportive treatment[13] are well-established attitudes in order to decrease mortality and morbidity due to FN. These attitudes have to be revised and adapted in order to face the ESKAPE bugs and to continue to use antimicrobials to treat severe infections jeopardizing the prognosis of potentially curable malignant diseases.

The resistance related to antibiotics is a natural phenomenon associated to the evolution of bacterial life and the genes of resistance are frequently issued

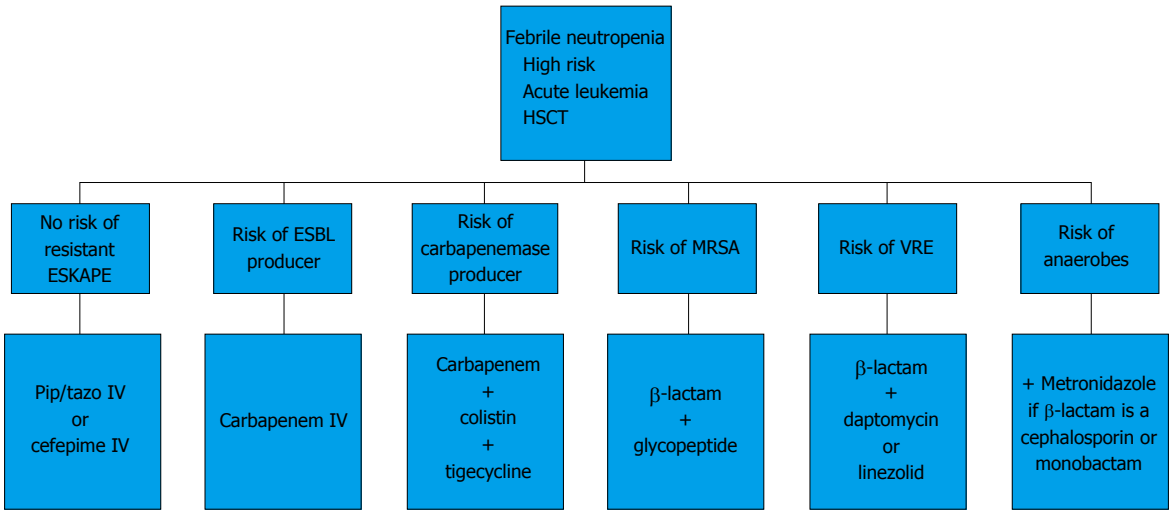

Figure 4 Decision tree for administration of antibiotics to high-risk patients with febrile neutropenia. ESKAPE: *E. coli*, *S. aureus*, *Klebsiella sp. Acinetobacter sp*, *P. aeruginosa*, *Enterococcus sp*; ESBL: Extended-spectrum β-lactamase; MRSA: Methicillin-resistant *S. aureus*; VRE: Vancomycin-resistant enterococci; HSCT: Hematopoietic stem cell transplant patients; *P. aeruginosa*: Pseudomonas aeruginosa.

from essential genes. Evidence exists that these genes pre-existed the era of antibiotics and they probably developed in antibiotic producing bacteria[93,94]. Bacteria, especially those of commensal and environmental flora use the mechanisms of resistance in order to survive in nature[95,96]. Antibiotics create a strong selective pressure on bacteria and create favorable conditions for the development of resistance; resistance to antibiotics is the final product of a complex process including multiple genetic maneuvers.

These genetic maneuvers include 3 levels. The first level is the point mutations (micro-evolutionary change) that occur in in a nucleotide base pair; the point mutations will create alterations in enzyme substrate specificity or the target site of an antibiotic, interfering with its activity. The second level of genomic variability (macro-evolutionary change) in bacteria results in massive modifications (inversions, duplications, insertions, deletions, or transpositions) of large portions of DNA as a single event. Specialized genetic elements called integrons, transposons, or insertion sequences generate these massive rearrangements independently from the rest of bacterial genome[95]. The third level of genetic variability is due to the acquisition of foreign DNA carried by plasmids, bacteriophages, isolated sequences of DNA and transposable genetic elements from other bacteria. The further inheritance of foreign DNA will contribute to enhance genetic variability of bacteria and increase their capacity to respond to selection pressures such as the use of antimicrobials[93].

Bacteria develop antibiotic resistance through (at least) eight different mechanisms: Enzymatic alteration (β-lactamases, extended-spectrum β-lactamases, carbapenemases), decreased permeability (outer/inner membrane permeability), efflux, alteration of the target site, protection of the target sight, overproduction of the target, bypass of the inhibited process and bind-up of the antibiotic. All classes of antibiotics may be affected *via* different mechanisms. The use of old (polymyxins, metronidazole) and new (linezolid, tigecycline) antibiotics when antibacterial resistance became important led to the apparition of resistant strains against these drugs, *via* the same mechanisms deployed against traditional antibiotics. Additionally to these mechanisms, bacteria may associate different mechanisms of antibiotic resistance resulting to MDR (multiple drug resistance)/Pan-resistance strains. In 2005, Deplano *et al*[97] described a Belgian out-break of Pan-resistant *Pseudomonas aeruginosa* (89% of the isolates belonged to serotype O:11). The Pan-resistance was due to the overexpression of AmpC chromosomal β-lactamases conferring resistance to multiple β-lactam antibiotics associated to the mutational loss of OrpD porin, conferring resistance to imipenem and the upregulation of the MexXY efflux system which exports fluoroquinolones, tetracycline, aminoglycosides and antipseudomonal β-lactam molecules[97]. Methodical transfer of multiple-resistance elements located on mobile genetic elements (transposons, plasmids) can help bacteria to acquire MDR/Pan-resistance[98,99]. The capacity of bacteria to seize numerous antibiotic resistance genes is illustrated by resistance integrons, which can insert resistance gene cassettes into their *attI* integration site and are often found on transposons carried on plasmids, with obviously endless recombinant capacity[100].

Moving in the inner circle of the ESKAPE bugs and their impact on the management of FN is strewn with pitfalls. Understanding the various mechanisms leading to resistance and being acquainted with the established epidemiological profiles will permit the quick and right choice of (empirical) antibiotic treatment in the advent of fever during neutropenia.

The *Enterococcus faecium* is actual the most important pathogen (among the *Enterococcus* sp.) in hospital acquired infections, followed by the *Enterococcus faecalis*. Enterococci are less virulent than other Gram-positive cocci and usually occur in the context of polymicrobial

infection in debilitated patients. The acquisition of resistance (to multiple antibiotics including vancomycin; VRE) allowed the emergence of superinfections in immunocompromised patients[101]. Acute outbreaks are usually monoclonal[101] and the hands of health workers spread Enterococci among patients. Patients may be colonized with *E. faecium* on the gastrointestinal tract and thus serve as a reservoir; adequate identification and management of these patients are the only way to prevent transmission to other patients and subsequent outbreaks[102]. Resistant strains to vancomycin (and to teicoplanin) appear when the production of peptidoglycan precursors is modified and therefor present a weak affinity for glycopeptides; Van A and VaB are the most frequent phenotypes associated to glycopeptide resistance[103]. Admission to intensive care and length of hospitalization, prior use of broad spectrum antibiotics, severity of illness and exposure to other patients colonized with VRE are well known factors for developing colonization/infection to VRE. Linezolid and daptomycin constitute the main therapeutic issues, but controlled trials lack actually[104].

The *Staphylococcus aureus* is well-known to be resistant to natural penicillins since the mid 40's; resistance to methicillin (a penicillinase-resistant penicillin) was first described in the mid 60's while the resistance to vancomycin was first reported in the mid-90's (Figure 1). The *mec* A gene, as part of the mobile genetic element named staphylococcal cassette chromosome is responsible for the synthesis of the penicillin-binding protein, PBP2a, located in the bacterial membrane and being able to catalyze the transpeptidation reactions of peptidoglycan during cell wall construction; it's an inducible protein and under the effect of regulatory genes implicated to its transcription (*mec R1*, *mecI, blaZ*, BlaR1 and BlaI), resistance towards β-lactams is observed[105,106]. The β-lactamases genes (*blaZ*, *BlaR1* and *Bla*) can produce hydrolyzing enzymes targeting the β-lactam ring[106]. Broad use of vancomycin provoked the emergence of intermediate (VISA)/resistant (VRSA) strains[107,108]. The mechanism of resistance in VISA is related to a thickening of the wall cell containing dipeptides that trap vancomycin and thus decrease the amount of drug directed against intracellular targets[109]. The mechanism of resistance in VRSA is related to a plasmid transfer containing the *van*A gene from Enterococci to *Staphylococcus aureus*[110]. While precise guidelines about treatment of MRSA infections exist[111], treatment against VISA/VRSA is mainly based on experimental trials using daptomycin, quinupristin-dalfopristin and linezolid[112,113].

The *Klebsiella pneumonia* and the *Enterobacteriaceae* represent the major providers of extended-spectrum β-lactamases (ESBLs) and carbapenemases. ESBLs include enzymes that have derived from narrow spectrum β-lactamases (TEM-1, TEM-2, SHV-1) or from chromosomally encoded β-lactamases produced by *Kluyvera sp*. (CTX-M type ESBLs)[114]. The broad use of carbapenems for serious infections due to ESBLs-producing bacteria selected the carbapenemases (mainly OXA-48, KPC, VIM, NDM); these plasmid-acquired enzymes hydrolyze most β-lactams including cabapenems. Their spread all over the world is spectacular[115,116] and worry about the outcome of serious infections due to these germs is more than real as therapeutic armamentarium is reduced to colistin, aminoglycosides and tigecycline. The detection of carbapenemases should be triggered when the *Enterobacteriaceae* have resistance or reduced susceptibility to carbapenems[117], while screening (stool, anal swabs) should be performed during outbreaks and endemic scenarios[116]. Mortality is mainly evaluated among bloodstream infections: It may vary from 39% to 53% but remains unacceptably high[74,118,119]. Well-identified risk factors (in multivariate analysis models) are the age of patient, APACHE Ⅱ (Ⅲ) score at infection onset, inappropriate antimicrobial therapy, onset of bacteremia while in the intensive care unit and malignancy; combination of antibiotics were more efficient than monotherapy and the emergence of strains resistant to colistin is already described[74,118-120].

The *Acinetobacter baumanii* and the *Pseudomonas aeruginosa* are the most popular and the most implicated in serious infections within immunocompromised patients between non-fermentative Gram-negative bacilli. Broad-spectrum empiric antibiotics always include coverage against Pseudomonas aeruginosa, in the setting of FN[75], while Acinetobacter baumanii is related to serious infections in the intensive care unit (ICU)[121]. *Pseudomonas aeruginosa* may acquire genes encoding a tremendous amount of β-Lactamases such as the OXA and PSE type β-Lactamases, KPC and the metallo-β-Lactamases. The metallo-β-Lactamases can induce resistance to all β-Lactam antibiotics (including carbapenems and excepting aztreonam) and the β-Lactamase inhibitors are inefficient; worst, the genes coding for theses enzymes may be linked to genes inducing resistance to other anti-pseudomonas drugs[122]. Nonetheless the most common mechanism of resistance to carbapenems is the loss of an outer-membrane protein called OrpD, following a mutation[123]. Other mechanisms such as upregulation of efflux pumps, outer-membrane impermeability, enzymatic alterations of the antibiotics and the 16S ribosomal RNA methylation may lead resistance to all class of anti-pseudomonas drugs including aminoglycosides[122-124]. The *Acinetobacter baumanii* infections occur more often in the ICU and the burn units and neutropenic patients seem to avoid reasonably this pathogen[69]. Besides intrinsic resistance (cephalosporinase: *bla* ADC, (OXA-69), *Acinetobacter baumanii* may acquire genes encoding different β-lactamases/carbapenemases; these enzymes are OXA-type β-lactamases (OXA-23) and metallo-β-lactamases (IMP, VIM, GIM, SPM)[125]. Fluoroquinolones are neutralized when point mutations in the in the quinolone resistance determining region of DNA gyrase gene occur[126] and upregulated efflux pumps may contribute to fluoroquinolone resistance. Aminoglycoside resistance results when enzymes capable of modyfing aminoglycosides are produced: Aph A6 3'-aminoglycoside phosphotransferase type Ⅵ will inactivate amikacin[126] and adenyltransferases (aadA1,

Table 7 Possible causes of fever in high risk neutropenic patients unresponsive to broad spectrum antimicrobials[139]

Infectious causes	Frequency
Fungal infections responding (40%)/resistant (5%) to empiric ATB	45%
Bacterial Infections (cryptic foci, biofilm, resistant organism)	10%
Toxoplasma gondii, mycobacteria, legionella, mycoplasma, chl.pneumoniae	5%
Viral infections (HSV, CMV, EBV, HHV6, VZ, parainfluenza, RSV, influenza)	5%
Graft *vs* host disease in hematopoietic stem cell transplantation	10%
Undefined (drug, toxic effects of chemotherapy, antitumor response, undefined pathogens)	25%

HSV: Herpes simplex virus; CMV: Cytomegalovirus; EBV: Epstein-Barr virus; HHV6: Human herpesvirus 6; RSV: Respiratory syncytial virus.

aadB) or acetyltranferases (aacC1, aacC2) will neutralize gentamycin and tobramycin[126,127]. Unfortunately, upregulated efflux pumps of the AdeABC type induced resistance to tigecycline[128].

Despite fascinating progress in treating serious bacterial diseases performed in the last century and since the discovery of penicillin, the emergence of resistant strains is the major threat in the 21st century. Frail patients undergoing sophisticated treatments (transplantations, CT, immunotherapy) for complex diseases such as cancer, autoimmune conditions are exposed to a supplementary risk of complications due to non-treatable bacterial infections[129,130].

The economic impact of infections due to resistant bacteria is well-known: The length of hospitalization is longer, the hospital charges are higher and the mortality/morbidity are increased[131,132]. The infection control team and the antimicrobial stewardship programs seem to be the most promising tools in fighting against resistant strains in the lack of new antibacterials; implementation of strategies preserving antibacterials may is the future in modern medicine if we don't want to lose the progress achieved in the past decades. Management of FN needs to be carefully thought in the advent of these disturbing elements and close collaboration with specialized teams in controlling infectious diseases is the only way to bring through the ESKAPE pathogens[98].

PERSISTING FN

Definition

Persistent febrile neutropenia (PFN) is FN that does not resolve in spite of the empirical administration of broad-spectrum antibacterial agents. It can concern 30%-40% of the patients presenting FN. The diagnosis of PFN requires at least 5 d of therapy in patients with haematological malignancy, including HSCT[133-135] but only 2 d in solid tumours[75,136], probably due to different immune response. Patients with haematological malignancies are usually more seriously ill, than patients with solid cancers[137].

Etiology of PFN

The most frequent cause of fever in high risk neutropenic patients unresponsive to broad spectrum antimicrobials is fungal infection (45%), followed by bacterial, viral infections, toxoplasmosis, drugs, toxic effects of CT and antitumor response (Table 7)[137].

Diagnostic approach

PFN for more than 3 d should prompt a thorough search for a source of infection. PFN with neutropenia lasting more than 7 d in high-risk hematological patients should lead to an evaluation for invasive fungal infection with a chest CT scan looking after pulmonary nodules or nodular pulmonary infiltrates and early assessment with bronchoscopy, bronchoalveolar lavage with cultures/stains, a sinus CT scan[75] and a regular *Aspergillus* galactomannan antigen testing and/or β-D-glucan detection. Repeated imaging may be required in patients with persistent pyrexia.

Procalcitonin (PCT) monitoring can be useful, a delayed PCT peak higher than 500 mg/mL suggest the early diagnosis of invasive fungal disease and PCT decrease reflects response to antifungal therapy[138].

Diarrhea, if present, should be assessed by analyzing a stool sample for *C. difficile* toxin. An abdominal CT may be helpful for the diagnosis of neutropenic enterocolitis[139]. Surveillance of IV catheters for possible skin bloodstream breakthrough infection is also indicated[75].

An evaluation for viral infections, by herpesviridae (Herpes, Varicella Zoster, HHV6, HHV8), Cytomegalovirus, Epstein Barr, but also respiratory virus, as guided by the local epidemiology (respiratory syncytial virus, influenza, parainfluenza) is recommended especially in high risk hematological patients. Eventually, exclusion of other non-infectious sources of recurrent or persistent fever like drugs, thrombophlebitis, cancer, resorption of hematoma is warranted[75].

Prospective trials are presently ongoing to evaluate the utility and cost-effectiveness of PET/CT in identifying sites of infection in cancer patients with PFN without an obvious source, in order to improve targeted therapy.

Therapeutic attitude

Modifications to the initial empirical antibiotic regimen should be guided[75] firstly by possible changes of the clinical stability, without a source of infection detected; in hemodynamically stable and asymptomatic patients, watchful waiting and re-evaluation for new possible infection is indicated, while in hemodynamically unstable patients, the antimicrobial regimen should be broadened to target drug-resistant bacteria. Delaying appropriate antibiotic therapy for such pathogens, is associated with increased mortality[140].

Unusual infections should be considered, particularly in the context of a rising C-reactive protein (CRP), in such cases proceeding to imaging of chest and abdomen is advisable. Sometimes the investigations may be directed by clinical findings[4,141].

Therapeutic approach for fungal infections

Empiric antifungal therapy should be considered in high-risk neutropenic patients who PFN after four to seven days and without identified source for the fever[75]. The incidence of fungal infection (especially *Candida* or *Aspergillus sp.*) rises after patients have experienced more than 7 d of PFN. In 1970s, already several studies have shown that invasive fungal infections were a common cause of PFN (9%-37.5%)[142-146] and was associated with significant mortality (69%)[145].

The IDSA guidelines recommend lipid formulation of amphotericin B, caspofungin, voriconazole, or itraconazole as suitable options for empiric antifungal therapy in PFN. The choice of the initial antifungal agent may vary based on epidemiology and local susceptibility patterns[133], toxicity and the cost of the antifungals.

Resolution of fever occurs in approximately 40%-50% of patients given empirical antifungal therapy[143,144,147,148], but such a successful outcome does not prove that the patient had indeed an occult fungal infection, since slow responses to empiric antibacterial therapy can occur.

Fluconazole can be given as first-line treatment provided that the patient is at low risk of invasive aspergillosis, has not received an azole antifungal as prophylaxis and local epidemiological data suggest low rates of azole-resistant *Candida*[19].

Liposomal amphotericin B or an echinocandin antifungal such as caspofungin are appropriate first-line treatments in high risk patients with PNF without an obvious site of infection and also in patients already exposed to an azole or known to be colonized with non-albicans *Candida*[19].

Addition of the newer antifungal agents active against possible azole-resistant *Candida sp*. Is also recommended, if the patient has been already treated with fluconazole prophylaxis.

In patients with nodular pulmonary infiltrates, invasive mold infection should be strongly suspected and prompt assessment with bronchoscopy, bronchoalveolar lavage for cultures and galactomannan testing should be performed; in those patients a preemptive treatment with voriconazole or a lipid formulation of amphotericin B is indicated.

PFN receiving anti-mold prophylaxis should be treated with a different class of antifungal than the one used for prophylaxis, in order to avoid cross resistance. The usual sensitivity and resistance of the common fungi are indicated in Table 8[149-151].

Pre-emptive antifungal therapy implies a diagnostic workup with chest and/or sinus computed tomography, serum galactomannan and/or β-D-glucan to evaluate fungal infections in patients with PFN[133]; that approach has been proposed in order to reduce unnecessary use of empirical antifungal therapy, associated toxicity and high cost[147]. Patients receiving pre-emptive antifungals are more likely to present a documented invasive fungal infection (IFI) compared to patients receiving empirical therapy by the time the antifungal agent is started[152].

Paediatric population with PFN are also at high risk for IFI. Prospective monitoring of serum galactomannan twice per week in high-risk hospitalized children for early diagnosis of invasive aspergillosis is probably indicated.

Computed tomography (CT) of the lungs and targeted imaging of other clinically suspected areas of infection, as well as other investigations, such as BAL and *trans*-bronchial or *trans*-thoracic biopsy are indicated in the case of pulmonary lesions[153]. CT of the sinuses is proposed in children of at least 2 years, although imaging during prolonged FN can be inconclusive and symptoms of sinonasal IFD in children are scarce[154,155].

Particular entities of PFN

Recurrent or recrudescent fever refers to a new episode of fever after an initial resolution of fever with antimicrobial therapy when the patient remains neutropenic[155]. This is relatively common, but it has not been adequately studied. Bacterial and fungal infections are common causes of this syndrome (around 30%)[156,157]. The various guidelines do not separate recurrent/recrudescent fever from persistent fever, although these two may be clinically and etiologically different.

Engraftment fever (myeloid reconstitution syndrome) consists of a new onset or worsening of inflammatory and/or infectious process, in temporal relationship to neutrophil recovery after aplasia[157,158]. This has to be differentiated from superinfection or the immune reconstitution syndrome. The engraftment syndrome is a diagnosis of exclusion, which presents particularly in the setting of stem cell transplantation (autologous or allogeneic) consisting in fever, rash and pulmonary infiltrates originally and is usually treated with corticosteroids when severe.

ECONOMIC AND COST ISSUES RELATED TO FN

General considerations and perspectives for clinical practice

Treatment of FN usually requires several days of hospitalization, diagnostic procedures, administration of intravenous empiric broad-spectrum antibiotics and hematopoietic growth factors[159,160]. Thus, such medical management is resource intensive. It is not surprising that FN has a considerable economic impact, particularly in the inpatient setting[51,161].

Our understanding of such a problematic issue is mainly derived from several seminal United States retrospective economic analyses, highlighting average costs per hospitalization for FN management, ranging from $18880 to $22086 (€15000-€24000). The direct costs for outpatient management were considerably lower, at $985 per episode. Patients with hematological malignancies usually have much higher hospitalization costs associated with each episode than those with solid tumors ($US23000-38600 *vs* $US7598-14900)[162-165]. In a recent review, a large variation in estimation among the cost of illness studies in lymphoma patients experiencing

Table 8 Usual sensitivity and resistances of fungi against the different antifungals[149-151]

Antifungal classes	Antifungal agent	Common resistances	Common sensitivity
Polyenes	Amphotericine B:	Candida lusitaniae	Candida
	Deoxycolate	Trichosporon	Aspergillus
	Liposomal	Fusarium	Zygomycetes
	Lipid complex	Scedosporium	
	Colloidal dispersion	Aspergillus terreus	
	5 Fluorocytosine	Zygomycetes	Candida
		Scedosporium	Torulopsis
		Fusarium	T. glabrata
		Cryptococcus	Cryptococcus
		Candida	Phialophora
			Cliadosporium
			Exophiala
Triazoles	Fluconazoles	Aspergillus	Candida albicans and others
		Candida kruzei	Candida glabrata[1]
		Candida glabrata	Cryptococcus neoformans
		Zygomycetes	Blastomyces dermatitidis
			Coccidioides
			Histoplasma capsulatum
	Itraconazole	Aspergillus niger	As itraconazole + Aspergillus flavus
		Aspergilus terreus	Aspergillus fumigatus
		Zygomycetes	Candida kruzei
		Mucor	Trichophyton
		Fusarium solani	
		Penicillium	
	Voriconazole	Zygomycetes	As itraconazole + Aspergillus niger
		Sisyrinchium inflatum	Aspergillus tereus
		Fusarium oxysporum, penicillium, Schedosporium apiospermum	
	Posaconazole	Trichosporon asahii	As voriconazole + Trichophyton
			Zygomycetes
Echinocandins	Caspofungin	Cryptococcus	
	Micafungin	Zygomycetes	
	Anidulafungin	Fusarium	
		Paecilomyces lilacinus	
		Trichosporon	
		Schedosporium	
		prolificans	
		Schedosporium inflatum	
		Candida parapsilosis	

[1]Are not always sensible to the antifungals.

FN have been reported, ranging from $5819 to $34756 (2013 $) per episode of FN[166]. It seems now well established that such previous exclusive estimations, based on hospitalization, may have underestimated costs by as much as 40% by ignoring important costs occurring after hospital discharge[167].

Similar trends with a different cost burden degree were observed in western European developed countries, with smaller studies providing estimates of the average charge for FN-related hospitalization ranging from €2619 in Spain to €4931 in France[168,169]. In a recent study conducted in Ireland, the mean cost per FN episode in the inpatient setting was estimated to be €8915[170]. It should be noted that results of cost-effectiveness studies may differ greatly across different countries and health care systems. Future cost evaluation studies should compare the cost of FN and intervention costs within the same health care system, and not between countries, so as to determine more accurately if the intervention is cost-effective.

Furthermore, results of studies that were conducted may not be directly applicable to other settings. Moreover, literature data based on clinical trials may carry the risk of representing care in overselected populations rather than "real life" practice. Many potential factors account for the large variation in estimating the cost of FN, such as the year of pricing, the perspective employed, and the cost estimation approach used. The public health care system is unique for each country, with different standards of care as well as different costing of health care resources.

Since FN is an acute condition, and typically produces temporary complete disability, the cost involved from the patient time lost from work was initially thought to be non-significant[171]. Thereafter, such indirect costs, including costs associated with patient work loss, caregiver work loss, paid caregiver and/or non-revenue-generating support centers, were estimated with great variations between studies, ranging from 11% to 44% of the total cost of FN management[161,166,172]. Future studies should place greater emphasis on improving the accuracy of providing a clearer description of these indirect costs.

The major economic impact of neutropenic complications is mainly related to the cost of hospitalization and the associated length of stay (LOS). In a retrospective analysis, it has been demonstrated that one-third of patients hospitalized for more than 10 d account for 78% of the total cost. The average LOS decreased over time by 10% while the cost per day increased by 28%, raising the total cost per episode of FN by 13%. The mean LOS was longer for patients with leukemia (19.0 d) compared to patients with lymphoma and solid tumors[51]. A recent publication on subpopulations of FN admissions with breast cancer in the United States between 2009 and 2011, showed, despite a shorter LOS than previously reported (5.7 d *vs* 8.0 d, $P < 0.05$), a significantly higher mean hospital charge ($ 37087)[173] than prior observation from former observations from Kuderer and colleagues ($ 12372)[8], suggesting that FN related hospitalizations continue to account for highly significant care expenditure.

Low risk patients generally have short hospitalizations and account for a relatively small proportion of the overall costs associated with FN[174]. There is also strong evidence suggesting that costs of in-hospital treatment are greater than the costs of ambulatory care for FN[166,175]. Therefore, strategies that support FN outpatient treatment may have important clinical and economic impacts[16,18,61]. However, these patients may have been selected for outpatient treatment because of their lower risk for complications. Future prediction risk models should not only include risk factors of FN to be considered for use of prophylactic therapies but also the predictors of higher cost of FN as well. Currently, the MASCC scoring system is widely used to prognosticate the severity of FN among cancer patients[18]. However, there is room to improve the sensitivity and specificity of the prognostic model. Considered that the management strategies of low-risk and high-risk FN are different, improving the current prognostic model to predict the severity of FN is worth to further explore in future studies.

Undoubtedly, recombinant G-CSFs represent a major clinical achievement[8]. Meta-analyses, which have shown that pegfilgrastim performs as well as or better than filgrastim in reducing FN rates for patients undergoing CT[176]. Consistently, several studies evaluated the relative cost effectiveness of pegfilgrastim, and showed that any incremental costs are justifiable given the clinical outcomes[177-180].

As already said, it is possible that these economic considerations have been the main incentive for international guidelines, justifying the use of primary prophylaxis, at a risk level > 20%[13,181,182]. However, considering only the cost of hospitalization for setting such threshold may not be optimal. Such guidelines do not consider all aspects of value in cancer patients, namely clinical impacts on QoL and mostly, potential effect of completing full dose CT therapeutic plan, with subsequent disease control and impact on survival, especially in the curative setting.

Both filgrastim and pegfilgrastim are expensive ($2600 and $3500 respectively for full treatment per cycle), and their economic burden is inseparable from the economics of FN. These agents will allow a greater relative dose intensity, less dose-delays and thereby, greater costs associated with the use of CT agents. Their high cost should be balanced not only against the cost of FN but also to the impact on increased clinical outcomes, such as QoL and survival. However, the exact economic benefits of such FN prophylaxis are not completely understood and established, mainly due to the lack of consistency in general use of G-CSFs among physicians. Indeed, under- and over-prophylaxis with G-CSFs remain a reality, being the consequences of either a bad knowledge and clinical applications of the guidelines, or the willingness for clinicians to overprotect their patients undergoing CT. It has been suggested that G-CSFs are underused for CT regimens with high risk of FN, and overused for those associated with low risk[183].

Actually, the risk of development of FN is not always easily determined on the basis of the type and dose of CT, and still many patients with a risk < 20% still develop FN, with a rate of complications similar to that of patients with a high risk[184]. Moreover, it seems that efficacy of G-CSF prophylaxis might be better in populations with low risk of FN ($\leqslant$ 10%)[8]. Current guidelines will have to be revisited to allow a larger number of patients to have access to primary prophylaxis, without compromising cost efficacy. Hence, other prophylaxis strategies have been explored, including in particular, limitation of primary prophylaxis to the first two cycles of CT only[185] or shorter duration of G-CSF primary prophylaxis (2 *vs* 7 daily injections)[49], but with reports of conflicting and ambiguous results in the literature. Further studies are needed and will be performed in this specific topic.

The great majority of previous large FN trials considered hematological malignancies, lymphomas, breast and lung cancers. Other groups, such head and neck cancer patients, may deserve special attention, because they truly represent a high risk group in terms of age, co-morbidities and aggressiveness of multimodal therapies. In this group, platinum and taxane-containing regimens (*i.e.*, induction TPF) have a reported FN incidence ranging from 5.2%-20%[186,187] and therefore, they are not considered as high risk to have access to primary prophylaxis with G-CSFs. It is now established and recognized that patients considered for clinical trials (with shorter therapy durations) are usually well selected (usually excluding high risk such elderly patients), and could be different from those unselected and managed in real-life daily clinical practice among the community setting.

A recent retrospective analysis from a Japanese group reported a 41%, 25% and 33% incidence of FN in the first, second and third cycles of taxane and platinum-based CT regimens. G-CSF was used in 58 out of 71 patients (82%) during the first cycle, but exclusively therapeutically and not prophylactically following health insurance rules for G-CSFs in Japan[188]. Their relative dose intensity was around 80% of other reports. Tube

feeding, diabetes mellitus and presence of CT-related gastrointestinal adverse effects (such mucositis, diarrhea and emesis) were significant predictors of FN. In this analysis, 62% and 70% of the patients had received prior CT and radiation respectively. The major interest of this retrospective analysis, and despite several limitations, is to show the much higher risk of FN in community setting than in clinical trials in a very specific group of tumors with high needs. Further investigations are needed for a better management and prophylaxis of FN in head and neck cancer patients.

Finally, a more comprehensive consideration of value should encompass not only the cost, but also potential survival benefit, QoL and equity between patients. More affordable G-CSFs, QoL through the use of biosimilars, might influence our prescribing to prevent FN in the future[189,190]. Several studies have demonstrated that the biosimilar G-CSF is equivalent in terms of efficacy and safety when compared against native G-CSF[191-193]. Although we dispose of encouraging clinical and safety outcomes, there is still a need for longer follow-up studies to confirm the safety, efficacy as well as cost effectiveness of these biosimilars.

FN AT THE EXTREME OF AGE (DAL LAGO L)

Elderly population

Due to the ageing, European population aged 65 years and older is projected to increase, leading to even older patients with cancer[194].

There is a paucity of evidence-based data for cancer management in older patients because of the underrepresentation in studies. Indeed, many clinical trials have tended to exclude older individuals, either on the basis of age alone, comorbidity, or both[195]. Consequently data about anti-cancer treatments are extrapolated from results in younger population, with a risk of overtreatment and/or complications such as FN following CT. Indeed, many clinical trials have tended to exclude older individuals, either on the basis of age alone, comorbidity, or both. The explanation for this situation is complex and associated with a biased approach by both physicians[196]. However, we do know that older patients are just as likely as younger ones to participate in clinical trials if given the opportunity.

Older age as risk factor for FN

Particular consideration should be given to the high risk of FN in elderly patients (aged 65 and over). Primary prophylaxis of FN is currently indicated for a risk > 20% of FN, but FN is more often complicated in older patients, even if the theoretical risk of FN is < 20%[13].

In a phase Ⅲ randomized trial in 509 metastatic breast cancer patients who received first-line CT with doxorubicin or a pegylated liposomal formulation. One of the risk factors for FN was advanced age[197].

FN prophylaxis

Elderly cancer patients cannot tolerate standard doses of CT but should probably benefit more from prophylaxis because of the frequency and severity of myelosuppressive complications.

One of the first randomized studies that demonstrated the benefit of primary prophylaxis of FN during CT evaluated the incidence of FN and related events in 852 older cancer patients (≥ 65 years of age) with either solid tumors or non-Hodgkin's lymphoma receiving pegfilgrastim; the administration of pegfilgrastim resulted in a significantly lower incidence of FN for both solid tumor and NHL patients compared with reactive use[198].

Cooper *et al*[9] meta-analysis of GCS-F for FN prophylaxis following CT demonstrated that there was no clear difference in GCS-F effectiveness in studies restricting to elderly population. Indeed, Lyman *et al*[51] meta-analysis of 59 individual randomized controlled trials involving nearly 25000 patients with solid tumors or lymphoma demonstrated significant reductions in all-cause mortality over the period of 2 years follow-up with GCS-F-supported CT (RR = 0.93), independent of the age group[17].

In a phase Ⅲ randomized trial of 175 NSCLC patients randomly assigned to CT with or without addition of G-CSF to antibiotic prophylaxis, it was shown a decreased incidence of FN with the addition of G-CSF, and older age was related to the risk of FN in cycle 1[199].

Phase Ⅲ results of 779 patients with ovarian cancer treated with carboplatin or cisplatin/paclitaxel were retrospectively analyzed according to feasibility, toxicity, and QoL in patients aged < 70 or ≥ 70 years; 13% of patients were aged ≥ 70 years. Toxicities were comparable between elderly and younger patients, except for FN (5% *vs* < 1%, $P = 0.005$)[200].

FN complications

It is therefore important to identify patients at risk for complications if FN appears using instruments like the MASCC score). This score identifies age 65 or older as an important risk factor for disease burden in case of FN[18].

Perspectives

Risk factors of CT toxicity (for example FN) other than chronological age should be identified and evaluated, as that chronologic age is often different from physiologic age. The next step in geriatric oncology will be to implement ongoing predictive models for CT toxicity that integrate patient age, and characteristics of the tumor and its treatment as well as laboratory values and overall geriatric assessment[201,202]. This might allow to better selection of patients who will benefit of primary GCS-F prophylaxis of FN.

CONCLUSION

During the past 50 years, FN prognosis has dramatically changed as a result of better supportive care in patients

with cancer and namely the use of empirical broad spectrum anti-microbial therapy. Nonetheless, FN is still diagnosed in 10% of the CT-treated patients and is responsible overall for a 10% mortality without taking into account the morbidity resulting from FN and the possible negative effect on cancer therapy.

A major advance in the management of FN has been the stratification of the population of patients with FN for the risk of complications and death. Using validated reliable predictive instruments, such as the MASCC score, it is possible to identify a population of "low risk" patients who can benefit from simplified and less expensive therapeutic approaches (*e.g.*, orally administered anti-microbial therapy and early home return).

Although the MASCC scoring index has been widely accepted, there is still room for improving its effectiveness, especially in some subset of the FN population, namely in patients with hematological malignancies. Similarly, attempts to improve the performance of the score by adding to it, some biological parameters are promising. Although the MASCC score can identify patients at high risk of complications during FN, more precise prediction of such patients is needed, to make possible earlier and closer monitoring of those patients who present still a high rate of death and complications, mainly because of uncontrolled sepsis. New paradigms for the diagnosis and management of non-low-risk patients with FN are urgently needed.

A major advance in the management of FN has been the introduction of the GCSFs, which efficacy for the prevention of CT-associated has been demonstrated beyond any doubt: 50%-80% of such episodes can now be avoided. Unfortunately GCSFs are expensive and this has led to restrictive algorithms for their use, to balance the cost of the prophylaxis and that of the management of FN; these considerations usually do not take into account the effect of FN on the well-being (QoL) of the patients. It is highly desirable that future research focuses on the definition of subset of patients who could benefit from GCSF prophylaxis, taking into account not only the type of CT used, but also many comorbid conditions making FN more common and more debilitating.

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MINIREVIEWS

Leptospirosis: A clinical review of evidence based diagnosis, treatment and prevention

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Abstract

Leptospirosis is a zoonotic disease with worldwide distribution and increasing prevalence. Infection is caused by the spirochete Leptospira, with common exposure being contaminated fresh water. Most infections are asymptomatic, but symptoms range from a mild, self-limiting, non-specific febrile illness to fulminant respiratory and renal failure with a high mortality rate. The combination of jaundice, renal failure, and hemorrhage is known as Weil's disease and is the most characteristic pattern associated with severe leptospirosis. Clinical suspicion alone may be enough to warrant empiric antibiotic treatment in many cases. Serological methods are the most commonly used means of confirming a diagnosis of leptospirosis. The "gold standard" is the microscopic agglutination test. Typical treatment for mild causes is oral doxycycline, though azithromycin and oral penicillins are reasonable alternatives. Intravenous penicillin G has long been the standard of care for severe cases though limited studies show no benefit compared to third generation cephalosporins. We review the clinical presentation, diagnosis, treatment and prevention of leptospirosis.

Key words: Leptospirosis; Tropical diseases; Infectious disease

Core tip: Leptospirosis is a zoonotic disease with worldwide distribution and increasing prevalence. Infection is caused by the spirochete Leptospira, with common exposure being contaminated fresh water. Most infections are asymptomatic, but symptoms range from a mild, self-limiting, non-specific febrile illness to fulminant respiratory and renal failure with a high mortality rate. Typical treatment for mild cases is oral doxycycline, though azithromycin and oral penicillins are reasonable alternatives. Intravenous penicillin G has long been the standard of care for severe cases though limited

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INTRODUCTION

Leptospirosis, caused by the spirochetal bacteria *Leptospira*, is a zoonotic disease with worldwide distribution and increasing prevalence. Clinical presentation is often nonspecific and can vary in severity from asymptomatic to fatal multi-system organ failure. The recent estimated worldwide incidence of leptospirosis is approximately 1.03 million cases with 58900 associated deaths[1]. Actual rates are likely higher: Many cases may go unrecognized due to their mild and nonspecific nature, definitive confirmation of diagnosis *via* laboratory testing is challenging, and in many countries (including the United States as well as many developing nations with high endemicity), leptospirosis is not a reportable disease[2,3]. The incidence in tropical areas is up to ten times higher, likely due to a combination of factors, including environmental (higher temperatures, humidity, and rainfall favoring organism survival) as well as socioeconomic (poor sanitation, closer human contact with both rodents and domestic animals)[4,5]. Occupations with exposure to animals or water (farmers/ranchers, vets, rice farmers, military personnel) have also been associated with higher risk of acquiring leptospirosis[6]. In developed countries, travel-related infections and recreational exposures have become increasingly recognized as a source of *Leptospira* infection. A 2009 review estimated that over half of leptospirosis cases in the United Kingdom were acquired abroad during travel to tropical regions[7]. Many cases have occurred in association with water-based activities such as swimming, triathlons, canoeing, and kayaking, including several outbreaks within the United States and abroad[6,8-10].

Leptospirosis is caused by bacterial spirochetes of the genus *Leptospira*. There are 21 identified *Leptospira* species (classified by genetic relatedness), 9 of which are known to be pathogenic[11]. Leptospires are also classified by serogroup, with over 26 pathogenic serogroups and 250 pathogenic serovars identified, as well as more than 60 nonpathogenic serovars[11,12]. The organisms are thin and corkscrew-shaped, with a characteristic end hook. Leptospires are motile, aerobic organisms that grow best between 28 ℃-30 ℃ and thus can remain viable for months in the environment (water or soil), where they are often widespread[2,12,13]. Additionally, animals are a natural reservoir for *Leptospira* species, as they live commensally in renal tubules of many species - most significantly rodents but also other mammals including livestock[12]. Shedding from kidneys and excretion in urine of colonized animals contributes to environmental perseverance of the organisms.

Transmission to humans is most commonly environmental *via* contact with water or damp soil contaminated with leptospires, but may also occur from direct contact with urine or blood from an infected or colonized animal[11]. The organisms typically enter the human body *via* cuts and abrasions or mucous membranes (oral mucosa, conjunctivae), and are likely unable to penetrate intact skin[13]. Water contaminated with pathogenic *Leptospira* may also rarely cause infection *via* the fecal-oral route (accidental ingestion) or respiratory route (inhalation of aerosolized organisms)[11,13]. Organisms then spread to the bloodstream and multiply, and hematogenous dissemination throughout the body occurs, with potential to affect nearly every organ system due to the ability of the spirochetes to easily cross tissue barriers before the host antibody response clears them from the blood[11,13,14].

CLINICAL PRESENTATION

The clinical features of leptospirosis are both highly variable and nonspecific, depending on both host and pathogen factors. A significant proportion of infections are likely asymptomatic or subclinical, and when symptoms do occur, onset is typically 2 to 30 d after exposure, with average incubation time of 7 to 12 d[13,14]. The majority of symptomatic cases (up to 90%) follow a biphasic pattern, consisting of an initial symptomatic leptospiremic phase lasting 5 to 7 d followed by an immune phase during which symptoms can gradually improve as the host mounts an antibody response, though clinically the two phases may be difficult to differentiate[11]. Symptoms typically begin with abrupt onset of fever, chills, myalgias, and headache, similar to many other febrile illnesses[2,11]. In leptospirosis, muscle pain is often focused in the calves and lower back, and headache is typically frontal and throbbing in character[13]. Conjunctival suffusion (erythema without exudate) is the most characteristic physical finding, but presence may be variable (seen in anywhere from 7% to 60% of patients based on review of several large case series)[11] Gastrointestinal symptoms (anorexia, nausea, vomiting, diarrhea) are common, and nonproductive cough occurs in approximately half of cases[13]. Aseptic meningitis is also relatively frequent (up to 80% of cases), and usually manifests approximately 7 d into the illness as the immune phase begins[11]. Less frequently, patients may have hepatosplenomegaly, lymphadenopathy, or pharyngitis. Of note, rash as a clinical manifestation of leptospirosis is very rare, and in fact is suggestive of other etiologies in a patient with febrile illness[13].

In a minority of cases, leptospirosis can progress to severe, fulminant disease with mortality rate from 5%-40%[11]. The combination of jaundice, renal failure, and hemorrhage is known as Weil's disease and is

the most characteristic pattern associated with severe leptospirosis, though any organ system in the body can be affected due to wide hematogenous dissemination during the leptospiremic phase. Kidney involvement is common because of the organism's predilection for renal tubules in their natural hosts, and renal failure occurs in 16%-40% of cases[15]. Renal dysfunction in leptospirosis is typically non-oliguric and associated with hypokalemia. Though renal function typically recovers with appropriate supportive care, its presence is associated with higher mortality[16]. Hepatic involvement typically occurs in a cholestatic pattern, with high conjugated bilirubin levels and more mild elevations in serum aminotransferases. Though improvement is slow, liver failure is generally reversible and not an independent contributor to increased mortality[13]. Coagulopathy and hemorrhagic complications can occur due to impaired synthetic function. Pulmonary manifestations of severe leptospirosis include alveolar hemorrhage (termed severe pulmonary hemorrhagic syndrome or SPHS) and pulmonary edema, both of which can result in acute respiratory distress syndrome (ARDS)[17]. Pulmonary involvement is associated with significantly higher mortality from leptospirosis, with case fatality rates estimated from 50%-70%[1,18]. *Leptospira* infection can also involve the heart, most commonly causing nonspecific echocardiogram abnormalities (even in mild disease). Myocarditis, pericarditis, heart block and arrhythmias may occur, and repolarization abnormalities are a poor prognostic sign[11,19]. Even after recovery, patients may have continued late sequelae including neuropsychiatric and ocular symptoms[14].

Laboratory findings commonly associated with leptospirosis are generally nonspecific, but may include mild leukocytosis often with left shift in up to 2/3 of patients, as well as thrombocytopenia[14]. Inflammatory markers (ESR, CRP) may be elevated. In cases with more severe renal manifestations, serum creatinine is often elevated, and both hypokalemia and hyponatremia may be present[13]. Even when clinical manifestations are mild, conjugated hyperbilirubinemia is often present, and can reach levels up to 40-80 mg/dL[2,13]. Mild elevations of serum transaminases are frequently seen[14]. Urinalysis may reveal proteinuria, pyuria, and occasional microscopic hematuria[2]. Creatine kinase and serum amylase may also be elevated. Examination of cerebrospinal fluid is typically consistent with aseptic meningitis, with a lymphocytic pleocytosis, moderately increased protein, and normal glucose levels[13].

DIAGNOSTIC TESTING

Because clinical manifestations of leptospirosis are very non-specific and have significant overlap with a variety of other febrile illnesses, a combination of exposure history and symptoms should prompt confirmatory testing. However, clinical suspicion alone may be enough to warrant empiric antibiotic treatment in many cases. In general, definitive diagnosis of leptospirosis can be made *via* either traditional microbiological methods (direct detection, culture) or serology. *Leptospira*, like other spirochetes, stains poorly with traditional staining methods and is best visualized with darkfield microscopy, however sensitivity and specificity are both poor when examining clinical samples[12,20]. Culture of *Leptospira* from patient samples is also challenging: The organisms typically take 1-2 wk to grow but may take over a month, and special growth media is required, often necessitating advance notice to the lab. Though specificity of culture is excellent, sensitivity is very poor (5%-50%)[11]. Blood and CSF cultures are most useful during the first 10 day of illness (leptospiremic phase), when organisms are spreading hematogenously[12,14]. However, as the immune phase begins, yield of blood cultures decreases significantly. After the second week of illness, urine cultures for *Leptospira* are more likely to be positive due to the organism's proclivity for renal tubules, and may remain positive for up to 30 d after resolution of symptoms.

Serological methods are the most commonly used means of confirming a diagnosis of leptospirosis. The "gold standard" is the microscopic agglutination test (MAT), in which acute and convalescent sera from a suspected case is mixed with a panel of live antigens from different serogroups of *Leptospira* organisms and examined for agglutination[11-13]. While there is some variability amongst labs/references, most commonly, a single titer of 1:100 (range is 1:100 to 1:800), or a four-fold rise in titer between acute and convalescent sera, serologically confirms the diagnosis of leptospirosis[12-14]. Though test characteristics are overall superior to culture and microscopy (90% sensitivity, > 90% specificity), this method has several limitations[11]. The test requires a panel of live organisms specific to the area the patient is suspected to have acquired the infection as well as specialized lab expertise, limiting use to reference laboratories[13]. Additionally, there is significant cross-reactivity both between different serogroups of *Leptospira*, as well as with other spirochetes (*Treponema* and *Borrelia* species)[11]. Because the antibody response required for MAT testing is often insufficient for detection until the second week of disease (when the immune phase begins), sensitivity when symptoms begin is limited. Several serologically-based methods to detect the early host response during the first week of disease have been developed; the most commonly used is enzyme-linked immunosorbent assay (ELISA). These assays use a general leptospiral antigen that will detect IgM to both pathogenic and non-pathogenic serogroups of *Leptospira*[14]. In addition to having greater sensitivity than the MAT during the first week of leptospiral infection, ELISA is more easily standardized, and several commercial products are available, so use is not restricted to reference laboratories.

As both culture and serological methods are limited in early detection (by leptospiral growth rate and host immune response development, respectively), newer molecular methods have been developed to facilitate

early detection. Both conventional and real-time PCR techniques are highly sensitive even early in disease, prior to development of antibody response[21]. Because this period correlates with the leptospiremic phase, blood is the best sample in which to detect leptospiral nucleic acid, though urine, CSF, or tissue may also have detectable levels later in disease[11]. Of note, because PCR detects nucleic acid and is not dependent on presence of live organisms, this technique can be used even after initiation of empiric antibiotics[21]. Other molecular techniques for early diagnosis of leptospirosis have been described, including *in-situ* hybridization and loop-mediated isothermal amplification, but though promising, the clinical applicability of these molecular methods has yet to be established[21]. Additionally, because specialized equipment is typically required, utility may be limited in resource-poor or field environments. In these situations, early IgM detection tests are likely to be the best balance of rapid results with suitable test characteristics, ease of use, and cost. In addition to ELISA as discussed above, several other rapid screening test methods have been developed including dipsticks, latex and slide agglutination tests, and immunochromatography[12-14]. Regardless of the method used, all positive tests require confirmatory testing, ideally with the MAT[14].

TREATMENT

Initial treatment depends on the severity of the illness at presentation. Most cases of leptospirosis are mild and self-limiting, and patients often do not present for care[22]. For milder cases, oral doxycycline, azithromycin, ampillicin or amoxicillin are all options based on *in vitro* testing though no randomized clinical trials comparing antibiotic regimens in mild cases have been performed[22,23]. In a small double blind randomized study by of 29 patients by McClain *et al*[24], antibiotic treatment has been shown to reduce symptoms including fever, malaise and headache by 2 d, and prevent leptospiruria, but there is insufficient evidence to conclude that treatment prevents progression to severe disease. Considerations for treatment should depend on cost, availability and differential diagnosis. Doxycycline should be avoided in pregnant women and children. In areas where rickettsial diseases are endemic, doxycycline or azithromycin are the drugs of choice[22]. Regardless of antibiotic choice, a Jarisch-Herxheimer reaction can develop, typically within the first few hours after antibiotic administration. For severe cases, intravenous penicillin G sodium has been the traditional recommended treatment based on a 1988 study by Watt *et al*[25], in which penicillin G treatment compared to placebo demonstrated significant reductions in fever duration, creatinine elevation and hospital duration in 42 patients and has been reinforced by expert opinion[14,23,25,26]. Due to emerging antibiotic resistance of bacterial pathogens, the narrow spectrum against other tropical infections, and several studies showing no clinical benefit including mortality with penicillin, there has been interest in evaluating other antibiotics[26-28]. In an open, randomized study by Suputtamingkot *et al*[26], 256 patients with confirmed leptospirosis were randomized to receive intravenous penicillin G, doxycycline or cefotaxime. There was no significant difference in mortality rate (1.2%, 1.2% and 0%), duration of fever (72, 72 and 60 h), and duration of hospitalization (6, 5 and 5.5 d)[26]. Similar findings were seen in an open-label, randomized study by Panaphut *et al*[29], which compared intravenous ceftriaxone to intravenous penicillin G in 173 patients with severe leptospirosis. There was no statistically significant difference in fever duration (3 d in each group), duration of renal impairment including failure (RR = 1.0; 95%CI: 0.7-1.4), or mortality (5 patients in each group, 5.8% overall case mortality rate)[29]. Interestingly, the role of any antibiotic in the treatment of leptospirosis has come into question. Both a 2012 Cochrane Review by Brett-Major and Coldren and in a 2013 meta-analysis by Charan *et al*[30] found insufficient evidence to recommend antibiotic treatment for both mild and severe cases of leptospirosis[29,30]. Specifically, Charan *et al*[30] demonstrated no statistically significant effect of penicillin G *vs* placebo on mortality or need for dialysis.

The reported mortality associated with severe pulmonary involvement is up to 50%-70%[1,18]. A proposed mechanism of pulmonary injury is immune-mediated inflammatory response, hence an interest in adjunctive treatment with steroids. Rodrigo *et al*[31] examined the role of steroids in patients with severe pulmonary infection in a 2013 meta-analysis. Of the five identified trials, four demonstrated benefits of early steroid administration; however, each was considered methodically flawed. The fifth trial was a double-blind, randomized control study, which demonstrated no mortality benefit and a potentially increased risk of infection[31]. Desmopressin has also been evaluated as adjunctive treatment, but a randomized study of 52 patients with confirmed leptospirosis by Niwattayaku *et al*[32] found no mortality benefit.

PREVENTION

There have been very few studies examining the efficacy of leptospirosis chemoprophylaxis. A 2000 Cochrane review article by Guidugli *et al*[33] identified two such studies, one of which was found to be flawed. The included study by Takafuji *et al*[34] was a double-blind, randomized study of 940 United States soldiers deployed to Panama. Subjects were randomized to either oral doxycycline 200 mg weekly or placebo. Twenty cases of leptospirosis occurred in the placebo group (incidence of 4.2%) *vs* 1 case in the doxycycline group (incidence of 0.2%), with an estimated protective efficacy of 95%[34]. The applicability of chemoprophylaxis in other situations is unclear[33].

CONCLUSION

Leptospirosis is a zoonotic disease with worldwide distribution and increasing prevalence. Infection is caused by the spirochete *Leptospira*, with common exposure

being contaminated fresh water. Incubation is typically 7-12 d but ranges from 2-30 d. Most infections are asymptomatic, but symptoms range from a mild, self-limiting, non-specific febrile illness to fulminant respiratory and renal failure with a high mortality rate. Laboratory confirmation of disease can be problematic, especially in resource poor areas. Serologic testing is most frequently performed, although newer diagnostic tests are becoming available. Oral doxycycline is the typical treatment for mild cases, though azithromycin and oral penicillins are reasonable alternatives. We favor doxycycline or azithromycin as confirmatory testing is often not available for mild cases and treatment will cover most rickettsial infections as well. Intravenous penicillin G has long been the standard of care for severe cases though limited studies show no benefit compared to third generation cephalosporins. While some controversy exists regarding the benefit of treatment of any cases of leptospirosis, we recommend treatment, particularly for severe cases until definitive studies are available, given the high mortality rates. Antibiotics should be chosen based on certainty of diagnosis, cost, availability and clinical support. Given the paucity of data, we cannot provide any evidenced based recommendations for chemoprophylaxis.

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ORIGINAL ARTICLE

Retrospective Study

Can the detection of IgA anti-*Mycoplasma pneumoniae* added to IgM increase diagnostic accuracy in patients with infections of the lower respiratory airways?

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Abstract

AIM

To evaluate the increase in diagnostic yield, by using IgA in addition to IgM, instead of IgM alone, in relation to the age of the patients.

METHODS

The study considered 1067 blood samples from patients with clinical signs of lower respiratory tract infections, tested for anti-Mycoplasma IgG, IgM and IgA antibody.

RESULTS

The increase in diagnostic yield with IgA, compared to IgM detection alone was of 3.5% with statistically significant differences between age groups (0.8% for those equal/under 50 years of age and 4.3% for those over 50).

CONCLUSION

Our findings demonstrate that IgA detection lead to a twofold increase in the number of diagnoses among the older age groups, but it did not result in relevant increase among the younger age groups.

Key words: Community-acquired infections; Diagnostic yield; Elderly patients; IgA; Mycoplasma pneumoniae

Core tip: Diagnosis of Mycoplasma pneumoniae infection relies on IgM detection but also IgA can be searched. There are few data on the range of increase of diagnosis adding the search for the IgA. Detection of IgA (without IgM) increases diagnosis of 3.5% compared to the detection of IgM alone. The greater increase is for the patients older than 50 years. Detection of IgA antibodies could be included in laboratory routine only in older patients.

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INTRODUCTION

Mycoplasma pneumoniae (*M. pneumoniae*) is one of the main causative agents for community-acquired infections of the lower and upper respiratory tract, especially during the first two decades of life[1-4]. Because symptoms can be commonly confused with those caused by other pathogens, diagnosis must rely on specific tests such as immunological assays[4-7]. Specific IgMs rapidly increase after the onset of the disease, reaching peak levels between 1-4 wk and then disappearing within a few months[8,9]. Compared to IgGs, which increase at slower rates and persist longer at high levels in the serum, the detection of IgM allows to diagnose acute infection[10-12].

However, while IgM values markedly increase in children and young patients[8,13-16] adult and elderly patients who might have repeatedly been exposed to the infection may have a less vigorous immune response, or no response at all[2,8,9,17-20]. In the event of reinfection, IgMs are produced less frequently and negative assay findings cannot exclude an ongoing infection especially in patients above the age of 45[14,18,21].

Because IgAs develop in a more predictable way and a more rapid rate compared to IgMs, and rapidly decrease during the second month from onset of the disease, these antibodies are considered reliable markers of infection[9,14,22]. Indeed, some authors emphasize that IgA detection can reveal to be useful in diagnosing infections[23,24] especially in IgM negative patients[9,22]. So far, different tests for IgA have been marketed and used in the laboratory routine[25], however the impact of the introduction of these tests in terms of increase in laboratory diagnosis accuracy must be fully reevaluated.

The aim of the present study was to assess the usefulness of IgA in confirming suspicion of infections by *M. pneumoniae* and the increase in diagnostic yield using IgA in addition to IgM - compared to IgM alone - in both younger and older patient groups.

MATERIALS AND METHODS

The present study was performed at the Microbiology Unit, Hospital of Legnano, which serves both patients hospitalized in the specialist medical and surgical departments, well as patients from the out-clinic. Between January 2012 and December 2014, 1067 samples collected from as many consecutive patients (49 out-patients and 1018 in-patients: 622 males and 445 females; mean age: 62.9 years, range 0.5-100) with clinical manifestations of infections of the lower respiratory airways who had been requested either by the hospital staff or by a GPs specifically for the search of anti-Mycoplasma IgG, IgM and IgA antibodies.

The IgG and IgM detection was performed by means of chemiluminescent assay (LIAISON Mycoplasma pneumoniae IgG and IgM; DiaSorin, Saluggia, Italy), whereas IgA detection was performed by immunoenzymatic assay (SeroMP recombinant IgA; Savyon Diagnostics Ltd, Ashdod, Israel). The tests were performed according to the manufacturers' instruction and the cut off for the three tests is 10 as index value (that is expressed for IgG and IgA as AU/mL or Arbitrary Units/mL and only as index value for IgM).

Statistical analysis

The data were statistically analyzed using the Fisher's exact test and linear regression method by SPSS software (Version 16.0, SPSS Inc. Chicago, IL).

RESULTS

The immunological assays of the 1067 samples yielded 178 (16.7%, 95%CI: 14.46-18.94) IgG positive, 66 (6.2%; 95%CI: 4.75-7.65) IgM positive and 50 (4.7%; 95%CI: 3.43-5.97) IgA positive with no statistical differences between out-patients and in-patients (P = 0.845 for IgG; 0.763 for IgM and 0.724 for IgA). Table 1 shows complete antibody profiles. Specifically, 53 individuals (groups C + E) (5.0%; 95%CI: 3.69-6.31) resulted positive for IgM but not for IgA, 37 (groups D + F) (3.5%; 95%CI: 2.40-4.60) IgA without IgM and 13 (groups G + H) (1.2%; 95%CI: 0.55-1.85) positive for both IgM and IgA. Overall, 103 subjects (groups C + D + E + F + G + H) (9.7%; 95%CI: 7.92-11.48) presented IgM and/or IgA antibodies.

The increase of diagnostic yield achieved by adding IgA investigation to IgM, compared to considering IgM alone resulted to be 3.5% (95%CI: 2.40-4.60).

Table 2 lists the positivity for antibodies classes for age groups and Table 3 shows the percentage increase of diagnosis (for each age group) adding IgA to IgM compared to cases that could be diagnosed considering IgM alone (with or without IgG). The data were analyzed by linear regression method, which pointed out a

Table 1 Serological profile for anti-*Mycoplasma pneumoniae* antibodies in patients with of lower respiratory airway infections

	Anti-*Mycoplasma pneumoniae* antibodies profiles			Patients	
Group	IgG	IgM	IgA	*n* (%)	95%CI
A	Negative	Negative	Negative	821 (76.9%)	74.37-79.43
B	Positive	Negative	Negative	143 (13.4%)	11.36-15.44
C	Negative	Positive	Negative	40 (3.7%)	2.57-4.83
D	Negative	Negative	Positive	21 (2.0%)	1.16-2.84
E	Positive	Positive	Negative	13 (1.2%)	0.55-1.85
F	Positive	Negative	Positive	16 (1.5%)	0.77-2.23
G	Negative	Positive	Positive	7 (0.7%)	0.20-1.20
H	Positive	Positive	Positive	6 (0.6%)	0.14-1.06

Table 2 Presence of serological markers for anti-*Mycoplasma pneumoniae* antibodies, divided per age group in patients with infections of the lower airway tract

	Age (yr)								
Antibodies	0-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	> 80
n	91	30	25	48	55	91	155	289	283
IgG	12	7	6	13	10	21	29	42	38
Positive	(13.2%)	(23.3%)	(24.0%)	(27.1%)	(18.2%)	(23.1%)	(18.7%)	(14.5%)	(13.4%)
95%CI	6.25-20.15	8.17-38.43	7.26-40.74	14.53-39.67	8.00-28.40	14.44-31.76	12.56-24.84	10.44-18.56	9.43-17.37
IgM	22	8	3	7	2	5	5	6	8
Positive	(24.2%)	(26.7%)	(12.0%)	(14.6%)	(3.6%)	(5.5%)	(3.2%)	(2.1%)	(2.8%)
95%CI	15.40-33.00	10.87-42.53	0.00-24.74	4.61-24.59	0.00-8.52	0.82-10.18	0.43-5.97	0.45-3.75	0.88-4.72
IgA	6	3	1	2	0	5	5	14	14
Positive	(6.6%)	(10.0%)	(4.0%)	(4.2%)	(0%)	(5.5%)	(3.2%)	(4.8%)	(4.9%)
95%CI	1.50-11.70	0.00-20.74	0.00-11.68	0.00-9.87	0.00-0.00	0.82-10.18	0.43-5.97	2.34-7.26	2.38-7.41
IgM and/or IgA	23	8	3	8	2	9	10	18	22
Positive	(25.3%)	(26.7%)	(12.0%)	(16.7%)	(3.6%)	(9.9%)	(6.5%)	(6.2%)	(7.8%)
95%CI	16.37-34.23	10.87-42.53	0.00-24.74	6.15-27.25	0.00-8.52	3.76-16.04	2.62-10.38	3.42-8.98	4.68-1.92

Table 3 Increase of diagnosis divided per age group adding search of IgA to IgM, compared to cases that can be diagnosed considering IgM alone

	Age (yr)								
Antibodies	0-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	> 80
n	91	30	25	48	55	91	155	289	283
IgM without IgA	17 (18.7%)	5 (16.7%)	2 (8.0%)	6 (12.5%)	2 (3.6%)	4 (4.4%)	5 (3.2%)	4 (1.4%)	8 (2.8%)
95%CI	10.69-26.71	3.35-30.05	0.00-18.63	3.14-21.86	0.00-8.52	0.19-8.61	0.43-5.97	0.05-2.75	0.88-4.72
IgA without IgM	1 (1.1%)	0 (0%)	0 (0%)	1 (2.1%)	0 (0%)	4 (4.4%)	5 (3.2%)	12 (4.1%)	14 (4.9%)
95%CI	0.00-3.24	0.00-0.00	0.00-0.00	0.00-6.16	0.00-0.00	0.19-8.61	0.43-5.97	1.81-6.39	2.38-7.41
IgM plus IgA	5 (5.5%)	3 (10.0%)	1 (4.0%)	1 (2.1%)	0 (0%)	1 (1.1%)	0 (0%)	2 (0.7%)	0 (0%)
95%CI	0.82-10.18	0.00-20.74	0.00-11.68	0.00-6.16	0.00-0.00	0.00-3.24	0.00-0.00	0.00-1.66	0.00-0.00
Increase of diagnosis	1.1%	0%	0%	2.1%	0%	4.4%	3.2%	4.1%	4.9%
95%CI	0.00-3.24	0.00-0.00	0.00-0.00	0.00-6.16	0.00-0.00	0.19-8.61	0.43-5.97	1.81-6.39	2.38-7.41

significant correlation with the patient age for the IgA (P = 0.048) and, stratified subjects according to two age groups: up to 50 years of age and > 50 years of age; a statistical difference was found (P = 0.035). Stratifying by the age groups (below/equal and above 50 years of age), a diagnostic increase of 0.8% was observed for individuals under/equal 50 years (95%CI: 0.00-1.91), and of 4.3% in those over 50 years of age (95%CI: 2.91-5.69) (P = 0.0052).

DISCUSSION

Overall, our results show that 9.7% of patients presenting lower respiratory airway infections were actually infected by *M. pneumoniae*, in agreement with data from literature, documenting it as the causative agent in 5%-30% of cases of community acquired pneumonia[18,26-29]. Our study revealed a higher percentage of infection in younger patients under the age of twenty, among which

Mycoplasma was associated to approximately one fifth of the overall infections.

Based on IgA screening, the detection of these antibodies (without IgM) led to a broad diagnostic increase of 3.5% compared to the detection of IgM alone. However, the greater increase was for the "over 50" group. Indeed, as suggested in literature, older patients may not produce IgM during infection by *M. pneumoniae*[8,13,16-21]; hence its inconsistent absence in this category of patients is a well-acknowledged limitation to *Mycoplasma* serology. In this setting, IgAs appear to be so far the only way to detect infection by this agent. Yet, the presence or absence of specific IgM in presence of specific IgA levels allows to differentiate between primary infection and reinfection, therefore, the estimation of both IgM and IgA is necessary for the maximal detection of an ongoing *M. pneumoniae* infection. Moreover, specific IgG levels in our patient population remained elevated for many weeks and were not useful from a diagnostic point of view.

In general, IgA were detected across all age groups; while these were associated to IgM in the younger age groups, this finding did not translate into an increase in diagnostic yield for such age groups. Nevertheless, IgA doubled the number of diagnoses in absolute values among the older age groups, suggesting that the search for IgA could be helpful whenever more sophisticated techniques, such as those of molecular biology, are not available.

Some authors have indicated the DNA detection by PCR as the gold standard for diagnosis of acute Mycoplasma infection[8,30-33], but other authors have emphasized the limits and have stressed the heterogeneity in sensitivity, the variability of results with regard to the time of collection (detection more frequent in early infection, less frequent during later stages of the disease) and positivity even in some healthy subjects[7,14,16,18,34,35]. Accordingly, the Authors suggest that serology should be combined with PCR, rather than be replaced by it[7,14,18]. Such observation is even more relevant considering that the molecular biology techniques may not be always available in some hospitals especially in countries and regions with limited resources[7].

In conclusion, IgA detection has demonstrated to be useful and reliable in confirming diagnoses of suspected *M. pneumoniae* infections in older patients, yielding higher diagnostic accuracy as compared to detection of IgM alone. This suggests detection of IgA antibodies could be included in laboratory routine in older patients showing clinical signs of lower respiratory tract infections.

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COMMENTS

Background

Mycoplasma pneumoniae (*M. pneumonia*) is one of the main causative agents for community-acquired infections of the lower and upper respiratory tract, especially during the first two decades of life. Because symptoms can be commonly confused with those caused by other pathogens, diagnosis must rely on specific tests such as immunological assays. Diagnosis of *M. pneumoniae* infection relies on detection of anti-Mycoplasma IgM; yet, while IgM values markedly increase in children and young patients, the immune response in adult and elderly patients who might have repeatedly been exposed to the infection may be less vigorous or even absent. Because IgAs develop in a more predictable way and at a more rapid rate compared to IgMs, the search of these antibodies in addition to IgM might add to diagnostic accuracy. However, so far, there is not enough evidence to support IgA as a reliable marker for infection, nor on the impact of its introduction in terms of increase in laboratory diagnosis accuracy so far has not been sufficiently evaluated.

Research frontiers

In the area of laboratory diagnosis, there is much interest in obtaining a higher diagnostic yield when there is a suspicion of infections by *M. pneumoniae*. Currently diagnoses are based on determination of IgMs alone for all age groups, and do not foresee routine determination of IgA in addition to IgM.

Innovations and Breakthroughs

Different tests for IgA have been marketed and used in the laboratory routine, but the impact of the introduction of these tests in terms of increase in laboratory diagnosis accuracy has not been sufficiently evaluated. In the present study, the authors evaluated the increase in diagnostic yield and the utility of this test in relation to the age of the patients.

Applications

IgA is a significantly useful marker in patient age groups > 50 years of age, increasing up to twofold the number of positive diagnoses.

Terminology

Specific IgM anti-Mycoplasma rapidly increase after the onset of the disease, reaching peak levels between 1-4 wk and then disappearing within a few months. Specific IgGs increase at slower rates and persist longer at high levels in the serum. The specific IgAs develop in a more predictable way and a more rapid rate compared to IgMs, and rapidly decrease during the second month from onset of the disease.

Peer-review

It is an interesting paper showing that IgA mycoplasma antibodies contribute to diagnostic yield increase, compared with usage of only IgM mycoplasma antibodies in older patients > 50 years old.

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