UNIVERSITE DE LA MEDITERRANEE AIX-MARSEILLE II
FACULTE DE MEDECINE DE MARSEILLE
ECOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

THESE DE DOCTORAT

Présentée par

Elisabeth BOTELHO-NEVERS
Née le 20 Février 1978 à Saint-Etienne

En vue d'obtention du grade de Docteur de l'Université de la Méditerranée
Spécialité: Maladies Infectieuses

Les facteurs de risque de sévérité liés à l'hôte et au traitement au cours de la fièvre boutonneuse Méditerranéenne

Soutenue le 8 Septembre 2011

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Pr Didier RAOULT	**Directeur de thèse** **Directeur du Laboratoire**

Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes
CNRS-IRD UMR 6236

Avant propos:

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille.
Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. Par ailleurs, la thèse est présentée sur article publié, accepté ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professeur Didier RAOULT

SOMMAIRE

Résumé

La fièvre boutonneuse Méditerranéenne (FBM) est due à *Rickettsia conorii* subsp. *conorii,* bactérie intracellulaire stricte. Cette maladie, initialement décrite comme bénigne, présente actuellement un taux de sévérité de l'ordre de 10% avec une augmentation de cette sévérité décrite dernièrement. Au cours d'une étude clinique rétrospective portant sur 161 cas de FBM, nous avons observé que le traitement par fluoroquinolones était associé à une évolution défavorable, ce qui à ce jour n'avait jamais été rapporté, alors que la doxycycline semble être protectrice. Nous avons également observé cet effet délétère des fluoroquinolones sur un modèle *in vitro* d'infection cellulaire à *R. conorii*, effet qui n'a pas été observé avec la doxycycline. Une des hypothèses pouvant expliquer cet effet est l'induction du module toxine-antitoxine par les fluoroquinolones. Ainsi nous avons montré que la ciprofloxacine modulait l'expression des gènes du couple toxine-antitoxine. Nous avons également montré que les statines pouvaient avoir un effet prophylactique au cours de l'infection par *R. conorii*. Enfin nous avons étudié la réponse de l'hôte au sein de l'escarre d'inoculation par une approche transcriptomique.

Cette thèse a mis en évidence que les traitements reçus au cours de la FBM peuvent modifier le pronostic de cette maladie. Le choix des antibiotiques est donc crucial et doit faire l'objet d'études complémentaires.

Mots clés: *R. conorii* subsp. *conorii*, fièvre boutonneuse Méditerranéenne, sévérité, fluoroquinolones, réponse de l'hôte, doxycycline, statines

Abstract

Mediterranean spotted fever (MSF) is caused by *Rickettsia conorii* subsp. *conorii*, a strict intracellular bacterium. The disease initially described as benign presents currently rates of severity around 10% with an increase described recently. In a retrospective clinical study of 161 cases of MSF, we observed that treatment with fluoroquinolones was associated with an unfavourable outcome whereas doxycycline appeared to be protective. We also observed this deleterious effect of fluoroquinolones *in vitro* in a cellular model of *R. conorii* infection, which was not observed with doxycycline. One hypothesis that could explain this effect is the induction of toxin-antitoxin module by fluoroquinolones. Thus we have shown that ciprofloxacin modulates the gene expression of toxin-antitoxin module. We have also shown that statins may have a prophylactic effect during infection by *R. conorii*. Finally, we have studied the host response within the inoculation eschar by a transcriptomic approach. This thesis has shown that the treatment received during the MSF can change the prognosis of this disease. The choice of antibiotics is crucial, and should be subject to further studies.

Keywords: *R. conorii* subsp *conorii*, Mediterranean spotted fever, severity, fluoroquinolones, host response, doxycycline, statins

Introduction

La fièvre boutonneuse Méditerranéenne (FBM) a été décrite pour la première fois par Conor et Bruch en 1910 (1). La fameuse «tâche noire» ou escarre d'inoculation a été décrite à Marseille en 1925 par Boinet et Pieri. Sept ans plus tard, *Rickettsia conorii*, bactérie intracellulaire stricte de la famille des *Rickettsiaceae* a été reconnue comme l'agent causal de la FBM par Blanc et Caminopetros qui ont dénommé ainsi la bactérie en l'honneur de Conor (1). Sur ces premières descriptions, la maladie avait une évolution spontanément favorable dans la très grande majorité des cas, survenant typiquement en été dans une zone d'endémie pour *R. conorii* et caractérisée par la présence de fièvre, d'une éruption maculo-papuleuse et d'une escarre d'inoculation (1;2). En effet, dénommée également initialement «typhus bénin d'été», la MSF a été longtemps considérée comme bénigne par rapport à son homologue américain, la fièvre pourprée des Montagnes Rocheuses ou Rocky Mountain spotted fever, avec une mortalité inférieure à 1% avant l'ère des antibiotiques (1).

Les premiers cas de FBM maligne ou sévère ont été rapportés en 1983 par Raoult *et coll.*(2). Bien que quelques cas de formes compliquées dits «cas atypiques» de FBM aient été décrits auparavant par Olmer (1), cette publication princeps (2) rapporte pour la première fois 6 cas de patients présentant des complications de type défaillance rénale, hépatique, neurologique, respiratoire et purpura dont 4 décèdent malgré une antibiothérapie adaptée. Entre 1983 et 1984, les formes sévères de FBM vont représenter 5% des cas totaux de FBM pris en charge à Marseille (3). Par la suite, les séries cliniques de FBM publiées ont rapporté de 5 à 12,7% de cas sévères avec des taux de mortalité entre 0 et 2,5% (4-7). Plus récemment des taux de sévérité et de mortalité anormalement élevés ont été observés au Portugal, avec jusqu'à 22% de cas sévères (8) et 18,1% de mortalité parmi les patients hospitalisés pour FBM entre 1994 et 1998 (9). En 1997, cette même équipe a rapporté un taux de mortalité de 32.3% chez les patients hospitalisés pour FBM (9).

Cette augmentation de la sévérité au cours de la FBM a été en partie mise sur le compte du pathogène (8;10). En effet depuis 2005, quatre sous-espèces ont été décrites au sein de l'espèce *R. conorii* (11). *R. conorii* subsp. *israelensis* semble induire des formes plus sévères de fièvre boutonneuse qu'il convient d'appeler «Israeli spotted fever» (1;11) et co-circule avec *R. conorii* subsp. *conorii,* l'agent de la FBM, dans certaines régions telles que le Portugal, Israël et la Sicile (1). Afin d'étudier les facteurs de risque de sévérité au cours de la FBM, nous nous sommes affranchis des facteurs liés au pathogène en nous intéressant exclusivement aux cas de FBM dus à *R. conorii* subsp. *conorii*. Ainsi, au cours de ce travail de thèse, nous avons étudié les facteurs de risque de sévérité liés à l'hôte et au traitement au cours de la FBM.

En guise d'introduction générale au cours de ce travail de thèse nous avons réalisé une revue sur les facteurs de risque de sévérité au cours des rickettsioses et notamment au cours de la FBM. Cette revue de la littérature nous a permis de faire une synthèse à la fois sur les facteurs de risques liés au pathogène, liés à l'hôte et liés au traitement au cours des rickettsioses. Ainsi, à l'heure actuelle, les facteurs associés à la sévérité liés au pathogène semblent être une évolution réductive des génomes des rickettsies les plus virulentes (12), avec perte des gènes de régulation (13). Cette revue a également été l'occasion de réactualiser les facteurs de risques liés à l'hôte, la dernière revue sur ce sujet datant de plus de vingt ans (14). Nous nous sommes également intéressés au rôle du traitement, notamment antibiotique, au cours des rickettsioses et de son impact sur le pronostic de ces pathologies, cet aspect ayant été peu étudié à ce jour.

Afin d'identifier les éléments pouvant expliquer la gravité actuelle de la FBM, la première partie de ce travail a consisté en une étude clinique portant sur les cas récents de

FBM ayant été diagnostiqués au Centre National de Référence des Rickettsioses à Marseille. Le but principal de cette étude était d'étudier les facteurs de risques de sévérité dans cette pathologie. Comme il a été dit plus haut les cas étudiés étaient des cas dus à *R. conorii* subsp. *conorii* afin de nous affranchir des facteurs de sévérité liés au pathogène. La dernière série clinique sur la FBM en France remontait à 1986 (4) et il nous a semblé nécessaire de réactualiser ces données. Par ailleurs, la définition même de forme «sévère» de FBM dans la littérature étant très variable et non standardisée, nous avons souhaité appliquer une définition stricte de la sévérité à savoir le syndrome de défaillance multiviscérale (SDMV) (15). Cette étude rétrospective a permis d'analyser les facteurs de risques de sévérité liés à l'hôte, notamment le terrain, les facteurs épidémiologiques mais également les facteurs liés aux traitements reçus, notamment antibiotique, au cours de la FBM. Cette approche générale sur les facteurs de risques de sévérité a mis en évidence que les fluoroquinolones pouvaient être associées aux formes sévères de la FBM.

Pour tenter de comprendre l'effet délétère des fluoroquinolones au cours de la FBM, nous avons réalisé un travail *in vitro* d'infection à *R. conorii* subsp. *conorii* sur modèle cellulaire. En effet, les fluoroquinolones sont des antibiotiques actifs *in vitro* sur *R. conorii* quelle que soit la sous-espèce (16;17). En pratique clinique les fluoroquinolones sont d'ailleurs proposées en alternative au traitement par doxycycline chez les adultes (1). Cette proposition est basée sur 3 études cliniques (18-20) portant sur des patients présentant des formes non sévères de FBM. Parmi ces études, une seule était randomisée, comparative à la doxycycline et réalisée en double aveugle (19) et mettait en évidence une réponse significativement plus lente dans le groupe ciprofloxacine par rapport au groupe doxycycline. L'utilisation des fluoroquinolones au cours des rickettsioses a également déjà été associée à des évolutions défavorables au cours du typhus épidémique, du typhus des broussailles et du

typhus murin (21-23). Cependant, aucune étude n'a été menée à ce jour pour comprendre cet effet délétère d'antibiotiques pourtant considérés efficaces sur les différentes espèces de Rickettsies, or les fluoroquinolones représentent 10 à 20% des traitements utilisés dans les séries cliniques récentes de FBM (8;9;24). En utilisant la technique des plages de lyse, une méthode ancienne mais très sensible que nous avons amélioré au cours de notre Master 2 Recherche, nous avons observé l'effet précoce des fluoroquinolones au cours de l'infection par *R. conorii* subsp. *conorii*. Nous avons également testé l'hypothèse d'un impact des fluoroquinolones sur l'expression du module toxine-antitoxine contenu dans le génome de *R. conorii*.

Etant donné l'existence de formes malignes de FBM, nous avons également testé *in vitro* l'effet des statines sur *R. conorii* subsp. *conorii.* En effet, les statines sont des inhibiteurs de l'HMG CoA réductase qui, au-delà de leur effet hypocholestérolémiant, ont des effets pleiotropiques très variés avec des actions anti-inflammatoires et anti-infectieuses reconnues (25;26) y compris chez des patients ayant des critères de sévérité admis en réanimation (27). Les statines semblent notamment avoir un effet anti-infectieux prophylactique observé à la fois *in vivo* (26;27) et également *in vitro* dans différents modèles. Toutefois, ces molécules n'ont jamais été testées sur les Rickettsies d'où cette démarche. Par ailleurs cette protection anti-infectieuse, lorsque les statines sont administrées de façon prophylactique, c'est-à-dire avant l'infection, semble passer par une modulation immune de l'hôte.

Enfin, au cours de ce travail de thèse, au-delà de l'étude clinique, nous avons souhaité appréhender la réponse de l'hôte au cours de la FBM au sein même de la lésion d'inoculation de *R. conorii*, à savoir au sein même de la «tâche noire». Cette escarre d'inoculation n'a longtemps été étudiée que d'un point de vue histologique (28). A ce jour un seul travail à

porté sur la réponse immune intralésionnelle de l'hôte dans la FBM (29). Cette étude n'a permis de tester l'expression que de quelques marqueurs de la réponse immune. Une étude plus large sans a priori concernant les gènes modulés au sein de l'escarre d'inoculation était donc souhaitable afin de comprendre les mécanismes initiaux entrant en jeu dans la réponse de l'hôte. Une approche transcriptomique a déjà été utilisée pour étudier les modifications transcriptionnelles intralésionnelles sur *R. conorii* (30). Nous avons donc dans notre travail de thèse utilisé cette approche transcriptomique pour étudier la réponse de l'hôte.

Par ces différentes approches, le but de ce travail est donc d'approfondir et d'analyser les facteurs de risque de sévérité au cours de la FBM en lien avec des facteurs intrinsèques à l'hôte mais également extrinsèques de par les traitements reçus par ce même hôte.

REVUE

Host, pathogen and treatment-related prognostic factors in rickettsiosis

E. Botelho-Nevers, D. Raoult

European Journal of Clinical Microbiology and Infectious Diseases

Accepté 26 Avril 2011

URMITE UMR 6236, CNRS-IRD, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille

cedex 05, France

Eur J Clin Microbiol Infect Dis
DOI 10.1007/s10096-011-1208-z

REVIEW

Host, pathogen and treatment-related prognostic factors in rickettsioses

E. Botelho-Nevers · D. Raoult

Received: 24 February 2011 / Accepted: 28 February 2011

Abstract Diseases caused by rickettsiae, which are vector-borne bacteria, vary widely from mild and self-limiting, to severe and life-threatening. Factors influencing this diversity of outcome are related to the host, to the infectious agent and to the treatment used to treat the infection. A literature search was conducted on PubMed using the phrases "factors-related severity, outcome, host, pathogen, *Rickettsia conorii*, *R. rickettsii*, *R. africae*, *R. felis*, *R. prowazekii*, *R. typhi*, genomics". Among host factors, old age and the male gender have been associated with poor outcome in rickettsioses. Co-morbidities, ethnical factors and the genetic background of the host also seem to influence the outcome of rickettsial diseases. Moreover, although the degree of the host response is beneficial, it could also partly explain the severity observed in some patients. Among pathogen-related factors, traditional concepts of factors of virulence had been challenged and genomic reductive evolution with loss of regulatory genes is the main hypothesis to explain virulence observed in some species, such as *Rickettsia prowazekii*, the agent of epidemic typhus. *R. prowazekii* is the more pathogenic rickettsiae and harbours the smaller genome size (1.1 Mb) compared to less or non-virulent species, and is not intracellularly motile, a factor considered as a virulence factor for other intracellular bacteria. The antibiotic regimen used to treat rickettsioses also has an influence on prognosis. Usual concepts of severity and virulence in rickettsioses are challenging and are frequently paradoxical. In this mini-review, we will describe factors currently thought to influence the outcome of the main rickettsioses responsible for illness in humans.

E. Botelho-Nevers · D. Raoult (✉)
URMITE, UMR CNRS 6236-IRD 198,
Université de la Méditerranée, Faculté de Médecine,
27 Bd. Jean Moulin,
13385 Marseille cedex 05, France
e-mail: didier.raoult@gmail.com

Introduction

Rickettsioses are diseases due to small intracellular bacteria that belong to the genus *Rickettsia*, associated with arthropod vectors. The main bacteria belonging to this genus and responsible for diseases in humans are usually separated into two groups: the spotted fever group (SFG) and the typhus group (TG) rickettsioses. Increasing pathogenic species of rickettsioses have been reported [1], but, here, we will focus on the most usual human pathogens: *Rickettsia rickettsii*, *Rickettsia conorii*, *Rickettsia africae*, *Rickettsia felis*, *Rickettsia typhi* and *Rickettsia prowazekii*. Among tick-borne SFG, some species cause greater death-to-case ratios than others. Indeed, *R. rickettsii*, the agent of Rocky Mountain spotted fever (RMSF) usually found in America, remains a fatal disease in more than 2% of cases [2, 3] and have had, in the past, death-to-case ratios near to 20% [4]. Around the Mediterranean basin, *R. conorii*, responsible for Mediterranean spotted fever (MSF), was considered for a long time a benign disease. In the early 1980s, severe cases of the disease, RMSF-like, were reported [5]. The current report showed that the mortality rate is 2.5%, in contrast to the MSF case fatality rate of 32% reported in hospitalised patients in Portugal [6]. The role of the subspecies *R. conorii israelensis* in the increase of disease severity in Portugal had been questioned [7, 8]. Among SFG, *R. africae*, the agent of African tick-bite fever (ATBF), is causing an increasing number of cases reported in travellers; however, no fatalities have been reported [9]. In 2010, *R. felis* was emerging and several cases have been described worldwide, specifically in Africa [10, 11].

Published online: 26 April 2011

Among TG, there are *R. typhi*, the agent of murine typhus, a flea-transmitted disease that occurs in warm and humid climates and *R prowazekii*, causing epidemic typhus, a louse-borne disease more common during cold months when heavy clothing and poor sanitary conditions favour lice proliferation. The mortality of murine typhus is around 1% with the use of appropriate treatment [12]. In epidemic typhus, mortality is variable and was estimated to be up to 20% in the pre-antibiotic era, but is now low when appropriate antibiotics are given [13, 14].

In this review, we propose to study the prognosis factors that influence the severity of rickettsioses. If host factors play an important role in severity, they remain poorly studied with mainly retrospective series studying epidemiological factors. Moreover, interspecies differences in rickettsial virulence may have an impact on severity and may represent pathogen-related factors of virulence. Finally, the antibiotic regimen used in the treatment of rickettsioses also seems to play a role in disease severity and some regimens have been associated with enhanced severity in TG and SFG rickettsioses.

Host-related prognostic factors

Host-related risk factors associated with severity and poor outcome during rickettsioses have been described mainly in retrospective studies. The source of data should be considered before in order to associate host factors and severity. Indeed, if in large nationwide studies, as for RMSF, only some risk factors could be studied (e.g. age, race) [2, 4, 15, 16], in series from one centre [6, 17–20], more precise risk factors could be studied but few cases are described, leading to possible bias in the interpretation of the results. We summarise in Table 1 the host-related factors associated with severity during rickettsioses reported in the literature.

Demographical host factors

Age

Early epidemiologic studies about spotted fever group rickettsiae such as Rocky Mountain spotted fever (RMSF) or Mediterranean spotted fever (MSF) showed that increasing age is associated with increased risk of severe disease and a fatal outcome. Indeed, in RMSF, a clear age-dependent mortality was reported before 1980, with a death-to-case ratio of around 5% for patients aged less than 15 years to around 20% for those aged 60 years and older [4, 19]. This age-dependant mortality was noticed before the availability of effective antibiotics for rickettsial diseases [21]. In the years 1981–1998, the risk of death from RMSF remained significantly higher in patients older than 40 years of age [22], with a case–fatality rate of 5.8% compared to 2% in younger people. In people older than 70 years, the case–fatality rate peaked at 9% [16]. This is supported by an experimental animal model, where adult guinea pigs infected with *R. rickettsii* suffer more severe disease with increased mortality than juvenile animals [21]. Recent reports, however, underline a significant increase of the case–fatality rate in children under 5 years of age (5%), which is higher than that of patients older than 60 years of age during years 1997–2002 [15] and also in children aged 5–9 years compared to patients older than 70 years of age [2]. There is no explanation for this increase in mortality among children; however, hypotheses of misdiagnosis of disease and rarer use of doxycycline in children could be raised [2].

In MSF, death and severe forms of disease occurred more frequently in patients older than 60 years of age [6, 17, 18, 23, 24] (Table 1). In contrast, paediatrics series of MSF did not report either fatal nor severe forms of disease [25–27]. In benign African tick bite fever, older patients also seem to have a more severe course of disease [28]. A similar effect is also seen in epidemic typhus, in which a clear age-dependent mortality was reported in large historical series, with no deaths before 20 years of age and a mortality rate of more than 60% in those older than 60 years of age [21]. Finally, murine typhus, though usually mild, is also more severe in elderly patients [20, 29–31].

Studies about the precise mechanisms inducing increased severity of rickettsial diseases in older patients remain warranted. Infectious diseases, in general, are known to be more frequent and more severe in older patients [32]. Immunosenescence, malnutrition, as well as a large number of co-morbidities which are age-associated have been advanced reasons to explain the association between ageing and increased severity in infectious diseases [32, 33]. Changes in the innate and acquired immunity associated with ageing and the increase of co-morbidities such as diabetes among the elderly [32, 33] may explain the increased severity observed in older patients during rickettsioses.

Sex

The male gender has been described as a risk factor in several infectious diseases, such as Q fever [34, 35]. In rickettsioses, a higher death-to-case ratio for males than females was described in RMSF between 1970 and 1974 [4]. This had also been observed in experimental *R. rickettsii* infection of guinea pigs, with a higher mortality rate among males than females [21]. However, in most recent series, this difference was not observed in RMSF [2, 22], but this may be related to confusion with other diseases

Table 1 Host-related factors of severity in the main rickettsioses

Rickettsial disease	Reference, period	Age	Sex	Ethnicity	Comorbidities
Rocky Mountain spotted fever	[4], period 1970–1971	CFR: 12.7%>30 years vs. 5.2%<30 years	CFR: 8.2% in males vs. 4.5% in females	CFR: 13.9% in non-white vs. 5.8% in white	ND
	[4], period 1970–1974	CFR: 13.9%>30 years vs. 5.3%<30 years			ND
	[19], period 1977–1980	CFR: 5.5%>15 years vs. 3.4%<15 years (NS); 69% of deaths occurred in patients >20 years	No difference in fatality	No difference	ND
	[22], period 1981–1998	CFR: 5.8%>40 years vs. 2%<40 years	No difference in CFR: 3.5% in females vs. 3.3% in males	CFR: 5.1% in blacks vs. 3.3% in whites	ND
	[22], period 1981–1989	CFR: 7.5%>40 years vs. 2%<40 years	–	–	–
	[22], period 1990–1998	CFR: 3.9%>40 years vs. 2.1%<40 years	–	–	–
	[16], period 1993–1996	CFR: 9%>70 years vs. 0.6% for patients aged 40–49 years	Incidence in males 56%	CFR: 4.3% in blacks vs. 2.6% in whites (NS)	–
	[15], period 1997–2002	CRF: 5% <5 years vs. 2.5%>60 years	ND	ND	ND
	[2], period 2000–2007	CRF: 2.6% in patients aged 5–9 years, 1.3% >70 years	No difference in fatality	CFR: 2.2% in American Indians vs. 0.5% in whites vs. 0.2% in blacks	ND
Mediterranean spotted fever	[5], period 1974–1981	Malignant form of MSF: 4/4>60 years	Malignant form of MSF: 4/4 males	ND	Diabetes, cardiac insufficiency, chronic alcoholism
	[18], period 1982–1984	5 deaths: 4/5>60 years	ND	ND	Deficiency in G6PD
	[23], period 1983–1984	Malignant form of MSF: 6/7>55 years	Malignant form of MSF: 4/7 males	ND	Alcoholism, heavy smoking, respiratory insufficiency, deficiency in G6PD
	[6], period 1994–1998	Median age of fatal cases 70 years in 1997, 80 years in 1994–1996, 1998	No difference in fatality	ND	Diabetes
	[24], period 2004–2005	11 cases of MODS: mean age=56.7 years	MODS: 10/11 males	ND	Smoking, alcoholism, diabetes, high blood pressure
Epidemic typhus	[21], from Wolbach in 1922	CFR: 0% before 20 years of age vs.>60% in>60 years	ND	ND	ND
Murine typhus	[20], period 1980–1987	Increasing age associated with severity (p=0.002)	ND	ND	ND
	[29], period 1980–1984	Two fatal cases, aged 41 and 70 years	ND	ND	ND
	[43]	ND		ND	Deficiency in G6PD

CFR=case–fatality rate; ND=not determined

[3]. MSF affected men significantly more frequently in France [18] and the first report of six malignant MSF cases [5] involved males. However, later, no difference in regard to gender was found by the same team [23]. Nowadays, the incidence of MSF in Portuguese cases seems to be the same between males and females, with no difference in the risk of dying according to gender [6, 7]. In Algeria, disease occurs more frequently in males and 10 of the 11 severe cases with multiorgan involvement described were males [24]. MSF is also more common among males in Spain [36] and Croatia [37]; however, association with severity could not be studied as no severe cases have been reported in these series. The only two severe cases reported of infection due to *R. felis*, usually considered benign, occurred in two young males presenting hepatitis and lung involvement [38]. In murine typhus, no association between severity and gender has been reported, and the sex ratio varies widely between series [20, 29, 39]. By contrast, in TIBOLA/SENLAT, a clinical entity characterised by scalp eschar and neck lymphadenopathy following tick bites and due to several bacteria such as *R. slovaca* or *R. raoultii*, the great majority of cases occur in females and in children [40, 41]. Therefore, an association of severity with gender may be tied to occupation and/or tick and vectors contact more than specific susceptibility, as it has been described in *R. conorii*, *R. felis* or *R. typhi* in Spain [42].

Comorbidities

Among comorbidities of the host affecting severity in rickettsioses, chronic alcoholism had been reported earlier to be associated with severe MSF [5, 23] and this association had been recently confirmed in fatal forms of MSF [7]. Based on the report of a fatal case of RMSF in an alcoholic man without any other predisposing conditions and on expert opinion [21], alcoholism was considered to also enhance severity in RMSF. This underlying condition was also reported to be associated with liver involvement in murine typhus [43]. Although experimental models of chronic alcoholism exist [44], until now, the impact of this condition on experimental rickettsioses has not been studied. Other comorbidities, such as diabetes, had also been reported to be associated with severe MSF. In their series of malignant MSF, Raoult et al. observed diabetes in 3 of 6 severe cases of MSF, all of which died. Recently, in a series of 105 hospitalised patients with MSF [6], diabetes was found to be associated with fatality, and this factor being adjusted by age. In a series of 525 consecutive cases of MSF, Bellissima et al. seemed to also find an association between diabetes and severe forms of MSF [25]. In other rickettsioses, the role of diabetes as a risk factor has not been studied. Heavy smoking had also been considered as a predisposing factor for severity in MSF in some series, and notably among French people [23], as in the series of Mouffok et al., where 6 of 11 severe MSF were chronic tobacco smokers [24]. To our knowledge, no experimental models support these findings and the explanation for these findings remains lacking.

Ethnical factors

Ethnical factors also seem to have an impact on severity in rickettsioses. Black people had been significantly associated with a higher death rate than white people in RMSF (13.9% vs. 5.8%) [4, 19]. In the series of Helmick et al. [19], non-white cases had a risk of dying that was four times higher than white cases. In recent studies, the difference in mortality between blacks and whites remain observed [16, 22]; however, the difference was not significant and was no longer seen when the analysis was controlled with confounding factors (less reported tick attachment and atypical presentation among black patients) [22]. It is conceivable that delays in seeking medical care or difficulties in diagnostics (e.g. rash on black skin) have played a role in finding this association in older studies. The fact that black females had a similar death ratio than white females with RMSF [4] could have suggested that a sex-linked condition might be the explanation for this difference. Moreover, black patients are underrepresented in series of RMSF [4, 19] and murine typhus [39] in the USA compared to the general population. Recently, an increasing incidence of RMSF has been reported among the American-Indian population in the United States from 2001, with a significantly higher case–fatality rate (1.8%) compared to white persons (0.4%, $p<0.02$) [45]. In this study, American-Indians also have a higher but non-significant case–fatality rate compared to black persons (0.6%, $p=0.2$) [45]. Three of the four fatal cases occurred in American-Indian children aged 5–9 years, a fatality rate (11.5%) that is significantly higher than the same age group among white children (1.7%, $p<0.03$) [45]. Openshaw et al. also reported an increased fatality rate in this population compared to other ethnical groups [2]. This increase in mortality among American-Indians has been suspected to be related to globally higher risk for infectious diseases and differences in access to healthcare facilities [45]. The role of ethnical factors on severity during the course of rickettsioses may result from genetic or environmental factors.

Genetic background

A possible explanation for the higher proportion of severe cases of rickettsial disease in black men was provided by the report of an unusual severe murine typhus in a 21-year-old black soldier [21]. This young

patient had G6PD deficiency, a genetic condition affecting 12% of American black men [21]. The report of haemolysis in murine typhus had further been described in patients with this deficiency [43] or other haemoglobinopathies. This condition is also associated with severe RMSF and, notably, with the fulminant form of the disease [21, 46]. In MSF, the G6PD deficiency had also been associated with severity. Two severe or fatal cases of MSF were described in previously healthy young men with this deficiency [23, 47], these cases being unusual in having a severe form of MSF despite their young age. In patients with G6PD deficiency, haemolysis triggered by rickettsial infection seems to be critical to explaining the enhanced severity of rickettsiosis and stimulating rickettsial virulence. In vitro data also support this finding as it has been shown that haemoglobin stimulates *R. conorii* invasiveness [21].

Genetic factors in patients also may influence the severity of rickettsial disease. In fact, the degree of cytokine expression depends not only on the intensity of stimulation but also on host genetic factors, such as polymorphisms in cytokine genes and, particularly, cytokine single nucleotide polymorphisms (SNP) are tools for the prediction of disease susceptibility [48]. Mansueto et al. showed that patients with MSF presented significantly less frequently a particular genotype of IFN-γ (+874TT) compared to patients without MSF. Moreover, interaction between particularly IFN-γ and IL-10 genotypes was also observed in MSF patients, suggesting that genetically tuned cytokine production may play a crucial role in the regulation of immune response against *R. conorii*, influencing, therefore, the susceptibility to this infection and their outcome [48]. In MSF patients, a particular polymorphism of toll-like receptor-4 (TLR4; G allele at +896 SNP) has been found to be overrepresented [49]. TLR-4 has a crucial role in limiting rickettsial growth [50]. This SNP of TLR-4 is known to attenuate receptor signalling and is associated with increased risk of severe bacterial infections [49].

In experimental models, the genetic background of animals seems also to have an impact on the severity of rickettsioses, as it had been demonstrated in a murine model of epidemic typhus: C57BL/6 mice infected with the same inoculum of *R. prowazekii* to BALB/c mice all succumbed, whereas the latter became sick but recovered after 6 days [51]. The death of C57BL/6 mice may be due to an exaggerated, cytokine-mediated, host acute phase reaction against these vasculopathic rickettsioses, which is more controlled in BALB/c mice [51].

Inter-individual differences in the endothelial inflammatory response to TNF have been proposed to explain the difference observed between patients presenting cerebral or uncomplicated malaria [52] and TNF-238 polymorphism has been found to be associated with severe malaria in the Gambian population [53].

Host response

Host-mediated events of inflammation and immunity probably also play a role in the severity of rickettsioses [21]. In severe cases of MSF, marked endothelial injury induced by *R. conorii* have been demonstrated, with higher levels of circulating endothelial cells and cell fragments found in severe cases compared to mild cases [54]. This higher endothelial injury suggests more severe systemic vasculitis and inflammation in severe cases [54], as it had also been reported in MSF compared to ATBF [55]. We summarise in Table 2 the host response found in severe cases described in the literature. Enhanced severity might be explained not only by defective host responses, but also

Table 2 Host response observed during severe MSF and during infection by *R. conorii*: the differences between severe and non-severe infections are summarised

Disease/reference	Host response in severe cases
Mediterranean spotted fever	
[56]	Significantly higher plasma TNF-α levels in severe cases compared to mild MSF
[58]	High levels of expression of mRNA of TNF-α within eschars in 100% of severe cases (not significant)
	RANTES mRNA expression significantly higher in severe cases
[55]	Marked increase in MCP-1, IL-8, adhesion molecules in response to *R. conorii* compared to *R. africae*
[90]	Higher values of two soluble TNF receptors in severe patients
Experimental models	
[91] mice, *R. conorii*	Lower levels of IL-2 and IFN-γ and higher levels of IL-10 in lethally infected compared to sublethally infected mice in splenic T-cells
[92] mice, *R. conorii*	Defective mice in TLR-4 died compared to mice with intact TLR-4
	Higher levels of IL-6, TNF-α, IL-12p40, IL-12p70 and IL-17 in the sera of intact mice (no fatalities)

by the hyperactive responses of some systems. Host responses had been particularly studied in MSF. Oristrell et al. showed that patients with severe MSF (significant dysfunction of a major organ) had significantly higher plasma TNF-α levels than those with mild MSF [56]. Besides its anti-rickettsial activity [57], TNF may also exert cytotoxic effects on host cells and have a role in rickettsial vasculitis, as it had been described recently in malaria [52]. Alternatively, these high levels of TNF could be an epiphenomenon in the context of inflammatory response in severe MSF cases [56]. This is related with the murine model of infection with *R. prowazekii*, in which BALB/c mice reproduce human epidemic typhus and exhibited high levels of IFN-γ and TNF gene transcripts and survived the infection, leading to the hypothesis that both cytokines are involved in the protection of BALB/c mice against *R. prowazekii* infection [51].

Within eschars of inoculation in MSF, high levels of expression of mRNA of TNF-α have also been found in 100% of severe cases studied; differences with the mild or moderate forms of disease were, however, not significant [58]. Thus, some authors considered that severe MSF could be due to an immuno-pathological condition rather than to an overwhelming condition [58]. Other actors of immune response also seem to play a dual role in MSF. Indeed, in the same study, RANTES mRNA expression was found to be significantly higher in severe MSF cases than in mild cases. This chemokine has a protective role in MSF; however, in excess, it could increase T-cell infiltration at the site of infection and lead to excessive amounts of proinflammatory cytokines, with indirect damage to endothelial cells [58]. In a study comparing the expression of chemokines in MSF and ATBF, Damås et al. showed that MSF induced a higher inflammatory response than ATBF [55]. This also contributes to consider that, while cytokine release is beneficial for the clearance of bacteria, in excess, it also contributes to an inappropriate local and systemic inflammation, potentially leading to tissue damage and harmful effects on the host.

Pathogen-related prognostic factors in rickettsioses

As described in the first part of this review, the severity and fatality rates of the various rickettsial diseases vary considerably from relatively mild diseases (ATBF, infection due to *R. felis*, murine typhus) to life-threatening illnesses (epidemic typhus, RMSF and, more recently, MSF). The virulence of the causative agent has a major influence on the outcome of the diseases [59, 60]. Many potential virulence factors have been described in the bacteria that belong to the genus of *Rickettsia* [61]; however, a lot of them are common between the different species of rickettsiae and do not explain the difference in severity observed between the species [62]. Factors thought to participate in the virulence of rickettsioses and common to SFG and TG rickettsioses are summarised in Table 3.

The underlying mechanisms explaining the differences of virulence observed between different rickettsioses remained poorly understood for a long time [61].

Differences in virulence among species

Comparative analysis of genomes between species revealed, surprisingly, a reductive evolution of rickettsiae from less virulent species (i.e. 1.5 Mb for *R. belli*) to most virulent species (i.e. 1.1 Mb for *R. prowazekii*) [1, 62, 63] (see Table 4). Rickettsial evolution challenges traditional concepts of pathogenesis usually associated with the idea of the acquisition of virulence factor. Indeed, paradoxically, comparison of the genomes of *R. conorii* and *R. prowazekii* showed that the genome of the latter is a quasi subset of *R. conorii* [1, 59, 61, 63, 64]. *R. prowazekii* did not exhibit genes directly identifiable as specific virulence determinants [1, 63]. Only four genes of *R. prowazekii*, also found in *R. typhi*, are not present in *R. conorii*. The role of these genes in the pathogenicity of typhus remains unknown [1]. The comparative analysis of the genomes of these two species also provided an explanation for the lack of motility of *R. prowazekii*. Indeed, it is a second paradox in

Table 3 Pathogen-related virulence factors found in both typhus group and spotted fever group rickettsioses. These factors are thought to participate in the pathogenesis of rickettsioses

Reference	Thought to be a 'virulence' factor	Mechanism of action
[93]	Phospholipase A2 (PLA2)	Mediate the escape from phagosomes. Role in haemolytic activity
[94]	Phospholipase D superfamily	Found in virulent strains of rickettsiae but also in strains with unknown pathogenicity, might account for the activity previously attributed to PLA2
[95]	Haemolysin	Haemolysis, involved in host-cell entry, exit from phagosome and host-cell lysis
[96, 97]	Type IV secretion system	Transport of protein and toxins from bacterial cytoplasm into the host or extracellular matrix
[98]	InvA	Invasins, facilitate bacterial survival during internalisation

Table 4 Size of genomes of rickettsioses. Reductive evolution in genomes is observed and correlate with the relative virulence of species. Relative virulence was compared to the more virulent species of each group (e.g. *R. rickettsii* Sheila Smith for spotted fever group rickettsioses and *R. prowazekii* for typhus group rickettsioses)

Species (strains)	Genome size of chromosome (bp)	Relative virulence	Reference
Spotted fever group			
R. rickettsii (Sheila Smith)	1,257,710	Virulent ++	[68]
R. rickettsii (Iowa)	1,268,175	Avirulent	[68]
R. conorii (Malish)	1,268,755	Virulent +	[63]
R. africae (ESF-5)	1,278,540	Virulent −	[62]
R. felis (URRWXCal2)	1,485,148	Virulent −	[99]
Typhus group			
R. prowazekii (Rp22)	1,111,612	Virulent ++	
R. prowazekii (Madrid E)	1,111,523	Avirulent	[74]
R. typhi (Wilmington)	1,111,496	Virulent -	[66]
Others			
R. belli (RML369-C)	1,522,076	Unknown	[100]

rickettsial virulence, motility that has been considered as an important virulence determinant in *Listeria* spp. and *Shigella* spp. [1], is present in *R. conorii* and lacking in *R. prowazekii* that is more virulent. Actin-based mobility present in *R. conorii* involves the protein RickA, which is encoded by a gene without an orthologue in *R. prowazekii* [61]. The gene encoding RickA is commonly found in other *Rickettsia* spp. without identified pathogenicity. Recently, Sca2 has been associated with actin mobility in SFG rickettsiae [65] and is truncated in *R. prowazekii* [1] but present in less virulent and slowly motile *R. typhi*. In an experimental model, guinea pigs infected with the Sca2 mutant of *R. rickettsii* did not present with fever, suggesting that Sca2 may be a virulence factor in *R. rickettsii* [65]; in contrast, it is obviously not the case in *R. prowazekii*. This suggests that motility is not a virulence factor *per se*, but can be found in pathogens as part of a virulence repertoire [59].

A comparative analysis of genomes of tick-borne SFG rickettsiae, *R. africae* causing a mild disease, *R. conorii* and *R. rickettsii* also showed a genomic reductive evolution of the more pathogenic rickettsiae (Table 4). Based on this observation, it had been speculated that the difference in virulence among *Rickettsia* species could be due to a loss of regulatory genes [62]. Some genes unique to *R. africae* have a regulatory role, notably during stress conditions. Therefore, higher regulatory ability in *R. africae* had been speculated to be linked to greater adaptive capabilities to its host and lower pathogenicity [62]. A comparative analysis of genomes of the two species belonging to TG, *R. typhi* and *R. prowazekii*, revealed a high degree of similarity, illustrating the small differences that can affect virulence [66]. One of these being Sca-2, a protein associated with actin-based motility that appears to be not functionally expressed in *R. prowazekii* [1]. These differences that are now better understood, were observed earlier in experimental models that revealed inherent differences in the virulence of various species of *Rickettsia* [60, 67]. However, the putative role of toxins in rickettsial diseases has not been elucidated. As they are considered to be central in bacterial pathogens [59], they should be actively searched for in rickettsioses.

Differences in virulence among strains of the same species of *Rickettsia*

Genomic comparison of virulent *R. rickettsii* Sheila Smith and avirulent *R. rickettsii* Iowa showed evidence for genomic reduction in the virulent strain compared to the latter [68] (see Table 4). In contrast, *R. rickettsii* Iowa has a truncate *rompA* gene leading to the absence of rOmpA, compared to the virulent strain [68]. The avirulent strain also produces a defective rOmpB, which is still present, however, in the outer membrane of this strain. The surface protein rOmpA, implicated in the attachment of *R. rickettsii* to host cells, does not seem to be absolutely required, as the avirulent strain also adheres to cells. The interaction between rOmpB and the host cell outer membrane Ku70 [69], in the absence of rOmpA, may be sufficient for the adherence and uptake of *R. rickettsii* Iowa in vitro [68]. Compared to virulent strains of *R. rickettsii* that produced clear plaques, the avirulent strain produces opaque plaques related to a deficiency in host cell lysis [68]. This indicates a correlation between the lyse of host cells and the virulence of organisms [68]. Early animal experiments by Price reported a variation in virulence among different strains of *R. rickettsii*, with a fatality rate in guinea pigs varying from 0 to 33% [70]. Eremeeva et al. later showed that injury induced in endothelial cells differs markedly among different *R. rickettsii* isolates [71], corroborating the correlation between the ability to lyse host cells and the virulence of the organism [71]. Variations in pathogenic

biotypes of *R. rickettsii* isolates have been described and potential correlations of these differences to various clinical manifestations of RMSF have been suggested [72]. The genotyping of different isolates of *R. rickettsii* leads to distinguishing four primary groups: isolates from Montana, where RMSF had the higher case fatality rate, isolates from *Rhipicephalus sanguineus* ticks and human infections in Arizona, and two other groups associated with tick isolates [72, 73]. Comparative genomic, proteomic and transcriptomic analysis of virulent and avirulent *R. prowazekii* also revealed several differences [74]. Multiple non-synonymous mutations in the *sca* family (cell surface antigen) genes have been found when avirulent and virulent strains of *R. prowazekii* have been compared, leading to a possible variation in virulence [74]. The gene *adr1*, encoding for adhesin Adr1, is notably conserved in virulent strains but altered among the avirulent Madrid E strain of *R. prowazekii*. Recently, a new adhesin, Adr2, has been found to be sufficient to mediate *R. prowazekii* (virulent strain Rp22) entry into cells at the early stage of mammalian cell infection [75]. Post-translational modifications, especially methylation, also play an important role in the virulence of *R. prowazekii*. Indeed, two methyltransferases have been shown to be overproduced in virulent strain Rp22 compared to avirulent Erus strain, whereas the avirulent Madrid E strain is known to have an inactivated gene for a methyltransferase [74]. These findings reveal the genomic plasticity of *R. prowazekii* and its adaptive mutation capabilities.

Differences in virulence among subspecies of the same species of *Rickettsia*

Among human diseases, patients infected by *R. conorii* subspecies *israelensis* presented more severe disease compared to those infected with *R. conorii conorii* [7, 8, 74, 76]. Indeed, patients infected by *R. conorii* subspecies *israelensis* had a risk of dying 2.81 times higher (95% confidence interval, 1.18–6.72, $p=0.020$) than patients infected by *R. conorii conorii* (respectively 29% vs. 13%) [7].

Differences in clinical features have also been described among subspecies of *R. sibirica* complex. *R. sibirica sibirica*, the agent of Siberian tick typhus, seems to be more pathogenic, with several severe and fatal cases reported compared with *R. sibirica* subspecies *mongolitimonae*, causing lymphangitis-associated rickettsiosis more recently described [77], and with no severe cases described [77–80]. These differences in virulence between subspecies of the same species are, however, not yet understood and comparative analysis of genomes of the subspecies will probably lead to a better comprehension, notably for *R. conorii*.

Treatment-related prognostic factors

The impact of treatment on the prognosis of rickettsial diseases has been studied episodically. Regimens used in the treatment of rickettsioses have usually been tested in vitro [81]. The delay in the instigation of treatment in rickettsial diseases has been reported, in some studies, to be associated with poor outcome. In RMSF, delayed diagnosis and delay in treatment have been associated with a case–fatality rate three times higher [22].

Doxycycline, chloramphenicol, fluoroquinolones and some macrolides are efficient in vitro [81], but cotrimoxazole should be avoided because of the absence of enzymes of the folic acid pathways [61, 66]. In MSF, a comparative study of cotrimoxazole versus doxycycline had to be suspended prematurely because of a deleterious effect of this regimen [82]. Later, cotrimoxazole has been reported to be associated with fatality in MSF [6]. In epidemic typhus, cotrimoxazole was also significantly less efficient than doxycycline at improving the symptoms of patients [83].

Chloramphenicol, which has been used for a number of years, is now considered to be less effective than doxycycline and has been associated with poor outcome in SFG and TG rickettsioses. Shaked et al. reported relapse in 10 of 24 patients with MSF or murine typhus treated by chloramphenicol compared to no relapse in 108 patients treated with doxycycline [84]. In epidemic typhus, chloramphenicol was also found to be less effective for the improvement of symptoms and was associated with a delay in apyrexia compared to doxycycline; moreover, the unique death in the series occurred in a patient receiving chloramphenicol [83]. More recently, in a nationwide study on RMSF, the use of chloramphenicol alone has been associated with a case–fatality rate three times higher than patients not treated with this regimen [22].

Fluoroquinolones have also been associated with poor outcome in the treatment of typhus, as a fatal case of imported epidemic typhus occurred in a nurse treated with ciprofloxacin [85]. This regimen had also been associated with poor response in two cases of murine typhus [86]. In our experience, fluoroquinolones are associated with higher severity in MSF (unpublished data).

Doxycycline remains the gold standard treatment in SFG and TG rickettsioses, and is associated with better outcome and earlier defervescence [22, 83], even in children [87]. The reluctance among healthcare providers to prescribe doxycycline to children may contribute to the recent increase in the fatality rate of young children with RMSF [2, 15]. In fact, in the USA, 50–75% of physicians would prescribe an antibiotic other than doxycycline for children suspected of RMSF [2]. Finally, doxycycline is the reference treatment for everyone except for pregnant women.

Conclusions and perspectives

As reviewed here, several host factors, such as age, comorbidities and deficiency in G6PD, have been found to influence the outcome of rickettsial diseases and are associated with severity. While observed in retrospective studies, some of these factors have been supported by experimental models. However, few factors have been independently associated with severity. Clinical prospective studies including severe and non-severe forms of the same disease are then warranted in order to better understand the role of each factor in severity. Moreover, recent experimental models have been developed and could be used to test the effect of some underlying conditions on the outcome of rickettsial diseases in order to explain the findings of retrospective studies. Host response has been only partially studied and only some effectors the of immune pathway have been studied. A microarray approach will allow obtaining a neutral and more extensive understanding of mechanisms associated with host response to rickettsial infection within eschars of inoculation. This transcriptomic analysis may also show differences in response leading to severity.

Pathogen-related prognosis factors seem, in fact, due mainly to a reductive evolution of genomes, to challenge then the usual concepts of pathogenesis that focussed primarily on the acquisition of virulence factors [59, 64]. The association between increased virulence and reductive evolution has been observed between species but also between subspecies [62]. The reductive evolution leads to a loss of regulatory genes, which has been speculated to explain virulence in rickettsiae. Comparative analysis of more genomes will confirm this hypothesis.

Finally, the role of antibiotic regimen used during the course of rickettsiosis have been poorly studied; nevertheless, some antibiotics considered, however efficient in vitro, have been reported to be associated with severity in rickettsioses. In our experience, chloramphenicol and fluoroquinolones in vitro seem to increase firstly the lytic activity of *R. conorii* in host cells before to undergoing their antibiotic activity (unpublished data). The role of stress caused by some antibiotics on the liberation of toxins of rickettsiae could then be questioned. More studies in vivo and in vitro are warranted in order to understand the underlying mechanisms. Adjunctive non-antibiotic treatment may probably play a role in the treatment of rickettsioses in the future. Statins have been suggested to have a beneficial effect on the outcome of different infections [88]. In vitro, lovastatin and pravastatin have been shown to limit infection in cells infected with *R. conorii* [89]. Clinical studies on the potential protective role of statins in patients with rickettsioses are then warranted.

Acknowledgements We are grateful to Dr. Silpak Biswas for editing the manuscript.

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ARTICLE 1

Analysis of risk factors for malignant Mediterranean spotted fever indicates that fluoroquinolone treatment has a deleterious effect

Elisabeth Botelho-Nevers[1,2], Clarisse Rovery[1], Hervé Richet[1] and Didier Raoult[1]

Journal of Antimicrobial Chemotherapy

2011 Aug;66(8):1821-30.

[1]URMITE UMR CNRS 6236, IRD 198, Université de la Méditerranée, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille cedex 05, France

[2] Pôle de Maladies Infectieuses et Tropicales. Assistance Publique des Hopitaux de Marseille, Marseille, France

Contribution de EBN: récupération des données cliniques, constitution de la base de données, analyses des données, analyses statistiques, écriture du manuscrit sous la direction de DR.

Commentaire

L'objectif de cet article était d'identifier les facteurs de risque de sévérité au cours de la fièvre boutonneuse Méditerranéenne (FBM) causée par *Rickettsia conorii* subsp. *conorii* et ainsi de faire abstraction des facteurs de risques liés au pathogène (autres sous espèces impliquées).

Nous avons étudié rétrospectivement les caractéristiques épidémiologiques, cliniques et biologiques et les traitements reçus par des patients présentant une FBM. Un patient souffrant de ≥ 2 dysfonctions d'organes ou le décès du patient a été défini comme un cas sévère. Nous avons procédé à une comparaison entre les caractéristiques des patients présentant une forme sévère et ceux présentant une forme non sévère de FBM.

Ainsi, pendant la période d'étude (Janvier 1999 à Décembre 2009), 161 cas de FBM ont été diagnostiqués au Centre National de Référence des rickettsioses. Dans 115 cas, la FBM avait été acquise en France et autour du bassin méditerranéen dans les autres cas. Vingt-six cas (16,1%) ont été considérés comme sévères, ce qui est 3 fois plus élevé que le taux de sévérité retrouvé dans les études précédentes réalisées dans ce même laboratoire. Les signes cliniques et biologiques associés aux formes sévères sont comparables à ceux rapportés dans la littérature. Nous avons cependant mis en évidence pour la première fois que le type de traitement antibiotique était associé à la sévérité de la maladie. L'administration de doxycycline préalable à la détérioration au cours de la FBM (31 patients) protège les patients du développement de formes sévères (risque relatif (RR) 0,248, intervalle de confiance 95% = 0,08 à 0,76). La doxycycline induit une défervescence plus précoce par rapport à d'autres traitements antibiotiques (3,02 jours +/ -2,2 vs 7,1 jours + / - 6,57, p = 0,021). En revanche, le traitement par fluoroquinolones (chez 21 patients) était significativement et indépendamment associé à la sévérité au cours de la FBM (RR = 2,53, IC 95% = 1,40 à 4,55) et était associé à

un séjour hospitalier prolongé, comparativement aux patients traités par d'autres antibiotiques.

En conclusion, dans cette étude rétrospective le traitement par fluoroquinolones a été associé à une augmentation de la sévérité au cours de la FBM. Les fluoroquinolones ont déjà été associées à des évolutions défavorables au cours du typhus endémique et du typhus épidémique. Nous ne recommandons donc pas l'utilisation des fluoroquinolones pour traiter les rickettsioses.

Journal of Antimicrobial Chemotherapy Advance Access published June 4, 2011

J Antimicrob Chemother
doi:10.1093/jac/dkr218

Journal of Antimicrobial Chemotherapy

Analysis of risk factors for malignant Mediterranean spotted fever indicates that fluoroquinolone treatment has a deleterious effect

Elisabeth Botelho-Nevers[1,2], Clarisse Rovery[1], Hervé Richet[1] and Didier Raoult[1]*

[1]*URMITE UMR CNRS 6236, IRD 198, Université de la Méditerranée, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille cedex 05, France;* [2]*Pôle de Maladies Infectieuses et Tropicales, Assistance Publique des Hopitaux de Marseille, Marseille, France*

*Corresponding author. Tel: +33-491-38-55-17; Fax: +33-491-83-03-90; E-mail: didier.raoult@gmail.com

Received 26 January 2011; returned 8 February 2011; revised 1 May 2011; accepted 5 May 2011

Objectives: To identify risk factors for malignant Mediterranean spotted fever (MSF) caused by *Rickettsia conorii conorii*.

Patients and methods: Epidemiological, clinical and biological characteristics as well as risk factors (including treatment regimens) for severe MSF cases were analysed retrospectively. A patient with two or more organ dysfunctions or patient death was defined as a severe case.

Results: During the study period (January 1999 to December 2009), 161 MSF cases were referred to our centre for rickettsioses. Twenty-six cases (16.1%) were considered severe, which is 3-fold higher than in our previous studies. The clinical and laboratory findings were comparable to those reported elsewhere except that the type of antibiotic treatment was associated with disease severity. Doxycycline administration prior to deterioration of disease (in 31 patients) protected patients from development of severe MSF [relative risk (RR) 0.248, 95% confidence interval (CI) 0.08–0.76] and induced earlier defervescence compared with the other treatment regimens (3.02 ± 2.2 days versus 7.1 ± 6.57 days, $P = 0.021$). In contrast, fluoroquinolone treatment (in 21 patients) was significantly and independently associated with MSF severity (RR 2.53, 95% CI 1.40–4.55) and was associated with a significantly longer hospital stay.

Conclusions: In this retrospective study fluoroquinolone treatment was associated with increased MSF disease severity. Fluoroquinolones have been previously associated with treatment failure in typhus and scrub typhus cases. Thus, we do not recommend the use of fluoroquinolones to treat rickettsial diseases.

Keywords: *Rickettsia conorii*, treatment-related, severity

Introduction

Mediterranean spotted fever (MSF), which was first described in 1910, is a tick-borne disease that is caused by the obligate intracellular bacterium *Rickettsia conorii*. Specifically, MSF is transmitted by the brown dog tick, *Rhipicephalus sanguineus*.[1] The disease is typically transmitted in the spring and summer months in geographical regions surrounding the Mediterranean basin and can be easily recognized clinically by the presence of a 'tache noire', which corresponds to the eschar of tick inoculation, and a febrile rash involving the palms and soles.[2] For more than 70 years, MSF was considered benign compared with Rocky Mountain spotted fever (RMSF), which is a similar disease prevalent in the USA that is caused by *Rickettsia rickettsii*. The first series of severe MSF cases that resulted in death were reported in France in 1983.[3] Since that report, severe forms and fatalities have been regularly described.[1,4–9] Current estimates suggest that in approximately 5%–10% of MSF cases the disease is malignant and can cause death in 50% of these cases.[2] Recently, MSF cases were reported to be more severe than RMSF,[10] with a case fatality rate of 32.3% in hospitalized patients in Portugal in 1997.[11] Since 2005, multilocus sequence typing and analysis has created a four-subspecies classification for *Rickettsia conorii*: the subspecies *R. conorii conorii* is now considered to be the aetiological agent of MSF.[12] However, infection with this subspecies has been reported to be less severe than with *R. conorii israelensis*, which causes Israeli spotted fever.[9,13] In the present study, we analysed MSF cases due to *R. conorii conorii*.

In previous studies, several severe forms of MSF have been reported;[3,4,7,8,11,14] however, there is no standardized definition of MSF severity. Aside from fatality, qualification as severe MSF is based upon: (i) clinical signs such as a purpuric rash and neurological involvement; or (ii) laboratory findings such as thrombocytopenia, hyponatraemia, hypocalcaemia, renal dysfunction and increased levels of transaminases.[2] Recently, de Sousa *et al.*[15] defined severity on the basis of admission to an intensive care unit or evidence of the involvement of three or more

organ systems (hepatic injury and/or respiratory and/or renal insufficiency, haematological and/or neurological involvement). They also defined moderately severe and mild cases as the involvement of two and one organ systems, respectively. To simplify and standardize the severe/malignant MSF cases in this study we used the definition of multiple organ dysfunction syndrome (MODS) that is commonly used clinically.[16]

Currently, few studies have assessed the association between the treatment regimen for MSF and the disease outcome. Doxycycline is considered to be the antibiotic of choice for MSF[2,17,18] and other rickettsioses,[17,19–22] although other antibiotics are also considered to be reliable alternatives. Chloramphenicol has been used for several years, although its efficacy has been questioned because it seems to be less effective than doxycycline in treating MSF[23] and other rickettsioses,[17,19,23–25] despite *in vitro* susceptibility.[17,26] Among the macrolides, clarithromycin, josamycin and azithromycin seem to be reliable alternatives for MSF treatment in children and pregnant women, as has been shown in randomized trials,[27,28] and to be effective in *in vitro* susceptibility tests,[26] unlike erythromycin.[18,26] Ciprofloxacin is considered to be an efficient alternative for MSF treatment,[17,29–31] although clinical data are not widely available.

In the present study, we evaluated the epidemiological and clinical characteristics and treatment of MSF cases to identify risk factors associated with severe MSF in a retrospective cohort.

Patients and methods

Cases of MSF were identified from the French National Reference Centre for Rickettsial Diseases in Marseilles, where samples from patients that were thought to have MSF were received. The epidemiological and clinical data were collected retrospectively using a standardized form. For confirmed and probable cases of MSF that were diagnosed between January 1999 and December 2009, the medical charts were reviewed retrospectively to obtain additional data about each case (specifically the epidemiological and demographic characteristics, clinical presentation, time between appearance of signs and treatment, laboratory findings, antibiotic regimen used and outcome data).

Inclusion criteria

The patients that were included in the study were classified as 'confirmed' or 'probable' MSF cases. A 'confirmed' MSF case was defined as the presence of signs and symptoms compatible with the disease (e.g. fever, tache noire and rash) and at least one confirmatory laboratory finding, such as a positive PCR assay with specific primers for *R. conorii conorii* from the blood or a skin biopsy of the eschar,[32] or isolation of *R. conorii conorii* from the clinical specimens.[33] The identification of rickettsial antigens by immunochemistry from the biopsy was also considered confirmatory, as described elsewhere.[34]

A 'probable' MSF case was defined when a patient had a score of >25 according to the MSF diagnostic score chart.[35] For the majority of the patients, a serum sample was obtained within 2 weeks following the onset of symptoms, and, when available, a late-phase sample (>2 weeks after the onset of symptoms) was obtained. Immunoglobulin (Ig) and IgM antibody estimation was performed with a microimmunofluorescence assay, which is a reference method for rickettsial diseases, as reported elsewhere.[36] *Rickettsia conorii* strain Malish was used as the antigen. Titres of 1:64 for IgG and 1:32 for IgM were defined as the cut-off values.

Western blotting was performed as described elsewhere,[36] when adequate blood samples were available. Cross-adsorption followed by a microimmunofluorescence assay and western blotting was performed as described elsewhere,[37] when the patients exhibited an antibody titre of >1:64 and when adequate serum volumes were available, in order to help discriminate cross-reactions. Samples were considered to be positive for *R. conorii* when the western blot profile showed only *R. conorii*-specific antibodies or when cross-adsorption demonstrated that the homologous antibodies were directed against *R. conorii*.

Definition of disease severity

Malignant/severe cases of MSF were defined as death during the course of MSF, or dysfunction of two or more organ systems,[16] including dysfunction of the respiratory, renal, cardiovascular, neurological, hepatic or haematological systems. As a secondary outcome we included change of the drug regimen caused by the appearance of organ dysfunction, non-response or worsening of signs and symptoms (i.e. persistent fever, appearance of new clinical signs).

Definition of regimens and outcome evaluation

We compared different therapeutic regimens that are commonly used to treat MSF. The various antibiotics were chosen based upon their effectiveness *in vitro*.[26] The antibiotics that were studied included doxycycline, fluoroquinolones, new macrolides and chloramphenicol. Ineffective regimens included the β-lactams and co-trimoxazole.

We compared outcomes in patients who were given different treatments in the first 5 days of the disease, and counted only outcomes that appeared after commencement of treatment (see Results).

Statistical analysis

Data were first analysed with EpiInfo software (version 3.5.1, Centers for Disease Control and Prevention, Atlanta, GA, USA) and with PASW statistics software version 17 (SPSS, Chicago, IL, USA). Proportions were compared by using the Yates χ^2 corrected test or the Fisher exact test. Continuous variables were compared by using analysis of variance or the Mann–Whitney two-sample test when data were not normally distributed. Significance was defined as $P<0.05$. Data for small groups ($n<16$, e.g. 10% of the population studied) were not analysed.

A binary logistic regression analysis was performed with the PASW software. The severe form of the disease was used as the dependent variable and tobacco use, dehydration, hyponatraemia at the start of the hospital stay, use of corticosteroids before or during the disease and treatment with doxycycline, fluoroquinolone or β-lactam regimens as independent variables. The Hosmer and Lemeshow test was used to assess whether or not the observed event rates match expected event rates in subgroups of the population.

Ethics

The study was approved by the ethics committee of our University under the reference 11-003. The study was included in a regional Programme Hospitalier de Recherche Clinique (UF 8046).

Results

During the study period (1 January 1999 to 31 December 2009), 161 confirmed or probable MSF cases were diagnosed at our centre.

Characteristics of MSF patients

There were 93 men included in this study and the mean age of the study population was 50.2 years (range 1–85 years). MSF was

Table 1. Comparison of patients from the present series with those of >3000 patients in the literature (references 5, 6, 8, 9, 11, 14 and 38–46)

Variable	Total patients in the literature	Present series
Total number of patients	3394	161
Deaths	1.5 (42/2806)	2.5
Severe cases	5 (149/2999)	16.1
Male/female ratio	1.5	1.36
Age (years, mean ± SD)	35.2	50.2 ± 18.9
Onset between July and September	84.8 (1826/2153)	75.5
Contact with dogs	55 (1507/2740)	80 (76/95)
Clinical findings		
fever	97.8 (2642/2702)	100 (158/158)
asthenia	71.4 (330/462)	81 (102/126)
arthromyalgias	50.1 (1387/2770)	57.3 (71/124)
eschar(s)	69.7 (1995/2861)	72.7 (109/150)
multiple eschars	3.7 (88/2330)	8.2 (12/147)
regional adenopathy	38.2 (694/1814)	15.2 (19/125)
rash	95.2 (2729/2866)	94.9 (150/158)
maculopapular rash	88.9 (867/975)	68.8 (108/157)
petechial rash	8.1 (200/2466)	32 (41/128)
rash, palm/soles	80.7 (916/1135)	54.9 (39/71)
headache	48.4 (1344/2776)	67.4 (93/138)
meningitis	2.4 (33/1381)	9.1 (13/143)
consciousness changes	5.6 (124/2208)	18.2 (27/148)
seizure	1.1 (12/1039)	2 (3/147)
hypoacusis	1.2 (16/1285)	6 (8/134)
vertigo	1.4 (16/1118)	11.1 (15/135)
cough	9.5 (175/1849)	26.2 (34/130)
dyspnoea	6.2 (88/1425)	16.1 (23/143)
pneumonia	1.2 (22/1810)	13.5 (18/133)
conjunctivitis	15.1 (376/2486)	10.6 (15/141)
abdominal pain	11.5 (185/1604)	19.8 (25/126)
nausea/vomiting	23.1 (326/1412)	16.3 (20/123)
diarrhoea	8.4 (72/856)	20.6 (26/126)
hepatomegaly	35.3 (890/2522)	17.4 (21/121)
splenomegaly	18.5 (362/1960)	8.3 (10/125)
jaundice	2.7 (14/520)	4 (5/125)
oedema	22.8 (62/272)	6.1 (8/131)
phlebitis	0.9 (3/321)	0.7 (1/136)
dehydration	21.3 (30/141)	16 (20/125)
Laboratory findings		
increased transaminase levels	29.1 (690/2368)	80.3 (110/137)
γ-glutamyl transferase >50 IU/L	36.4 (181/497)	54.4 (56/103)
alkaline phosphatase >190 IU/L	20.6 (133/645)	54.4 (56/103)
creatinine phosphokinase >170 IU/L	20.4 (108/529)	46.6 (27/58)
lactate dehydrogenase>480 IU/L	77.4 (537/694)	89.9 (62/69)
albuminaemia <30 mg/L	11.5 (54/471)	35.2 (19/54)
creatininaemia >1.3 mg/dL	13.8 (192/1385)	25 (31/124)
urea nitrogen >25 mg/L	20.7 (80/386)	23 (28/122)
hyponatraemia	31.5 (309/981)	39.6 (44/111)
kalaemia <3.5 mmol/L	15.6 (47/302)	19.8 (21/106)
leucocytes $<4.5 \times 10^9$/L	20.6 (409/1986)	12.8 (14/109)
leucocytes $>10 \times 10^9$/L	14.1 (255/1810)	29.4 (32/109)
haemoglobin <11 g/dL	7.7 (55/718)	17.6 (19/108)
platelets $<150 \times 10^9$/L	25 (429/1716)	61.7 (74/120)

Continued

Table 1. *Continued*

Variable	Total patients in the literature	Present series
interval between onset of fever and initiation of treatment (days)	4.9	7.2±3.9
time to defervescence (days)	2.4	3.9±4
Treatment		
doxycycline	80.7 (856/1060)	76.7 (112/146)
chloramphenicol	19.3 (383/1983)	2.5 (3/118)
fluoroquinolones	2.2 (16/707)	28.8 (38/132)
co-trimoxazole	2.7 (13/486)	3.2 (4/123)
macrolides	27 (372/1377)	12.2 (15/123)
Co-morbidities		
alcoholism	5.3 (14/322)	13.8 (18/130)
tobacco use	6.1 (19/312)	26.6 (34/128)
hypertension	7 (22/314)	22.1 (29/131)
cardiac insufficiency	10.3 (16/155)	4.5 (6/132)
diabetes	9.3 (30/322)	7.8 (10/129)

Data are % (*n*) unless indicated otherwise.

acquired in the south of France for 115 of the cases and in the Mediterranean basin by French travellers for the remaining cases (Portugal $n=12$ cases, Morocco $n=11$, Algeria $n=10$, Italy $n=4$, Spain $=1$ and Tunisia $n=1$). MSF was considered confirmed in 41 cases and probable in 120 cases and the median diagnostic MSF score was 36. The clinical and main biological findings of all the MSF patients are described in Table 1. The summarized characteristics of more than 3000 MSF patients previously described in the literature[5,6,8,9,11,14,38–46] are also listed in Table 1 for comparison. The clinical and laboratory findings for our patients are comparable to the literature values. However, the severity rate was significantly higher in our study compared with the literature (16.1% in our patients versus 5% in the literature, $P=0.019$), although in our study a more selective definition was applied.

Global outcome and treatment

In the study, 26 cases were considered to have severe/malignant MSF (16.1%). Twenty-two patients were hospitalized in the intensive care unit (ICU) and four were managed in medical wards. The distribution of organ dysfunction in the 26 severe cases is reported in Table 2. Twenty-three additional cases were considered to be moderately severe and would have been considered severe according to the definition of severity that has been reported in the literature until recently (e.g. without MODS but with one organ system failure or with atypical manifestations). There were four deaths (2.5%), three of which were in the ICU and one was in the medical ward.

Table 2. Organ dysfunction involved in malignant cases of MSF (at least one dysfunction for each type)

Variable	No. (%)
Respiratory dysfunction	
PaO_2 <60 mmHg	16 (61.5)
mechanical ventilation	12 (46.1)
Cardiovascular dysfunction	
systolic blood pressure <90 mmHg	16 (61.5)
need for inotropes	7 (26.9)
Renal dysfunction	
creatinine >300 μmol/L	8 (30.7)
diuresis <500 mL	8 (30.7)
haemodialysis	2 (7.7)
Neurological dysfunction	
acute confusion	15 (57.7)
Glasgow scale <6	
Hepatic dysfunction	
bilirubin >100 mg/dL	1 (3.8)
alkaline phosphatase level, 3-fold higher than normal range	8 (30.7)
Haematological dysfunction	
haematocrit <20%	1 (3.8)
leucocyte count $<2\times10^9$/L	1 (3.8)
thrombocytopenia $<40\times10^9$/L	11 (42.3)

Comparison between severe and non-severe cases

There were 17 men in the severe group and the severe patients were significantly older than the non-severe group (mean age±SD 58.3±16.2 versus 48.6±19.02 years, respectively, $P=0.031$). Regarding the co-morbidities (see Table 3), only tobacco use was significantly associated with disease severity. The characteristics of patients with severe and non-severe MSF are summarized in Table 4. Classical abnormalities found in severe MSF cases were also present in our patients, although hypocalcaemia was not found to be associated with severity in our study.[47] Multivariate analysis indicated that tobacco use,

Table 3. Univariate analysis of demographic characteristics and treatment received in severe and non-severe cases of Mediterranean spotted fever

Finding, by type	Type of infection, no. (%) of patients of each group		RR (95% CI)	*P* value
	non-severe ($n=135$)	severe ($n=26$)		
Co-morbidity				
alcoholism	13/107 (12.1)	5/23 (21.7)	1.73 (0.73–4.07)	NS
tobacco use	24/107 (22.4)	10/21 (47.6)	2.51 (1.17–5.38)	0.020
chronic bronchitis	2/108 (1.9)	1/22 (4.5)	2.01 (0.39–10.47)	NS
diabetes mellitus	7/106 (6.6)	3/23 (13)	1.78 (0.63–4.99)	NS
obesity (BMI > 30)	8/108 (7.4)	1/22 (4.5)	0.64 (0.09–4.23)	NS
cardiac insufficiency	4/109 (3.7)	2/23 (8.7)	2.00 (0.60–6.62)	NS
hypertension	25/108 (23.1)	4/23 (17.4)	0.74 (0.27–2.00)	NS
Epidemiological findings				
MSF acquired in France	97/131 (74)	18/26 (69.2)	0.82 (0.39–1.75)	NS
tick bite	53/101 (52.5)	10/20 (50)	0.92 (0.41–2.05)	NS
contact with dog	64/80 (80)	11/14 (78.6)	0.93 (0.29–3.00)	NS
Treatment[a]				
doxycycline	97/121 (80.2)	16/26 (61.5)	0.48 (0.24–0.96)	0.041
β-lactam (in association or alone)	36/103 (35)	16/26 (61.5)	2.37 (1.17–4.08)	0.013
macrolides	12/102 (11.8)	3/24 (12.5)	1.06 (0.36–3.12)	NS
co-trimoxazole	3/102 (2.9)	1/24 (4.2)	1.32 (0.23–7.53)	NS
chloramphenicol	1/99 (1)	2/21 (9.5)	4.10 (1.67–10.09)	NS
fluoroquinolones	24/107 (22.4)	15/26 (57.7)	3.29 (1.66–6.50)	0.0007
initiation of corticosteroids during MSF	3/66 (4.5)	2/26 (7.7)	1.45 (0.47–4.47)	NS
use of corticosteroids (initiation + usual treatment)	3/66 (4.5)	5/26 (19.2)	2.5 (1.30–4.80)	0.038

BMI, body mass index; NS, not significant ($P>0.05$).
The denominator for each finding is variable as some data may be missing.
[a]Treatment received by the patients during the entire course of MSF. For details about treatment received prior to the appearance of severity, see the Results section.

dehydration and hyponatraemia were independently associated with disease severity (see Table 5).

Treatment

The treatment details are reported in Table 3. There was no delay in the commencement of efficient treatment in patients with a severe outcome compared with the non-severe group (6.75 ± 3.11 versus 7.05 ± 4.08 days, $P=0.809$). During treatment, 64/133 patients (48.1%) received more than one antibiotic (in combination or successively).

Outcome versus treatment

For patients with a severe outcome, we counted the treatment received prior to the outcome. The median interval from start of disease to outcome was 5 days. In order to compare with the treatment received by patients without severity or worsening of the disease (control group), we assessed the treatment received by this control group in the first 5 days of the course of MSF. Data about treatment received prior to the outcome was available for 26 severe patients (doxycycline 3, fluoroquinolones 8, β-lactams 9, no treatment 11), 13 patients with worsening of the disease (doxycycline 1, fluoroquinolones 8, β-lactams 11, no treatment 0) and treatment received in the first 5 days of the disease was available for 61 controls (doxycycline 27, fluoroquinolones 5, β-lactams 21, no treatment 16). The absence of treatment prior to evaluation was not different between the severe patients ($n=11$) and the control group ($n=16$) [$P=0.130$, relative risk (RR) 1.630, 95% confidence interval (CI) 0.866–3.065].

Treatment with doxycycline and fluoroquinolones

Doxycycline administration was protective against severity ($P=0.015$, RR 0.248, 95% CI 0.081–0.759) and worsening of the disease ($P=0.05$, RR 0.137, 95% CI 0.019–0.997). Patients treated with doxycycline became afebrile significantly earlier compared with patients treated with other efficient regimens (mean ± SD 3.0 ± 2.2 days versus 7.1 ± 6.6 days, respectively, $P=0.021$). The prior use of fluoroquinolones was significantly associated with an increased risk of severe outcome (RR 2.53, 95% CI 1.405–4.557). The same increased risk was observed in patients with worsening of the disease compared with controls (RR 7.51, 95% CI 2.923–19.285). The association between fluoroquinolones and increased disease severity was not explained by a delay in the start of treatment in

Table 4. Univariate analysis of clinical and laboratory findings in patients with severe and non-severe Mediterranean spotted fever

Finding, by type	Type of infection, no. (%) of patients of each group		RR (95% CI)	*P* value
	non-severe ($n=135$)	severe ($n=26$)		
Clinical findings				
fever	131/131 (100)	26/26 (100)	/	/
eschar	91/125 (72.8)	18/25 (72)	0.96 (0.43–2.14)	NS
multiple eschars	8/124 (6.5)	4/23 (17.4)	2.37 (0.96–5.83)	NS
rash	123/131 (93.9)	26/26 (100)	/	NS
maculopapular	98/109 (89.9)	10/15 (66.6)	0.30 (0.12–0.76)	0.011
purpuric	25/105 (23.8)	16/23 (69.6)	4.85 (2.16–10.86)	<0.001
palm and sole	34/62 (54.8)	5/9 (55.6)	1.02 (0.30–4.50)	NS
asthenia	79/101 (78.2)	22/24 (91.7)	2.61 (0.66–10.36)	NS
myalgia	56/100 (56)	15/24 (62.5)	1.24 (0.59–2.62)	NS
arthralgia	43/95 (45.3)	13/24 (54.2)	1.33 (0.65–2.72)	NS
anorexia	35/91 (38.5)	10/18 (55.6)	1.78 (0.76–4.15)	NS
dehydration	8/99 (8.1)	12/25 (48)	4.80 (2.57–8.93)	<0.001
loss of weight	27/78 (34.6)	5/10 (50)	1.75 (0.55–5.58)	NS
abdominal pain	15/105 (14.3)	10/21 (47.6)	3.67 (1.76–7.67)	0.001
nausea	14/102 (13.7)	6/21 (28.6)	2.06 (0.91–4.66)	NS
vomiting	12/104 (11.5)	5/21 (23.8)	1.98 (0.84–4.71)	NS
diarrhoea	19/105 (18.1)	7/21 (33.3)	1.92 (0.87–4.27)	NS
digestive bleeding	0/117	5/22 (22.7)	7.88 (5.05–12.29)	<0.001
hepatomegaly	17/101 (16.8)	4/20 (20)	1.19 (0.44–3.20)	NS
splenomegaly	7/101 (6.9)	3/20 (15)	1.96 (0.69–5.56)	NS
jaundice	1/105 (1)	4/20 (20)	6.00 (3.18–11.29)	0.002
cough	23/110 (20.9)	11/20 (55)	3.45 (1.57–7.60)	0.003
dyspnoea	8/120 (6.7)	15/23 (65.2)	9.78 (4.70–20.36)	<0.001
pneumonia	9/111 (8.1)	9/22 (40.9)	4.42 (2.22–8.81)	<0.001
pulmonary oedema	2/122 (1.6)	7/23 (30.4)	6.61 (3.71–11.78)	<0.001
hypotension	0/122	3/23 (13)	/	0.003
arrhythmia	3/107 (2.8)	4/22 (18.2)	3.87 (1.79–8.37)	0.016
deep venous thrombosis	0/114	1/22 (4.5)	6.43 (4.34–9.52)	NS
pulmonary embolism	3/114 (2.6)	1/22 (4.5)	1.57 (0.27–8.97)	NS
arterial thrombosis	2/114 (1.8)	3/22 (13.6)	4.14 (1.81–9.47)	0.030
headache	78/116 (67.2)	15/22 (68.2)	1.04 (0.45–2.36)	NS
delirium	16/124 (12.9)	15/25 (60)	5.71 (2.84–11.45)	<0.001
stupor	12/123 (9.8)	15/25 (60)	6.72 (3.40–13.31)	<0.001
seizures	0/123	3/24 (12.5)	6.86 (4.62–10.18)	0.004
focal neurological deficits	3/122 (2.5)	6/24 (25)	5.07 (2.70–9.54)	<0.001
meningitis	9/119 (7.6)	4/24 (16.7)	2.00 (0.80–4.97)	NS
hypoacusis	7/113 (6.2)	1/21 (4.8)	0.79 (0.12–5.14)	NS
cerebellar ataxia (vertigo)	11/114 (9.6)	4/21 (19)	1.88 (0.73–4.86)	NS
Laboratory findings				
hyponatraemia	29/90 (32.2)	15/21 (71.4)	3.81 (1.60–9.06)	0.001
hypokalaemia	16/87 (18.4)	5/19 (26.3)	1.44 (0.59–3.56)	NS
creatinine >140 μmol/L	12/98 (12.2)	19/26 (73.1)	8.14 (3.79–17.50)	<0.001
urea >7.5 mg/L	11/97 (11.3)	17/25 (68)	7.13 (3.45–14.75)	<0.001
hypoalbuminaemia	12/45 (26.7)	7/9 (77.8)	6.45 (1.48–28.01)	0.006
hypocalcaemia (corrected)	17/52 (32.7)	5/11 (45.5)	1.55 (0.53–4.52)	NS
increased transaminases	87/114 (76.3)	23/23 (100)	/	0.004
cholestasis	41/83 (49.4)	15/20 (75)	2.52 (0.99–6.41)	0.033
creatine kinase >1.5 normal range of each laboratory	17/45 (37.8)	10/13 (76.9)	3.83 (1.17–12.49)	0.014
leucopenia, $<4\times10^9$/L	13/87 (14.9)	1/22 (4.5)	0.32 (0.05–2.22)	NS

Continued

Table 4. *Continued*

Finding, by type	Type of infection, no. (%) of patients of each group		RR (95% CI)	*P* value
	non-severe ($n=135$)	severe ($n=26$)		
leucocytosis, $>10.5\times10^9$/L	24/88 (27.3)	8/21 (38.1)	1.48 (0.68–3.22)	NS
anaemia, haemoglobin <11 g/dL	10/85 (11.8)	9/23 (39.1)	3.01 (1.53–5.91)	0.005
thrombocytopenia, platelets $<140\times10^9$/L	52/97 (53.6)	22/23 (95.7)	13.67 (1.91–98.06)	<0.001
prothrombin ratio <70%	6/71 (8.5)	5/19 (26.5)	2.56 (1.15–5.73)	0.050
activated partial thromboplastin time (ratio >1.2)	11/67 (16.4)	11/17 (64.7)	5.17 (2.17–12.30)	<0.001
circulating anticoagulant	2/8 (25)	5/5 (100)	/	0.02
fibrinogen <2 g/L	3/64 (4.7)	6/19 (31.6)	3.79 (1.93–7.46)	0.004

NS, not significant ($P>0.05$).
The denominator for each finding is variable as some data could be missing.

Table 5. Results of the multivariate analysis

Variable	*P* value	Odds ratio	95% Confidence interval
Tobacco use	0.037	7.008	1.120–43.867
Dehydration	0.011	11.795	1.764–78.853
Hyponatraemia	0.021	7.972	1.369–46.435
Use of corticosteroids	0.707	1.697	0.108–26.701
Doxycycline regimen	0.616	1.635	0.239–11.187
Fluoroquinolone regimen	0.031	9.767	1.234–77.291
β-Lactam regimen	0.344	2.215	0.427–11.489

The severe form of the disease was used as the dependent variable and tobacco use, dehydration, hyponatraemia, use of corticosteroids, doxycycline, fluoroquinolones and β-lactam regimens as independent variables.
Hosmer and Lemeshow test: $P=0.990$.

patients treated with these compounds compared with patients treated with other efficient regimens (7.72 ± 3.78 versus 6.68 ± 3.95, respectively, $P=0.341$). A longer stay in hospital (14.23 ± 11.72 days versus 7.31 ± 5.72 days, $P=0.002$) was observed in patients treated with fluoroquinolones compared with the controls. Logistic regression indicated that fluoroquinolone treatment was independently associated with MSF disease severity [odds ratio (OR) 9.767, 95% CI 1.234–77.291].

Other treatments

Macrolides were used to treat 15 patients (9.3%), chloramphenicol 3 patients (1.8%) and co-trimoxazole 4 patients (2.5%); the small sample sizes of these groups did not allow us to analyse the impact of these regimens on prognosis. The use of β-lactams, which is typically considered an inefficient treatment for MSF, was not associated with an increase in severity compared with controls ($P=0.986$, RR 1.00, 95% CI 0.51–1.98) but was associated with worsening of the disease compared with controls ($P=0.007$, RR 7.22, 95% CI 1.72–30.31); however, this association was not independent according to multivariate analysis, as shown in Table 5.

Discussion

We report a large study of severe MSF cases caused by *R. conorii conorii*, including 26 patients that presented with MODS. The use of a standardized definition of disease severity may allow a more reliable characterization compared with other studies and may serve as a basis for future comparisons. Recently, 40 severe MSF cases have been reported: only 15 of these were caused by *R. conorii conorii*, while the remaining cases were caused by *R. conorii israelensis*,[9] which is considered more virulent.[2,9,13]

In our study, we analysed host- and regimen-related risk factors, but not severity due to subspecies-related risk factors; we assumed that our patients were infected only by the subspecies *R. conorii conorii*, based on the geographical area where the tick bites were sustained.[2] Among patients who acquired MSF in Portugal (where *R. conorii israelensis* is present),[9] only one had a severe form. The co-morbidities, clinical signs and biological findings associated with disease severity in our study are comparable to those previously reported (Table 1).[3,4,7–9] However, the rate of severity reported in our study is 3-fold higher than in our previous series.[4,5,38] Moreover, we and others reported severe cases of MSF in patients without underlying conditions,[7,48] leading us to question whether increased MSF severity is caused by pathogen virulence[1] or other human factors. Indeed, the global severity rate for MSF has increased from 6.1% in the older studies[5,14,38–40] to 13.1% in recent studies,[8,9,11] as is shown in Figure 1. When compared with the >3000 MSF patients in the literature, our patients received fluoroquinolones significantly more frequently ($P<0.0005$) and chloramphenicol and macrolides less frequently ($P<0.0005$ and $P=0.023$. respectively) (Table 1). Indeed, there is a difference between the old and recent studies regarding the use of newer antibiotic regimens, specifically the fluoroquinolones, which we hypothesize may be associated with an increase in disease severity and an unfavourable outcome in MSF cases treated with this regimen.

The use of fluoroquinolones to treat MSF is based upon previously reported *in vitro* studies.[26,49] Fluoroquinolone treatment was proposed as an alternative to tetracycline and chloramphenicol in a non-comparative study.[30] Later, Gudiol *et al.*[31] performed a double-blind randomized study that compared a

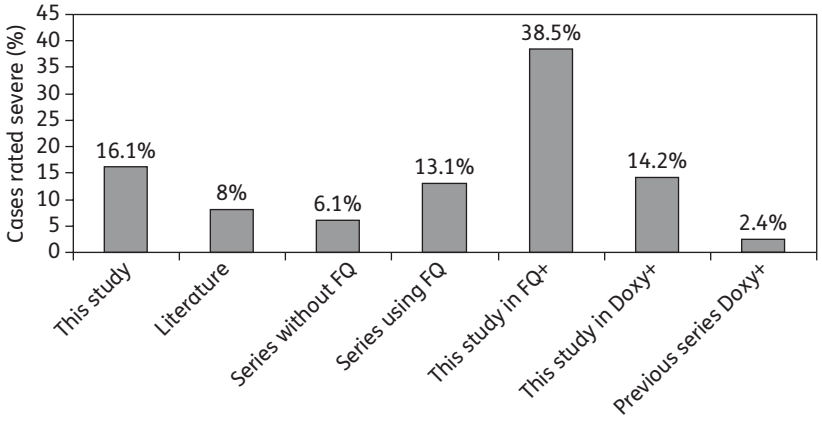

Figure 1. Comparison of proportion of MSF cases rated as severe according to the treatment regimen used and its chronological course. The grey bars represent the percentage of severe cases of MSF. 'Literature' includes references 5, 8, 9, 11, 14 and 38–40; 'Series without FQ' (fluoroquinolones) includes references 5, 14 and 38–40 (from 1983 to 2003); 'Series using FQ' includes references 8, 9 and 11 (since 2003). 'This study in FQ+' represents the percentage of severe cases among patients treated with fluoroquinolones in our study; 'This study in Doxy+' represents the percentage of severe cases among patients treated with doxycycline in our study; 'Previous series Doxy+' represents severity in patients treated with doxycycline and is taken from reference 38.

2 day course of oral ciprofloxacin (500 mg twice a day) with oral doxycycline (100 mg twice a day) in 43 patients with non-severe MSF.[31] They showed that fever and major complaints persisted significantly longer in the ciprofloxacin group. In a non-blinded comparative study of ciprofloxacin (750 mg every 12 h) and doxycycline in non-severe MSF, Ruiz Beltran *et al.*[29] did not observe a significant difference between the two regimens. The use of fluoroquinolones to treat MSF has significantly increased, as reported here (see Table 1): this treatment was used in 10–20% of cases in recent studies[9,11,50] and was associated with a concomitant increase in severity rate from 6.6% to 22% (Figure 1).

In our study, we found that fluoroquinolone treatment was significantly and independently associated with MSF severity. This effect was not due to the use of fluoroquinolones in already severe patients as we studied the use of this regimen prior to the deterioration of the disease. Fluoroquinolones have been previously associated with a poor outcome and death in other rickettsial diseases, as was reported for a fatal case of epidemic typhus in a nurse who was treated with ciprofloxacin,[51] and is associated with a poor response in murine typhus.[52] Similar to the previous results with typhus, a worse prognosis was then seen in MSF despite *in vitro* susceptibility. In the literature MSF severity was increased in series where fluoroquinolones were used compared with series not using them (13.1% versus 6.1%, respectively, $P=0.146$) (Figure 1).

We hypothesized that the association between fluoroquinolone treatment and poor MSF outcome may be caused by toxin liberation. In fact fluoroquinolones have been previously associated with toxin-related diseases such as *Clostridium difficile* infection.[53] Moreover, toxin/antitoxin modules exist in many pathogenic bacteria, including *Rickettsia felis*, and one module is found in the genome of *R. conorii*.[54] Although the precise function of these modules remains unknown, they may be involved in bacterial stress response and may function when the elimination of a short-lived antitoxin allows the cognate toxin to interfere with the bacterial transcription/translational machinery.[55] Several types of stress, including fluoroquinolones, result in toxin-mediated cell death. Administration of fluoroquinolones at a subinhibitory concentration has been shown to increase the production of toxins that are encoded by phages in diverse bacterial models.[56–58] In intracellular bacteria such as the Rickettsiae, the effect of potentially released toxins after antibiotic exposure on the host cells remains unknown. Studies regarding the *in vitro* effects of ciprofloxacin on toxin/antitoxin expression in *R. conorii* are needed to understand our *in vivo* observations.

Doxycycline, which is considered to be the gold standard treatment for spotted[2,18,19,22] and typhus group[17,20,21] rickettsioses, decreased disease severity and led to earlier defervescence compared with the other treatment regimens in this study. However, the rate of disease severity with doxycycline treatment was higher than was previously reported in our centre.[38]

Our studies have a possible bias of recruitment because all patients were recruited at the French referral centre, which may receive more severely ill patients. This was an observational study and we do not know the reasons for the different treatment choices. The retrospective nature of the study, the fact that severe patients are a small group and that some data are missing are also limitations that should be considered. However, severity in MSF is rare and to obtain a large series of severe cases in a prospective way may take a long time.

In conclusion, we found that fluoroquinolone administration is associated with increased MSF severity. Therefore, we do not recommend the use of this regimen for the treatment of spotted fever group rickettsiosis. We recommend using doxycycline as a first line of treatment in all patients with suspected rickettsiosis, including children.[59]

Acknowledgements

The authors are grateful to all of the physicians for providing the clinical information.

Funding

This work was supported by a regional 'Programme Hospitalier de Recherche Clinique' UF 8046 titled: 'Study of severity factors in Mediterranean spotted fever by transcriptomal analysis of eucaryote cells and *Rickettsia conorii* in skin biopsy'.

Transparency declarations

None to declare.

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ARTICLE 2

Deleterious effect of ciprofloxacin on *Rickettsia conorii* infected cells is linked to toxin-antitoxin module up-regulation

E. Botelho-Nevers, S. Edouard, Q. Leroy, D. Raoult

Soumis

URMITE UMR 6236, CNRS-IRD, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille

cedex 05, France

Contribution de EBN: réalisation de toutes les expérimentations, analyses des données, écriture du manuscrit sous la direction de DR.

Commentaire

L'objectif de cet article de recherche était d'essayer de confirmer et de mieux comprendre l'effet délétère des fluoroquinolones observé précédemment *in vivo* au cours de l'infection par *Rickettsia conorii*.

Pour cela, nous avons utilisé la méthode des plages de lyse «revisitée» afin de pouvoir tester l'effet précoce de la ciprofloxacine sur les cellules infectées par *R. conorii*. Cette technique des plages de lyse a été modifiée d'une part car la lecture se fait sans aucune coloration, directement par lecture microscopique et d'autre part car l'acquisition des images et leur analyse se fait de façon numérique. Ainsi l'analyse des résultats s'est faite avec ImageJ® après prise de photographies avec suivi des mêmes plages de lyse dans le temps grâce à l'utilisation de boites de Petri quadrillées. Nous avons ainsi comparé les effets observés sur des cellules infectées non traitées ou traitées par ciprofloxacine ou bien par doxycycline.

Par ailleurs, on sait que les fluoroquinolones sont capables d'induire l'expression de toxines à la fois plasmidiques (31-34) et chromosomiques (35) et ceci à des concentrations sous ou supra-inhibitrices. De plus, *R. conorii* contient un module toxine-antitoxine *VapC1-VapB1* dont les fonctions sont inconnues à ce jour (36). Nous avons donc testé l'hypothèse d'une induction de ce module par les fluoroquinolones. Pour cela, nous avons réalisé une reverse transcriptase PCR quantitative sur des cellules infectées par *R. conorii* traitées par ciprofloxacine à dose sous ou supra-inhibitrice afin de quantifier l'expression de *VapC1* et *VapB1* sous l'effet de la ciprofloxacine. Ces résultats ont été comparés au témoin (cellules infectées non traitées) et après normalisation par rapport à l'expression du gène conservé *GroEL*.

Bien que comparable à H0, la taille des plages de lyse était significativement augmentée dans le groupe traité par ciprofloxacine à 0,5 et 50µg/ mL par rapport au contrôle à H2, H4, H6 ($p<0,0001$ dans tous les cas) et H24 (avec ciprofloxacine 0.5µg/mL $p= <0,0001$, avec

ciprofloxacine à 50µg/mL p= 0,035). Le traitement par doxycycline à 5µg/mL n'a pas induit de différence significative dans la taille des plages de lyse par rapport au contrôle à H0, H2, H4, H6, H24 (p> 0.05). L'analyse des qRT-PCR a montré que l'expression des gènes *VapC1* et *VapB1* était considérablement augmentée à H2 et H4 dans les cellules traitées par ciprofloxacine 50µg/mL mais pas par ciprofloxacine 0.5µg/mL, par rapport au contrôle à la même heure.

En conclusion, ces résultats *in vitro* sont venus confirmer microscopiquement nos constatations cliniques préalables: les fluoroquinolones ont un effet délétère sur les cellules infectées par *R. conorii*, cet effet n'étant pas observable avec la doxycycline. Pour comprendre le mécanisme à l'origine de cet effet, nous avons émis l'hypothèse d'un rôle de la toxine bactérienne. Dans ce sens nous avons montré que la ciprofloxacine induisait la transcription du module toxine-antitoxine. Toutefois, il reste à savoir *(i)* comment la toxine (dont l'antitoxine est également exprimée sous l'effet des fluoroquinolones) peut avoir une action toxinique *(ii)* si la toxine est relarguée dans la cellule hôte comme il a été observé récemment au laboratoire dans un modèle d'infection à *Rickettsia felis* (37), et est ainsi à l'origine des effets délétères observés. Enfin les fluoroquinolones sont connues pour leur action immuno-modulatrice qui pourrait également participer à l'effet délétère observé chez l'hôte (38).

Synopsis

A deleterious effect of fluoroquinolones during *Rickettsia conorii* infection has been reported in humans. Using a new plaque assay, we show here that ciprofloxacin at 0.5 and 50 µg/mL induced a significant increase in lytic areas compared to the control at 2 hours (H2), H4, H6 ($p<0.0001$) and H24 (respectively $p<0.0001$, $p= 0.035$) when not induced with doxycycline. By quantitative reverse transcriptase-PCR, ciprofloxacin was found to cause an up-regulation of toxin-antitoxin module transcription. Indeed, the mRNA levels of *VapC* and *VapB* were significantly increased at H2 and H4 in cells treated with 50 µg/mL ciprofloxacin (not with 0.5 µg/mL ciprofloxacin) compared to control levels (fold change >2.9). We speculate that the toxic effect of fluoroquinolones on *R. conorii*-infected cells is mediated by the overexpression of toxin-antitoxin, followed by their release into the host cytoplasm.

Key words: fluoroquinolones, deleterious effect, doxycycline, toxin-antitoxin module, *Rickettsia conorii*, host

Introduction

Since their commercialization in 1980, fluoroquinolones have become an increasingly popular class of antibiotic used for a large range of infections. Their mechanisms of action, inhibiting bacterial DNA synthesis, confers a bactericidal effect on a large variety of bacteria [1]. Previously, the susceptibility of Rickettsial species to fluoroquinolones has been tested. In vitro testing of pefloxacin, ciprofloxacin, and ofloxacin on spotted fever group rickettsiae and typhus group rickettsiae has shown good efficiency with Minimal Inhibitory Concentration (MIC) around 1 µg/mL [2]. Fluoroquinolones have been considered to be a possible alternative to doxycycline and chloramphenicol for the treatment of Rickettsioses, notably for the treatment of Mediterranean spotted fever (MSF). We previously published a non-comparative study of a small series of patients with MSF that was successfully treated with ciprofloxacin [3]. In a non-double-blind comparative study of ciprofloxacin (750 mg/12 h) and doxycycline in non-severe MSF, Ruiz-Beltran et al. did not observe a significant difference between the two regimens [4]. Gudiol et al. performed a double-blind randomized study that compared a two-day course of oral ciprofloxacin (500 mg twice a day) to oral doxycycline (100 mg twice a day) in 43 patients with non-severe MSF. They saw that the fever and major complaints persisted significantly longer in the ciprofloxacin group [5]. In other Rickettsioses, such as murine typhus [6], epidemic typhus [7] and scrub typhus [8], patients treated with fluoroquinolones demonstrated a poor or fatal outcome. Nonetheless, fluoroquinolones are recommended as an alternative regimen in the treatment of MSF [9]. Recently, in a large series of MSF cases, we observed that patients treated with fluoroquinolones had deleterious outcome, with treatment being significantly associated with severity [10]. The mechanism of this deleterious and unexpected effect is unknown.

Toxin-antitoxin (TA) systems were initially identified as plasmid stabilization factors, also called plasmid-encoded ‘addiction systems’, comprising a long-lived toxin and a short-lived

anti-toxin [11]. The lack of expression of the toxin-antitoxin module due to loss of the plasmid leads to the liberation of toxin not sequestered by anti-toxin and induces bacterial death. Therefore, the TA modules are selfish genes and make it impossible to cure the bacteria from their plasmids. These modules have also been found on chromosomes in multiple copies and seem to have a role in the stabilization of integrons in bacterial chromosomes [12]. TA modules have been found to be in larger numbers in "bad bugs," i.e., bacteria associated with the worst human pandemics, compared to their respective controls, i.e., closely related bacteria that are not epidemic and/or virulent [12]. Therefore, TA systems seem to be part of the virulence repertoire of epidemic bacteria [12]. Interestingly, bacterial TA, whose toxins are mostly RNAse [13], have been showed to be toxic for eukaryotic cells as well [14, 15]. In Rickettsia species, the function of TA modules in their genomes remain unknown [16], and *R. conorii* harbors one module TA. Fluoroquinolones have been shown to induce toxin release in different models at sub-inhibitory concentrations for phage toxins [17-19] and at supra-inhibitory concentrations for chromosomal toxins [20].

To understand the mechanism leading to a deleterious effect of fluoroquinolones during *R. conorii* infection, we tested the effect of ciprofloxacin at sub and supra-inhibitory concentration on *R. conorii*-infected cells in vitro. We tested the hypothesis that rickettsial toxin-antitoxin could play a role in this deleterious effect observed in the host cells.

Material and Methods

Bacterial strain. *R. conorii* (Malish strain, ATCC number VR-613) was cultured in L929 cells as previously described [21]. Fresh inoculum was used after titration for each experiment. Briefly, bacteria were harvested after 48 to 72 hours of incubation in cells. After low speed followed by high speed centrifugation, the pellet was kept and resuspended in Eagle's minimal essential medium (MEM) (Invitrogen) supplemented with 4% fetal calf serum (FCS) and 1% L-glutamine, and serial dilutions were performed. After Gimenez staining, titration of inoculum was performed microscopically at X 100 magnification [22].

Antibiotic. Ciprofloxacin (Bayer santé, France) was diluted in Phosphate Buffered Saline (PBS) to obtain a stock solution of 2 mg/mL and was stored at -20°C until use. Further dilution to obtain the final concentration of ciprofloxacin was made in MEM, 4% FCS and glutamine. Doxycycline (Sigma-Aldrich) was stocked at 1 mg/mL, stored at -20°C and diluted to obtain a final concentration of 5 µg/mL.

Plaque assay. L929 cell monolayers were seeded in squared Petri dishes 24 hours before use (60 mm; Nucleon Δ surface, Nunc, Denmark). After 24 to 48 hours, the confluent monolayer was infected with 1 mL of a solution containing 10^3 bacteria/mL. After 1 hour of incubation at 32°C, 5% CO_2, the inoculum was removed, and the dishes were overlaid with 5 mL of a medium containing MEM, 4% FCS, 1% L-glutamine, and agar 0.5 %. The dishes were incubated for 3 days at 32 °C in a 5% CO_2 incubator until lytic plaques started to appear under 40X magnification. Ciprofloxacin at a final concentration of 0.5 µg/mL or 50 µg/mL was added onto the agar (200 µl). In control dishes, the same quantity of MEM 4% FCS, 1% L-glutamine was added. At H0, H2, H4, H6 and H24 after adjunction of ciprofloxacin, pictures of at least 10 plaques for each condition were acquired with a camera (NIKON coolpix 950) at 40X magnification. We performed the same experiment with doxycycline at 5 µg/mL and compared the results to mock-treated cells. At the different time points, the same plaques

were followed. During the time intervals, the petri dishes were replaced in incubator. All conditions were assayed at least in duplicate. Obtained images were analyzed with ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2007). The area of each plaque was measured as lytic area. The lytic area of petri dishes treated with ciprofloxacin or doxycycline was compared to the lytic area of control dishes at the same time.

Real Time Quantitative reverse transcriptase PCR. Briefly, L929 cells were seeded in 12-well plates. At 24 hours, cells were infected with 0.5 mL fresh inoculum (10^5 bacteria/mL) for 1 hour at 32°C with 5% CO_2, aspirated and incubated for 48 hours in an incubator. Ciprofloxacin at a final concentration of 0.5 and 50 μg/mL was added to the infected monolayer, and MEM was added in control wells. At H0, H2, H4, H24, monolayers and supernatant were harvested and transferred to eppendorf tubes (Eppendorf, Germany) for centrifugation at 7500 rpm for 10 minutes. Pellets were kept frozen in azote and stored at -80°C until RNA extraction was performed.

Total RNA was extracted from pellets and treated as described previously [23]. Quantitative real-time RT-PCR (Superscript III Platinum, Invitrogen) was performed to assay the mRNA expression of *VapC*, *VapB* of *R. conorii* and the housekeeping gene *GroEL* using the 7900 HT system (Applied biosystem). Primers and probes are described in Table 1. Each condition was analyzed in triplicate, and each experiment was repeated twice, and the average was used to calculate relative mRNA expression. We used the delta-delta method (i.e., comparative CT [ΔCT method) for quantitative analysis of gene expression. We considered significant differential expression when the fold change was > 2.8.

Statistical Analysis. Data analysis was performed using Graphpad® software version prism 5.0 to compare mean values of lytic area between each condition tested and respective control. A difference was considered statistically significant at P-value < 0.05.

Results

Plaque assay. Ciprofloxacin used at 50 µg/mL (supra–inhibitory concentration) did not induce toxicity or plaque formation on uninfected L929 cells.

At baseline (H0), there was no difference in the lytic area of plaques between controls and cells receiving ciprofloxacin at 0.5 or 50 µg/mL ($p=0.742$ and $p=0.176$, respectively). At 0.5 µg/mL (sub-inhibitory concentration), ciprofloxacin induced significantly higher lytic areas at H2, H4, H6 and H24, compared to respective controls ($p<0.0001$) (Table 2). The percentages of increase in lytic areas between H0 and H2, H0 and H4, H0 and H6 were significantly higher in cells treated with 0.5 µg/mL ciprofloxacin compared to controls (respectively 83.2 +/- 13.6%, 127.6 +/- 16.7%, 191.6 +/- 24.1%, vs. 29.5+/- 14.7%, 77.6 +/- 23.9%, 111.9 +/- 24.5% in respective controls). The percentage of increase in lytic areas between H0 and H24 was not statistically significant (207.1 +/- 25.3% in ciprofloxacin 0.5 µg/mL vs. 192.2 +/- 34.2% in control).

At 50 µg/mL, ciprofloxacin induced significantly higher lytic area at H2, H4, H6 compared to respective controls ($p<0.0001$) as well as at H24 ($p=0.033$), see Table 2 and Figure 1. The percentages of increase in lytic areas between H0 and H2, H0 and H4, H0 and H6, H0 and H24 in the group treated with 50 µg/mL ciprofloxacin were as follows: 65.5 +/-26.8%; 112 +/-33.6%; 142.2 +/- 45.7%; 167.7 +/-40.9%, respectively. When compared to respective controls, the percentages of increase in lytic areas in cells treated with 50 µg/mL ciprofloxacin tended to be higher between H0 and H2, H0 and H4 and H0 and H6, but the differences observed were not significant.

Doxycycline used at 5 µg/mL (supra-inhibitory) did not induce a significant increase in lytic areas compared to mock-treated controls at H0 (0.040+/-0.001 vs. 0.040+/-0.001, $p=0.976$), at H2 (0.059+/-0.002 vs. 0.057+/-0.002, $p=0.425$), at H4 (0.068+/-0.002 vs. 0.067+/-0.002,

p=0.620), at H6 (0.079+/-0.002 vs. 0.078+/-0.002, p=0.753) or at H24 (0.090+/-0.002 vs. 0.092+/-0.003, p=0.902).

Quantitative Real Time reverse transcriptase PCR. When compared to the respective controls and normalized to *GroEL* expression levels, we observed that the expression level of the *R. conorii* genes *VapB* and *VapC* was higher in cells treated for several hours with 50 µg/mL ciprofloxacin. Fold changes for *VapB* under 50 µg/mL ciprofloxacin were of 2.97 +/- 1.13 , 4.84 +/- 1.06 and 1.27 +/- 1.18 at H2, H4, H24, respectively, whereas for *VapC,* the fold change was 3.01 +/- 0.11, 6.18 +/- 1.59 and 2.36 +/- 0.35, respectively. At 0.5 µg/mL ciprofloxacin, the expression level of *VapB* and *VapC* were not significantly modified compared to control at any time point (H2, H4, H24). These results are shown in Figure 2 and expressed as log2 fold change. The same results were observed when compared to the H0 control.

Discussion

For the first time, we have shown that ciprofloxacin, an efficient in vitro treatment of *R. conorii* [2] has a deleterious effect on *R. conorii*-infected cells, inducing higher lytic areas compared to mock-treated controls in the first hours after adjunction. This finding correlates with the clinical association between severity in Mediterranean spotted fever and treatment with fluoroquinolones [10]. In contrast, we found that doxycycline did not induce deleterious effects on infected cells, corroborating the results of our previous clinical study of MSF [10].

Antibiotics are known to interfere with proteic bacterial toxin release, but all antibiotics do not seem to have the same effect during infections. This effect of fluoroquinolones has been largely studied in *Staphylococcus aureus,* a bacterium that is able to produce a large number of toxins [17]. Sub-inhibitory concentrations of ciprofloxacin and trimethoprim have been associated with phage induction and expression of phage-related toxins in an in vitro model of *Staphylococcus aureus* infection [17]. In this model, ciprofloxacin, but not trimethoprim, enhanced the transcription of *RecA*, indicating involvement of the SOS response by autocleavage of the phage repressor [17]. Zhang et al. studied the effect of ciprofloxacin and fosfomycin in a model of *Escherichia coli* producing Shiga toxin [18]. They observed that only ciprofloxacin used at sub-inhibitory concentrations caused induction of the Shiga toxin-encoding bacteriophage and enhanced Shiga toxin production. Interestingly, they observed that mice treated with ciprofloxacin, but not fosfomycin, had a marked increase in free Shiga toxin in feces and a higher mortality rate whereas both antibiotics reduced fecal Shiga toxin-producing *E. coli* in mice [18]. Therefore, induction of bacterial toxins by fluoroquinolones seems to be associated with a deleterious effect in host in some cases. Antibiotic-related modulation of bacterial toxins seems to be linked to transcriptional up-regulation [24].

Previous studies have described toxic substances associated with *R. conorii* infection [25]. However, genome sequencing did not identify gene-encoding toxin proteins in *R. conorii* [26] but did identify one complete toxin-antitoxin module [16, 27]. We hypothesized that the association between fluoroquinolone treatment and the deleterious effect on *R. conorii* infection may be caused by the up-regulation of toxin-antitoxin genes.

In this study, we showed that ciprofloxacin at supra-inhibitory concentrations modulated the expression of genes of the TA module, *VapC-VapB,* present in *R. conorii.* Although the precise function of these chromosomal modules remains uncertain, they may be involved in bacterial stress response and may function when the elimination of a short-lived anti-toxin allows the cognate toxin to interfere with the bacterial transcription-translational machinery [16]. Toxin anti-toxin modules have been shown to control growth under amino acid and glucose starvation through a ppGpp-independent mechanism [28]. Several other types of stress, including antibiotics, result in toxin-mediated cell death. In intracellular bacteria such as Rickettsiae, the effect of cytoplasm-released toxins after antibiotic exposure on the host-cells is unknown. In the literature, TA modules located on the bacterial chromosome can be induced by fluoroquinolones [20]. The chromosomal toxin-antitoxin module *mazEF* is present in gram-negative and gram-positive bacteria. Nalidixic acid, a 4-quinolone agent that damages DNA by inhibiting topoisomerase gyrase, can induce *Escherichia coli* chromosomal-*mazEF*-mediated cell death and decrease the number of CFU when used at supra-inhibitory concentrations [20]. The drug-induced lethality was prevented when high expression levels of antitoxin *mazE* were induced within 90 minutes of drug treatment. Interestingly, the antitoxin is ineffective if its expression is induced later [20]. Similar results were obtained in *Streptococcus pneumoniae* cultures submitted to antibiotic exposure [29]; erythromycin and streptomycin more efficiently reduced the number of CFU in the wild-type strain compared to a mutant strain that lacked the *RelBE* module, indicating that the RelE toxin induces cell stasis

or death [29]. In *E. coli*, it has been shown that chloramphenicol at supra-inhibitory concentrations conferred a dramatic activation of *relBE* transcription [28].

In conclusion, fluoroquinolones usually considered efficient against *R. conorii* infection have deleterious effect on host cells. This effect is associated with the induction of the toxin-antitoxin module of *R. conorii.* However, the causal role of VapC toxin in eukaryotic cell death remains to be demonstrated and is the subject of ongoing work.

Funding statement: we have no funding source to declare

Acknowledgments

We are grateful to Mr. Guy Vestris for his technical help.

Table 1. Primers and probes used in real-time reverse transcriptase PCR in L929 cells infected with *Rickettsia conorii* treated with or without ciprofloxacin. (Genes of *R. conorii*).

Genes	Primers sequences	Probe sequences
GroEL	Reverse: 5'-AGATGCTGCTTCCGTTGCTTCG-3'	CCGCCACGCATTGGCATTGGCTCTGCC
	Forward: 5'-TCCATACCGCCCATACCTCCCA-3'	
VapB	Reverse: 5'-TCGGCATTTTTCATGAATTG-3'	TGGTCCACATCTTGATCCTGAACATGC
	Forward: 5'-CCTGCAGAAATCAAGCCTTC-3'	
VapC	Reverse: 5'-CCTCTAGTCCCTGGATTCTTTT-3'	TGATATGCTTATTGCAGCAACGGC
	Forward: 5'-TTACTCATGATTTATACACACAACG-3'	

Table 2. Comparison of lytic area of plaques between untreated cells (controls), cells treated with ciprofloxacin (CIP) at 0.5 µg/mL or 50 µg/mL.

	Lytic area		
	Control	CIP 0.5µg/mL	CIP 50µg/mL
H0	0.049 +/- 0.001	0.048 +/- 0.001	0.051+/- 0.002
H2	0.062 +/- 0.002	0.098 +/- 0.005	0.087 +/- 0.003
H4	0.082 +/- 0.002	0.126 +/- 0.006	0.112 +/- 0.004
H6	0.104 +/- 0.003	0.151 +/- 0.005	0.129 +/- 0.005
H24	0.132 +/- 0.002	0.159 +/- 0.005	0.143 +/- 0.005

At H0, the lytic area of control vs. CIP 0.5 µg/mL was p=0.742 and vs. CIP 50 µg/mL, p=0.176

For differences at H2, H4, H6, H24, see Figure 1.

Figure 1. Comparative lytic areas between controls (mock-treated) and cells treated with ciprofloxacin at 0.5 µg/mL and 50 µg/mL at H2, H4, H6 and H24.

* p<0.0001

**p=0.035

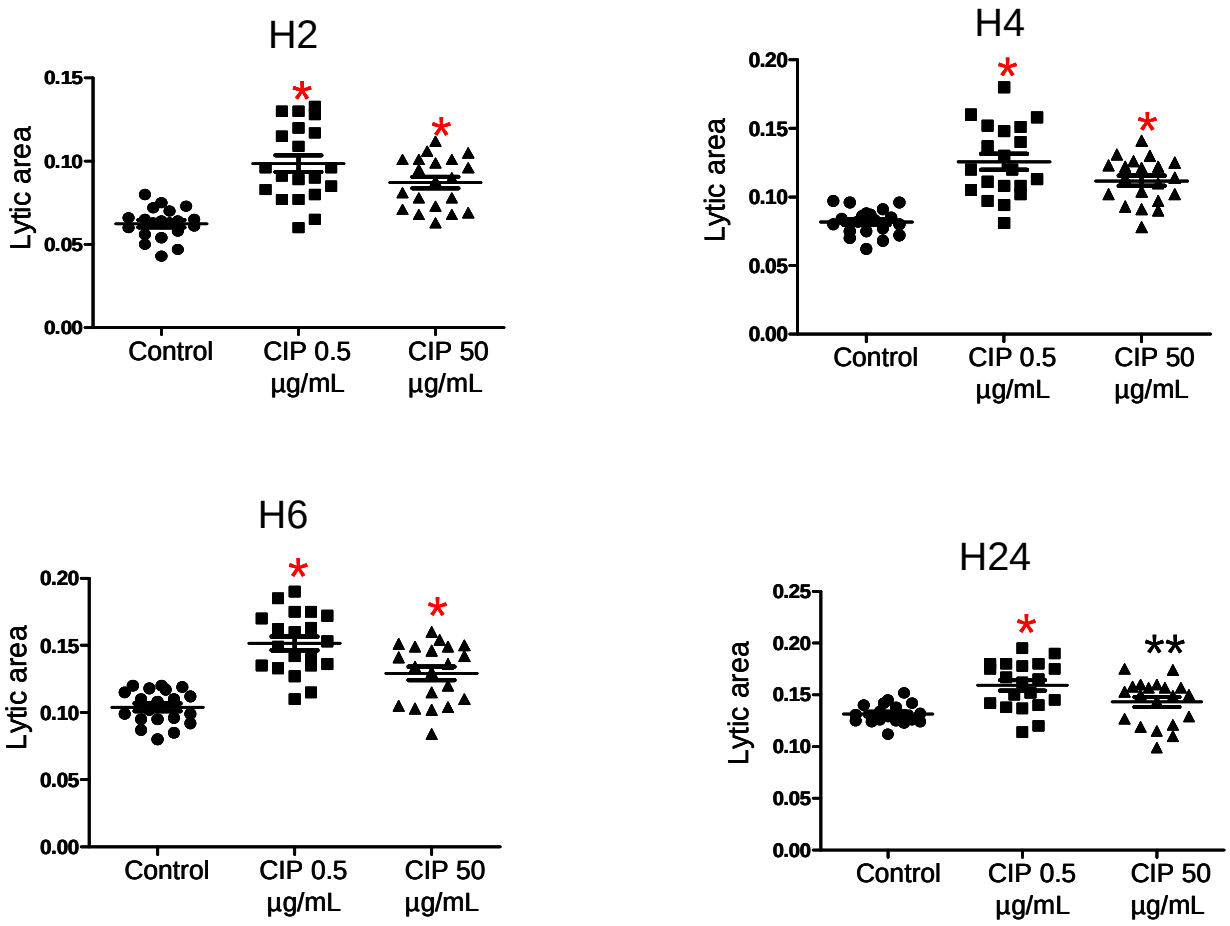
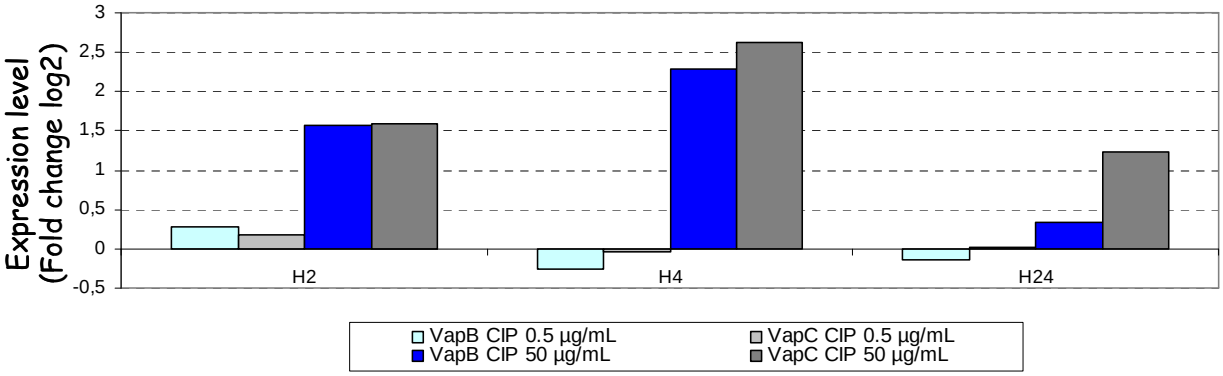


Figure 2: Expression levels of *VapB* and *VapC* of *Rickettsia conorii* compared to respective time controls and normalized to *GroEL*.



Fold change is expressed in Log2.

CIP= ciprofloxacin

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ARTICLE 3

Statins limit *Rickettsia conorii* infection in cells

E. Botelho-Nevers, J.M Rolain, L. Espinosa and D. Raoult

International Journal of Antimicrobial Agents

Volume 32, Issue 4, Octobre 2008, pages 344–348

URMITE UMR 6236, CNRS-IRD, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille

cedex 05, France

Contribution de EBN: développement du concept d'analyse des plages de lyse par acquisition numérique, réalisation de toutes les expérimentations, analyses statistiques des données, écriture du manuscrit sous la direction de JMR.

Commentaire

Des données récentes et nombreuses suggèrent que les statines pourraient avoir un effet bénéfique lors d'un sepsis. Nous avons testé l'effet de la lovastatine et la pravastatine sur un modèle d'infection cellulaire à *Rickettsia conorii* en utilisant la méthode des plages de lyse couplée pour la 1ère fois à un algorithme d'analyse d'image permettant d'avoir une analyse objective et quantitative de ces données.

Ainsi, nous avons observé que les statines ajoutées de façon concomitante à l'infection ne modifiaient pas la formation des plages de lyse, alors que les cellules qui étaient pré-incubées avec des statines pendant 48 h avant l'infection avaient une réduction significative de 30% à 68% du nombre de plages de lyse par rapport au témoin, fonction des composés testés et des concentrations. Ces résultats préliminaires soulèvent l'hypothèse que les statines peuvent avoir une action prophylactique et/ou moduler l'infection par *R. conorii* chez les personnes exposées.

Depuis ce travail nous avons cherché au cours de l'étude clinique (article 1) un impact des statines sur le pronostic de la FBM. Malheureusement le nombre de patients traités préalablement par statines était trop faible (13 au total) pour pouvoir espérer observer un quelconque effet ou non sur la protection de la forme sévère de la maladie.

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International Journal of Antimicrobial Agents 32 (2008) 344–348

INTERNATIONAL JOURNAL OF Antimicrobial Agents

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Short communication

Statins limit *Rickettsia conorii* infection in cells

E. Botelho-Nevers, J.M. Rolain, L. Espinosa, D. Raoult *

Unité des Rickettsies, URMITE CNRS UMR 6236, IRD 198, Marseille, France

Received 25 April 2008; accepted 28 April 2008

Abstract

Recent data suggest that statins may have a beneficial effect during sepsis. In this study, we tested the effect of lovastatin and pravastatin on the cellular culture of *Rickettsia conorii* using a quantitative plaque assay model associated with an original image analysis algorithm. Statins added at the time of infection did not modify plaque formation, whereas pre-incubation with statins for 48 h resulted in a significant 30–68% plaque reduction, depending on the tested compounds and doses. These preliminary findings raise the hypothesis that statins may prevent or moderate rickettsial disease in exposed people.

Keywords: Statins; Prevention; Rickettsioses; *Rickettsia conorii*

1. Introduction

Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase, which reduces the biosynthesis of cholesterol and also many non-steroidal isoprenoid compounds [1], resulting in pleiotropic effects [2]. The anti-inflammatory actions of statins are now recognised to play a role on multiple levels, including cell signalling and expression of various cytokines [2]. Statins may reduce the overall magnitude of the systemic response and modulate the host response to sepsis [2]. Cholesterol present in cell membranes is a critical molecule, affecting interactions of microorganisms with mammalian cells, and this cholesterol may be lowered by statin treatment [3]. Microorganisms such as *Rickettsia conorii* [4,5] target compounds in cholesterol-rich lipid rafts as cellular receptors [3], therefore lowering cholesterol might limit infection by these bacteria. To test this hypothesis, we have studied the effect of lovastatin and pravastatin on the cellular culture of *R. conorii*. This bacterium is a strict intracellular pathogen responsible for Mediterranean spotted fever (MSF). In 5–10% of cases MSF occurs as a malignant form, leading to death in one-half of those cases. To show the potential inhibitory effect of statins on the cellular culture of *R. conorii*, we used a plaque assay (previously described as a reference method to study sensitivity to antibiotics [6]) as well as real-time polymerase chain reaction (PCR) [6]. We designed an original digitalised analysis to reach sensitivity compatible with the activity level of statins.

2. Materials and methods

Lovastatin and pravastatin were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France) [7]. Lovastatin was hydrolysed as described elsewhere [8] and a stock solution (1 g/L) was stored at −20 °C until use. Hydrolysation was not required for pravastatin, which was stored as described (stock solution 2 g/L). Doxycycline was purchased from Sigma–Aldrich and stored as described above.

Rickettsia conorii (Malish strain, ATCC VR-613) was cultured in L929 cells (murine fibroblasts) as previously described [7]. Titration of bacteria was done as described elsewhere [9] and aliquots calibrated at 4×10^5 plaque-forming unit (PFU)/mL were stored at −80 °C until infection.

L929 cells were maintained in minimal essential medium (MEM) (Invitrogen, Paisley, UK) supplemented with 4% foetal calf serum (FCS) and 2 mM L-glutamine. The plaque assay was performed as described elsewhere [6]. L929 cell

* Corresponding author. Present address: Université de la Méditerranée, Faculté de Médecine, 27 Blvd Jean Moulin, 13005 Marseille, France. Tel.: +33 4 91 38 55 17; fax: +33 4 91 38 77 72.
E-mail address: didier.raoult@gmail.com (D. Raoult).

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doi:10.1016/j.ijantimicag.2008.04.027

monolayers were seeded 24 h before use in 60-mm Petri dishes (Falcon; Becton Dickinson, Cowley, UK) and were then incubated for 48 h with 200 μL of a non-cytotoxic final concentration of lovastatin (0.04 mg/L, 0.2 mg/L or 0.4 mg/L) or pravastatin (0.4 mg/L or 4 mg/L) or with statin-free medium as a control group. At Day 3, the dishes were infected with 1 mL of a solution containing 4×10^3 PFU/mL. After 1 h of incubation at 37 °C in 5% CO_2, the inoculum was removed and the dishes were overlaid with 4.8 mL of a medium containing MEM, 4% FCS, 1% L-glutamine and 0.5% agar. Statin or doxycycline (1 mg/L) solutions were added to the medium. Statin- and antibiotic-free dishes were also included and served as positive controls. All conditions were assayed in triplicate at least. The dishes were incubated for 8 days at 37 °C in a 5% CO_2 incubator. Colouration with crystal violet and visualisation were as previously reported [6].

An image of dry dishes was acquired with a photo scanner (ScanJet G 3670, 400 dpi; Hewlett-Packard, Issy-les-Moulineaux, France). Obtained images were analysed with ImageJ software (US National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/). Detection of lytic plaques was performed using the 'local threshold' algorithm (R. Couture 11-14-03, Department of Radiology, Washington University School of Medicine (couture@wuerl.wustl.edu), based on macro by Gabriel Landini (G.Landini@bham.ac.uk), published in the ImageJ NIH ListServer archives available at http://list.nih.gov/cgi-bin/wa?A2=ind0311&L=IMAGEJ&P=R4199&I=(3). Images were analysed with the macro command as follows: RGB (Red, Green, Blue) split; only the green component was kept; a local threshold was applied with the median filter radius set to seven; an ellipse region of interest (ROI) was set to fit the dish shape; a threshold was applied with fixed values. The area of detected particles was measured after elimination of noise and a 'morphological open' operation. To isolate the maximum lytic plaques, the morphological 'open' operation was applied on a mask generated by the Analyze particles command. First, Analyze gives the total area of lytic plaque and the ratio with respect to the ROI area; this is the quantification parameter noted as lytic area fraction (LAF). Our study was based on the variations of

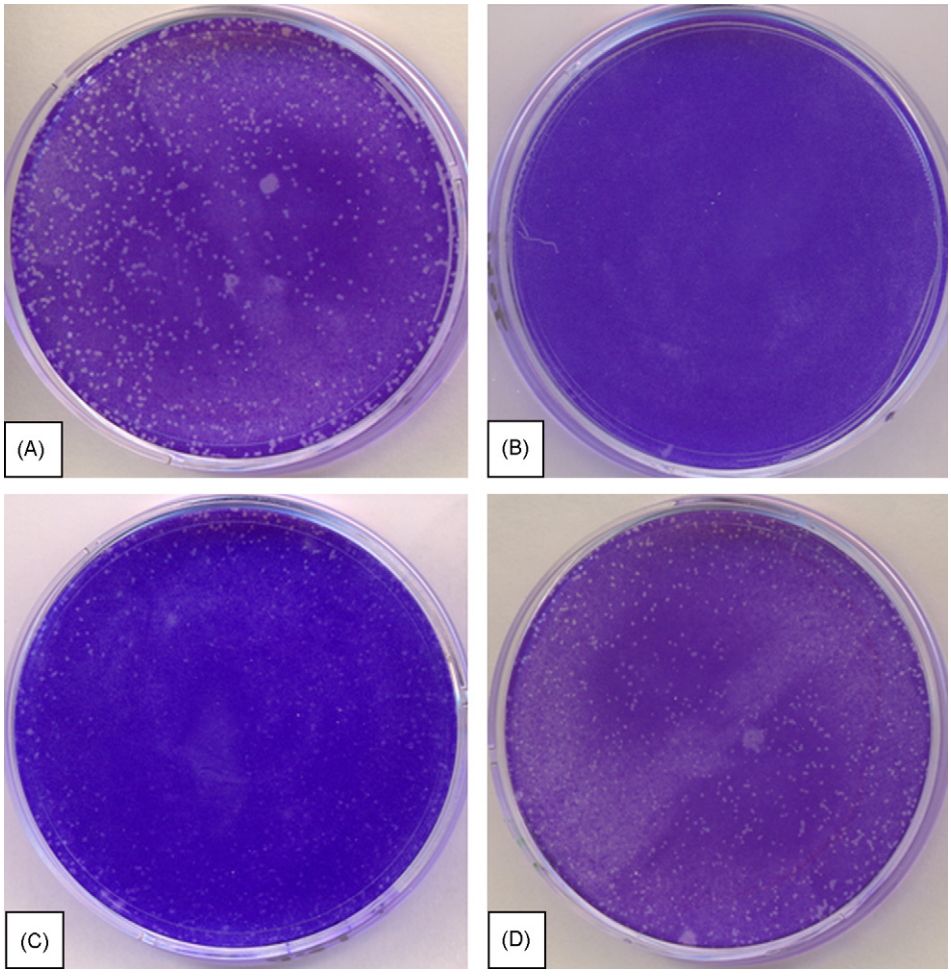

Fig. 1. Plaque assay at 8 days post infection: (A) control group; (B) cells treated with doxycycline at 1 mg/L; (C) cells treated with lovastatin at 0.4 mg/L; and (D) cells treated with pravastatin at 4 mg/L.

LAF in different conditions of infection. The mean LAF of the control group was compared with that of the lovastatin or pravastatin groups. Statins were classified as effective in reducing infection if the mean LAF of the statin group was significantly reduced compared with that of the control group. The mean average size between the control group and the statin groups was also compared. The same analysis was performed with dishes in which cells were not treated with statins prior to infection to test whether pre-incubation was necessary to achieve an effect.

To evaluate bacterial growth in the different conditions, a quantitative real-time PCR was used with primers and TaqMan* probe that targeted a portion of the rickettsial *gltA* gene. Bacterial growth in the control group (Days 0–4) was compared with growth of the 0.04 mg/L and 0.4 mg/L lovastatin groups and the doxycycline group.

Statistical analysis was performed using Epi Info software version 3.4.1 (CDC, Atlanta, GA) to compare mean values. A difference was considered statistically significant at $P < 0.05$.

3. Results

Quantitative real-time PCR with the TaqMan* probe failed to show a significant effect of 0.04 mg/L or 0.4 mg/L lovastatin on cells treated prior to infection compared with the control ($P = 0.732$). Indeed, when cells were treated with statins before infection, 0.4 mg/L lovastatin reduced bacterial growth by 13% compared with controls ($P = 0.827$), whereas 0.04 mg/L lovastatin induced a 41% increase in growth compared with controls ($P = 0.564$). Using a plaque assay, it was visually determined that statins induced slightly fewer lytic plaques that were smaller in size than the control group (Fig. 1). To showcase this weak difference, we have improved the reference plaque assay method to increase its sensitivity. The classic plaque assay, as well as quantitative real-time PCR or the dye-uptake assay, are well adapted to test variations of a factor of ten, but not lower. This is enough for testing antibiotic inhibition on *Rickettsia* [6] but it failed to show any significant difference in *R. conorii* infection in cells treated with statins compared with the control (data not shown). Indeed, the classic plaque assay was not sufficiently sensitive as it usually tests bacterial antibiotic susceptibility showing differences of the order of 90%. Because it was time consuming and labour intensive to count the number of lytic plaques or the diameter of the plaques (see Fig. 1), we coupled the classical plaque assay with software analysis to achieve an objective count of plaques, which allowed us to show the efficiency of statins in reducing infection due to *R. conorii*. Moreover, this method was easily reproducible and showed differences of the order of 30%. Using this modified method, it was found that among cells not incubated with statins prior to infection, only lovastatin at 0.4 mg/L induced a slightly significant reduction of LAF compared with the control ($P = 0.03$; see Table 1). There was no effect on average plaque size. Pravastatin failed to show any effect

Table 1
Data obtained with ImageJ software analysis using the 'local threshold' algorithm

Data	Control		Lovastatin				Pravastatin			Doxycycline
	4×10^3 (PFU/mL) ($n = 11$)	4×10^2 (PFU/mL) ($n = 5$)	0.04 μg/mL I[a] ($n = 6$)	0.2 μg/mL I ($n = 5$)	0.4 μg/mL I ($n = 13$)	0.4 μg/mL NI[b] ($n = 5$)	0.4 μg/mL I ($n = 6$)	4 μg/mL I ($n = 5$)	4 μg/mL NI ($n = 5$)	(1 μg/mL) ($n = 5$)
Mean of LAF	3.3	0.46*	2.28	1.58[c]	1.06*	1.58[d]	2.62	1.94[c]	2.72[d]	0.06
% reduction compared with control (4×10^3 PFU/mL)	–	−86	−30	−64	−68	−33	−20	−56	+15	−98
P-value (comparison with control, 4×10^3 PFU/mL)	–	–	0.23	0.003**	0.008**	0.03	0.45	0.0088	0.75	0.0012
Mean of average plaque size (pixel^2)	29.9	24.3	36	19.8	22.4	28.7[e]	34.1	21.1	42[e]	14.9
P-value (comparison with control, 4×10^3 PFU/mL)	–	–	0.02	0.0006[f]	0.0001	0.09	0.1	0.009[f]	0.32	0.0001

PFU, plaque-forming units; LAF, lytic area fraction.

* lovastatin at 0.4 mg/L vs. control 4×10^2 PFU/mL ($P = 0.07$).

** lovastatin at 0.4 mg/L vs. lovastatin at 0.2 mg/L ($P = 0.0084$); concentration effect.

[a] I indicates that lovastatin or pravastatin was added to the cellular culture before infection (incubation).

[b] NI indicates that statins were not added before infection (not incubated).

[c] For each condition, results of statins were matched with the control of the same experimentation: these two conditions have been compared with mean LAF of control = 4.4.

[d] When statins were not added before infection, statin groups were compared with LAF of control = 2.36.

[e] When statins were not added before infection, statin groups were compare with average size of control = 33.4.

[f] A match has been done for the average size compared with those of their control = 25.75.

on LAF or plaque size (Table 1). When cells were treated with statins prior to infection, lovastatin at 0.2 mg/L and 0.4 mg/L induced a significant reduction in LAF compared with the control (−64% ($P = 0.003$) and −68% ($P = 0.008$), respectively) (Table 1) in a dose-dependent manner. lovastatin at a lower concentration (0.04 mg/L) did not cause a reduction in infection (Table 1). However, lovastatin at 0.2 mg/L and 0.4 mg/L did significantly reduce the average plaque size compared with the control (−33.8% and −25.1%, respectively). In the same way, for cells incubated with pravastatin prior to infection, only the concentration of 4 mg/L was effective at significantly reducing LAF and average plaque size compared with the control (−56% ($P = 0.0088$) and −29.4% ($P = 0.009$), respectively) (Table 1). The lower concentration of pravastatin (0.4 mg/L) was not efficient (Table 1). Doxycycline was significantly more effective at reducing infection than statins. It reduced infection by the order of 3 log, whereas the most efficient concentration of lovastatin reduced infection by the order of 1 log.

4. Discussion

In this study, we have demonstrated for the first time that statins significantly limit infection due to *R. conorii*. We showed that lovastatin was effective at concentrations achievable in sera of patients [1]. Pravastatin reduced infection at a concentration 10 times greater than those achievable in patients [1]. Reduction in LAF by lovastatin and pravastatin could result from a reduction in the infection rate. Statins also reduce the plaque size, which may result from decreased cell toxicity induced by *R. conorii* [10].

To the best of our knowledge, we show for the first time an anti-infectious effect of statins on rickettsial species. Such effects of statins have been previously reported in vitro in other intracellular bacteria including *Salmonella enterica* serovar Typhimurium [11] and *Coxiella burnetii* [12]. In these models, statins induced a reduction of infection at the same amplitude. Moreover, statins have been tested on viruses and zygomycetes and inhibited their growth [2]. Other inhibitors of cholesterol, such as progesterone, have also been reported as a growth inhibitor of *C. burnetii* [12].

To explain the observed effect, we hypothesise that statins could have an effect on bacteria or an effect on host cells. However, since the steroid biosynthesis pathway is not found in the genome of *R. conorii*, we believe that statins do not directly target bacteria (http://www.genome.jp/kegg/pathway/map/map00100.html). Moreover, we showed that to be effective, statins should be incubated with cells before infection. We hypothesised that statins limit the entry of bacteria in cells and have an indirect effect on bacteria. Indeed, entry of *R. conorii* into cells is cholesterol-dependent, with protein KU70 known as its cellular receptor [5]. Moreover, Cdc42, a prenylated protein, is also necessary for *R. conorii* cell entry [4]. By inhibiting cholesterol and protein isoprenylation, statins may limit this step in infection and reduce infection by targeting the host. Moreover, statins may also have an effect on host cells by reducing the plaque size. Statins may impact the polymerisation of actin tails, as Cdc42 is involved in this function [4], and reduce cell-to-cell spread of bacteria. Finally, rickettsiae cytopathic effects are related to oxidative injury [13], which could be limited by the anti-oxidative stress activity of statins [2]. However, these hypotheses need to be confirmed.

The possible preventive action of statins could be confirmed using an animal model [14]. Epidemiological studies on rickettsial diseases (notably malignant forms) comparing untreated patients with patients treated with statins may reveal a protective effect of statins. If this is confirmed, a prospective study should be performed to improve the prognosis of malignant rickettsial diseases. Moreover, statins should be tested on other rickettsioses as their inhibitory effect has also been shown on *C. burnetii* [12].

In conclusion, we found a significant protective effect of some statins on rickettsial infection in vitro. These preliminary findings correlate well with data recorded for other pathogens and raise the hypothesis that statins may help to prevent severe infection in exposed people.

Acknowledgment

The authors thank Guy Vestris for technical help.

Funding: Supported by the Centre National de la Recherche Scientifique, France.

Competing interests: None declared.

Ethical approval: Not required.

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ARTICLE 4

Intralesional immune response in Mediterranean spotted fever

Botelho-Nevers E[1], Ben Amara A[1], El Filali A[1], Mouffok N[2], Boudebouch N[3], Merhaj V[1], Raoult D[1], Mège J.L[1].

En préparation

[1]URMITE UMR 6236, CNRS-IRD, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille cedex 05, France
[2] Service de Maladies Infectieuses, Centre Hospitalier Universitaire d'Oran, Algeria
[3] Laboratoire des Maladies Vectorielles, Institut Pasteur du Maroc, Casablanca, Morocco

Contribution de EBN: extraction des ARNm des biopsies d'escarre, aide à la réalisation du microarray, réalisation de toutes les RT-PCR de confirmation, analyse partielle des données, écriture du manuscrit sous la direction de JLM.

Commentaire

L'objectif de ce travail était d'étudier la réponse de l'hôte au sein même de l'escarre d'inoculation, qui représente la porte d'entrée de *R. conorii* subsp *conorii* et le site du déclenchement de la réponse de l'hôte (28). Pour cela, nous avons utilisé une approche de transcriptomique pan-génome humain. Ce type d'approche puissante permet d'obtenir une signature transcriptomique globale. Elle permet donc d'analyser la réponse induite chez l'hôte par la bactérie sans à priori, sans avoir une hypothèse précise, ce qui n'est le cas lorsque la réponse est étudiée de façon sélective (immuno-histo-chimie (39), RT-PCR (29)…).

Nous avons donc inclus 19 cas de FBM, pris en charge au Maroc et en Algérie, chez lesquels une escarre d'inoculation a été biospiée. Parmi ces 19 escarres, 3 étaient issues de patients ayant des formes sévères de FBM (souffrant de syndrome de défaillance multi-viscérale). Nous avons comparé le profil d'expression au sein de ces escarres à celui exprimé au sein de biopsies de peau saine. Après avoir démontré qu'il existait une reproductibilité inter et intra-individus, nous avons montré que la signature escarre était représentée par la modulation de 698 gènes (niveau d'expression ou «fold change» (FC) >3 et $p<0.01$). Parmi ces gènes, 494 étaient surexprimés (FC entre 3 et 399), et 204 étaient sous-exprimés par rapport aux témoins (FC entre 0,023 et 0,25). Les 698 gènes différentiellement modulés étaient classés en 11 groupes selon leur appartenance à un même processus biologique (annotation Gene Ontology (GO)). Le groupe le plus important était représenté par 219 gènes surexprimés et étant impliqués dans la réponse aux stimuli, c'est-à-dire la réponse immune et inflammatoire notamment. Le second groupe était composé de 127 gènes surexprimés et impliqués dans la signalisation. Le troisième groupe incluait 70 gènes sous-exprimés et impliqués dans la régulation biologique.

Nous avons également recherché une réponse spécifique de l'hôte au sein des escarres issues de forme sévère de FBM. Ainsi 102 gènes étaient modulés, dont 97 gènes étaient surexprimés

et 5 gènes étaient sous-exprimés spécifiquement dans le groupe des escarres issues de formes sévères. Nous avons observé que certains gènes tels que CCL18, S100A9, ICAM1 étaient différentiellement exprimés dans les escarres issues de formes sévères de FBM par rapport aux escarres issues de formes non sévères. La confirmation de l'expression d'une dizaine de gènes a été faite par reverse-transcriptase PCR en temps réel.

L'approche transcriptomique nous a permis de vérifier la surexpression de certains marqueurs immuns ayant déjà été décrits dans la littérature au cours de la FBM tels que l'interféron-γ, le «tumor necrosis factor», l'interleukine 10, CCL-5 ou IDO1 (29). En outre, cette approche nous a permis de mettre en évidence que certaines molécules pro-inflammatoires comme S100A9, étaient exprimées de façon plus importante au sein des escarres issues de formes non sévères de FBM par rapport aux escarres issues de formes sévères. Elle a également mis en avant certains marqueurs immuns tel que CCL-18, qui semble être davantage exprimé dans les escarres issues de formes sévères et n'a jamais été exploré à ce jour dans la FBM, il s'agit pourtant d'un attractant de lymphocytes T, cellules prédominantes dans les escarres (39). Il serait intéressant d'évaluer ces molécules à visée pronostique chez les patients souffrant de FBM. La meilleure compréhension des mécanismes conduisant ou non à la forme sévère de la FBM pourrait à terme permettre un ciblage des molécules clés à des fins thérapeutiques et/ou prophylactiques.

Abstract

The host response within the eschar of inoculation during Mediterranean spotted fever (MSF) has been poorly studied. Our objective was to evaluate the host response by comparing transcriptional profiles of eschars to controls using a whole-genome microarray. Hierarchical clustering revealed a signature of eschars consisting of 698 genes. The genes included in this signature were mainly up-regulated and were predominantly associated with immune response and signalling. New molecules have also been found up-regulated in eschars such as the proinflammatory S100A9, and the T cell attracting CCL-18. Genes down-regulated were mainly associated with biological regulation. We also observed that eschars from severe cases of MSF displayed a specific signature with notably difference in degree of modulation compared to eschars from non severe MSF cases. Some parameters identified in this work should be tested as biomarkers for prognostic assessment in future studies.

Introduction

Mediterranean spotted fever (MSF) is an acute rickettsiosis due to *R. conorii conorii* characterized by the association of an eschar of inoculation also called "tâche noire", fever and a maculo-papular rash (1). Inoculation of *R. conorii* to human beings by the bite of *Rhipicephalus sanguineus* leads to the invasion and multiplication of the bacteria in the endothelial cells of the small vessels, leading to a vasculitis and lesions at the site of tick bite (2;3). The "tâche noire" is then the result of a focus of epidermal and dermal necrosis due to severe injury of the cutaneous small vessels (2). Recently we reported that 72.7 % of patients with MSF have an eschar at the site of tick-bite and 16% of the cases were severe suffering of multiple-organ dysfunction syndrome or death (4). The increase in the severity rate observed since the first description of the disease in 1910 raised concern and suggests the involvement of host response in the MSF prognosis.

The eschar has long been considered as an excellent and accessible model for the study of the interaction host-pathogen in MSF, including the pathogenic mechanisms leading to necrosis and the immune mechanisms resulting in killing bacteria. The lesions observed in eschars consist in lymphohistiocytic vasculitis associated with dermal edema and in some cases with necrosis but only occasionally with nonocclusive thrombosis (2;3). Immunohistochemical analysis of the tâche noire suggests the importance of T cell-mediated immunity in local immune response to MSF (3). More recently De Sousa *et al*., studied the expression mRNA of some immune and inflammatory molecules within eschars (5). They observed increased levels of intralesional expression of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-10 and indoleamine-2,3-dioxugenase (IDO) in eschars compared to control subjects (safe skin) (5). A positive correlation between TNF-α, IFN-γ, inducible nitric oxide synthase (INOS), IDO and mild MSF have been also observed suggesting that type 1 polarization plays a protective role in MSF (5). More recently the pathogen transcriptional response has been

41 studied in the inoculation skin lesion using a microarray approach (6). Interestingly this study
42 showed that the modulated genes were mostly down-regulated and encoded proteins essential
43 for bacterial replication, whereas several of up-regulated genes seem to be involved in escape to
44 the host response (6). This global transcriptomic approach have been recently described as very
45 helpful and powerful to increase our knowledge about host-pathogen interactions notably for
46 obligate intracellular bacteria (7-9) and in patients with infectious diseases (10).
47 We therefore performed a study about the host response within the eschar of inoculation
48 obtained from patients suffering from MSF using a microarray approach. This approach
49 enabled us to describe a unique transcriptional response characteristic of “tâche noire”. In
50 addition, we identified a transcriptional signature associated with patients with severe form of
51 MSF.

Patients and Methods

Inclusion of patients

Patients included in this study were recruited at the Infectious Diseases Department of Oran, Algeria or at the Infectious Diseases Division of the Ibn Rochd Casablanca Teaching Hospital and at the Dermatology Department of Militar Hospital Mohamed V of Rabat, Morocco. All patients presented with clinical characteristics of MSF were diagnosed using serology as described elsewhere (4) and/or confirmed by positive PCR assay with specific primers for *R. conorii* subsp. *conorii* of the blood or the skin biopsy of the eschar (4). Severe cases were defined as having multiple organ deficiency systems syndrome (MODS) or death as previously described (4).

Control skin-biopsy samples were obtained from discarded skin tissues from patients admitted for plastic surgery.

All patients were volunteers and the study has been approved by the Ethic committee of Federative Research Institute 48, Mediterranée University, Marseille and included in regional "Programme Hospitalier de Recherche Clinique" UF 8046.

RNA extraction and microarray procedure

RNAs were extracted from skin biopsies using RNeasy Mini Kit (Qiagen) with a DNase I step to eliminate DNA contaminants and according to the manufacturing's recommendations as described previously (11). The quantity and the quality of RNAs were assessed using Nanodrop (Thermo Science) and 2100 Bioanalyseur (Agilent Technologies), respectively. The microarray study was performed using the technology provided by Agilent Technologies including microarrays chips including 45,000 probes (4X44K Whole Human Genome and One Color Microarray Based Gene Expression Analysis). In brief, 400 ng of RNA were labeled with cyanine-3 CTP using low RNA Input Fluorescent Amplification kit. Samples were deposited on microarray slides and the hybridization was carried out for 17 hours at 65°C using the QIamp

77 labeling kit. The microarray slides were scanned with a pixel size of 5 µm with the DNA
78 Microarray scanner G2505 B. The scanned images were analyzed with Feature Extraction
79 Software 10.5.1. The data processing and analyses were carried out using R software.
80 *Microarray analysis*
81 Microarray data were analyzed using Significance Analysis of Microarrays that gives a score
82 that represents the statistical significance for each gene and analyzed using R software, version
83 2.11.1 and Principal Component Analysis (PCA), as recently described (9). The modulated
84 biological processes were identified through an analysis of Gene Ontology (GO) and Kyoto
85 Encyclopedia of Genes and Genomes (KEGG) terms using GO stats package to test GO and
86 KEGG terms that were over- or under-represented. The data were generated in compliance with
87 the Minimum Information About a Microarray Experiment guidelines and were deposited in the
88 National Center for Biotechnology Information's Gene Expression Omnibus (accession number
89 XX). Finally, network pathways of differentially expressed genes were drawn by graph package
90 (http://www.bioconductor.org/packages/2.4/bioc/html/KEGGgraph.html) according KEGG.
91 Differentially expressed gene sets consisted of genes matching for an absolute fold change (FC)
92 >3 and a P-value <0.01 as determined by 2-tailed Students *t* test.
93 *Real-time RT-PCR*
94 Real-time quantitative reverse transcriptase PCR (qRT-PCR) was carried out as recently
95 described (9). In brief, reverse transcription of 150 ng of RNA was performed with the M-
96 MLV-RT kit (Invitrogen). cDNA was obtained using oligo(dT) primers and M-MLV reverse
97 transcriptase (Invitrogen) and qPCR experiments were performed using SYBR Green Fast
98 Master Mix (Applied biosystems) and a ABI PRISM® 7900HT Sequence Detection System
99 (Applied biosystems). The gene sequences were obtained using the database SOURCE
100 (http://smd.stanford.edu/cgi-bin/source/sourceSearch). The primers were designed using
101 Primer3 (http://frodo.wi.mit.edu/primer3), their sequences are provided in Table 1. Results

were normalized using the housekeeping gene β-actin and are expressed as fold change (FC) = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (CtTarget - CtActin)_{condition} - (CtTarget – CtActin)_{control}$. Genes having a FC > 1.5 were considered up-regulated whereas genes having a FC< 0.5 were considered down-regulated.

Results

Study population and Clinical data.

Nineteen patients were included in the study. Seven patients were enrolled in Algeria and the remaining twelve were enrolled in Morocco. Their mean age was 39.5 +/- 13.3 years (range 20-58 years). Sex ratio was 2 males for 1 female. Clinical presentation was high grade fever, maculopapular rash and eschar in all the patients. Rash was purpuric in 4 cases. Laboratory findings were known in 9 cases, and showed low grade platelets in 5 cases and increase level of transaminases in 7 cases of them. Clinical diagnosis of MSF was confirmed by positive PCR for *R. conorii* in the biopsy skin of the eschar in 11 cases and by serology in 9 cases. Three patients have a severe form of MSF with at least 2 organ systems dysfunctions (neurological and respiratory involvement). No death was observed.

An illustrative histological section of eschar from a patient suffering of MSF was described in Figure 1.

Transcriptional study of eschars.

Using a whole genome approach, we investigated the transcriptional profiles of eschars of inoculation from 19 patients with MSF and 6 controls represented by safe skin biopsies. We observed that 9734 genes were modulated in the eschars compared to the controls. Using principal component analysis, we showed that the 2 tested groups (eschars and controls) were well separately clustered. Furthermore good reproductibility inter individuals of each group and intra-individual for the control group, were observed, see Figure 2.

Using a p-value <0.01 and an absolute fold change >3.0 the eschar signature consisted of 698 genes, 494 being up-regulated with fold change (FC) ranging from 3.0 to 399.1 (expressed in log2 = 1.59 to 8.64) and 204 genes down-regulated with FC from 0.023 to 0.25 (expressed in log2 = -5.48 to -2.0).

The hierarchical clustering analysis of the 300 genes with the higher variance showed that the transcriptional profiles of patients were organized in 2 clusters (Figure 3). One branch consisted of controls and 4 patients whereas the other branch consisted of all other patients.

The analysis of the 698 eschar-associated genes revealed a specific profile that could be organized in 11 clusters using Gene Ontology (GO) terms (Figure 4). Three clusters were predominant of which, two were up-regulated and one was down-regulated. The other less representative clusters are described in Figure 4. The most enriched GO term was related to 'response to stimulus' and included 219 up-regulated genes in the eschars. This biological function is related to immune response, inflammatory response and response to wounding but also to induction of apoptosis. The second most enriched GO term was 'signaling' that included 127 up-regulated genes also included in neighbour GO terms such as cell-cell signalling or signal transduction. The third cluster contained 70 genes down-regulated in eschars and showed enrichment for genes involved in 'biological regulation'.

Furthermore, we analyzed the pathways involved in the eschar signature. The building of network based on gene interactions revealed a dense network in which some clusters were prominent. Hence, chemokines and chemokine receptors were over-represented around JAK3; most of them were upregulated. In addition, Wnt (wingless-type MMTV integration site family) pathway constituted a dense subnetwork in which Wnt5A and Wnt6 were upregulated whereas the other members were downregulated (see Figure 5).

Some modulated genes were also confirmed using real time RT-PCR (see Table 2).

Response of the host within eschars from patients with severe MSF. To better characterize response within eschars from severe cases of MSF, we analysed genes that were only found modulated in eschars from severe cases of MSF compared to controls. We found 102 modulated genes. They consisted of 97 up-regulated genes (FC>3) only within the eschars from severe cases of MSF. Only 84 genes had annotation with GO terms. These genes showed

enrichment for Go terms of ‘primary metabolic process’ (n=27 genes) and ‘immune response’ (n=17 genes). The other biological processes involved are described in Figure 6. We also observed that only 5 genes were down-regulated using a FC <0.25, for details see Figure 6. Using real time RT-PCR, we observed that some common modulated genes have different degree of modulation between eschars from severe and non severe MSF cases (Table 2). Indeed, the gene encoding CCL-18 (chemokine ligand 18) was more up-regulated in severe cases and the gene encoding S100A9 (S100 calcium-binding protein A9) was less up-regulated in severe cases. Concerning the specific genes, surprisingly using real RT-PCR, we observed that some genes were also modulated in eschars from non severe MSF cases. They included the genes encoding FLG (epidermal filigrin), ICAM-1 (intercellular adhesion molecule 1) and IL-17 RE (Interleukin 17 receptor E) (Table 2). The gene-encoding IL-22RA1 (Interleukin 22 receptor α 1) was found to be down-regulated only in eschars from severe MSF cases, see Table 2.

DISCUSSION

The aim of this study was to explore the response of the host within the eschar of inoculation in Mediterranean spotted fever, using a powerful technology, whole genome microarray, in order to identify new genes implicated in the response beyond those reported in literature. To our knowledge, this approach had never been used before to investigate the local host response in MSF.

We found that infection by *R. conorii* induced a specific signature with mainly up-regulation of genes implicated in immune and inflammatory response whereas the down regulated genes are involved in biological regulation. This finding was correlated to the first pathological evaluations performed in the eschars and showing a perivascular infiltrate of lymphocytes and mononuclear cells (2) with an important role of T cell-mediated immunity (3). Indeed, we found that CXCL-9 and CXCL-10, T-cell targeting chemokines were up-regulated in eschars. Valbuena has demonstrated that these chemokines play a role early during the immune response against rickettsial infections with a peak of expression occurring 4 days before CD8+ T cells infiltrated the infected tissues (12). More recently, De Sousa team have studied in MSF the response of the host within the eschar of inoculation using qRT- PCR and testing a selected panel of immune effectors (5). As in the present study, they observed that IFN-γ, IL-10, RANTES and IDO were higher in patients than in controls (5). Numerous systemic immune effectors have already been studied in human or animal studies of *R. conorii* infection. Some of these molecules have been also involved in the local response of the host to *R. conorii* at the inoculation site. Therefore we confirmed that CCL-2 (also called MCP-1), that has been found to be higher in MSF patients than in patients suffering from African tick-bite fever (ATBF), a less pathogenic rickettsiosis due to *Rickettsia africae,* (13) was also up-regulated in eschars. Interleukin 8, a pro-inflammatory cytokine, that has been found in higher

192 levels in MSF than in ATBF patients (13) had also an up-regulation of its gene in our study
193 within eschars.
194 We did not found the expression of some cytokines such as IL-12p and IL-17 within the eschar
195 of inoculation whereas high levels of these molecules have been recently found in sera of mice
196 that survived to challenge with *R. conorii* infection (14). The release of the proinflammatory
197 cytokine IL-6 have been found increased in plasma of MSF patients compared to healthy
198 controls and also in severe case of MSF compared to non severe cases (however difference was
199 not significant between severe and non severe patients) (15). In our study, RT-PCR did not
200 confirm any association between the expression of IL6 and the outcome of MSF.
201 For the first time the transcriptomic profile associated with severity of MSF was also
202 studied. The eschars associated with severe forms of MSF induced a specific signature, which
203 is different, notably in intensity, of the signature observed in eschars associated with non-severe
204 form of MSF. Indeed for the first time, we observed that CCL-18, a T-cell attracting
205 chemokine, inducing a M2 polarization of macrophages (10), was relatively more up-regulated
206 in eschars from severe form of MSF than in eschars from non severe patients. This chemokine
207 may participate in severe form of the disease by reducing microbicidal activity of the host and
208 subsequently persistence of bacteria leading to severity. We also observed that the
209 inflammatory mediator S100A9 was relatively more up-regulated in eschars from non severe
210 MSF than in eschars from severe form. This mediator have already been implicated in other
211 infectious conditions (8). Moreover expression of S100A8/9 have been associated with an
212 alteration of the rate of cell proliferation and differentiation in keratinocytes (16). This finding
213 illustrates the importance of the remodeling tissue within eschar. The adhesive molecule
214 ICAM-1 was up-regulated in eschars, particularly in non severe cases of MSF. This adhesion
215 molecule has previously been found to be highly expressed in MSF patients and regulates
216 leukocyte movement (13;17). Regarding IL-22RA1, that appears to play an important role in

pathogen defense, wound healing, tissue reorganisation and found in reduced level in chronic inflammatory conditions (18), its gene-encoding was found down-regulated only in eschars from severe MSF cases. This down-regulation may participate to an impaired immune response in severe form of MSF. Therefore this molecule could represent a biomarker for prognostic assessment in MSF and should be tested in future studies.

Some cytokines and chemokines have been previously associated with severity during *R. conorii* infection. In fact, lower levels of IL-2 have been found in dead mice infected with *R. conorii* comparatively to mice that survived (19), however, we did not found modulation in the IL-2 gene in our series. De Sousa found that CCL-5 was significantly higher in eschars from severe patients than in eschars from mild cases of MSF (5), fact that was not observed in our study.

Our study had several limits: eschars from severe form of MSF represented a small group, it was therefore difficult to reach significant differences. The diagnosis of MSF is usually based on clinical and serological data; sampling biopsies of eschars is rare. Moreover, severe form of the disease represent 5 to 16% of the cases of MSF (4) and lead to a small number of eschars from severe cases comparing to mild form.

In conclusion, we report here for the first time the transcriptomic profile associated with eschars of inoculation of MSF. This profile included mainly genes involved in immune response. Moreover, eschars associated with severe cases of MSF displayed a specific signature with notably relative up-regulation of CCL-18.

Acknowledgements

We are grateful to Dr H. Lépidi for performing histological analysis and Dr Y. Bechah for editing the manuscript.

240 **Table 1**. List of primers used in real-time reverse transcriptase PCR experiments

GENES	PRIMERS
CCL18 (Chemokine C-C ligand 18)	Forward 5'-CTCTGCTGCCTCGTCTATACCT-3'
	Reverse 5'-TCAGGTCGCTGATGTATTTCTG
S100A9 (S100 calcium binding protein A9)	Forward 5'-CTAAGACCACAGTGGCCAAGAT-3'
	Reverse 5'-CACAGCCAAGACAGTTTGACAT-3'
FABP7 (fatty acid binding protein 7, brain)	Forward 5'-TTGCTGAGGTGTAAAGGGTCTT-3'
	Reverse 5'-CTTGAATGTGCTGAGAGTCCTG-3'
IL34 (Interleukine 34)	Forward 5'-AACAAAGCTCCGTCCTAAACTG-3'
	Reverse 5'-GTCTCAGACCCAGTTGAACTCC-3'
KRT27 (keratin 27)	Forward 5'-CTCCTTGACAGAGACCGAGAGT-3'
	Reverse 5'-GCCATCTTCTCCATCTATCAGG-3'
IL6 (Interleukine 6)	Forward 5'-CCAGGAGAAGATTCCAAAGATG-3'
	Reverse 5'-GGAAGGTTCAGGTTGTTTTCTG-3'
ICAM1 (intercellular adhesion molecule 1)	Forward 5'-GCTCCCAGTCCTAATCACATTC-3'
	Reverse 5'-GTGCCGATAGAAAAATCACTCC-3'
FLG (filaggrin)	Forward 5'-GAGCTGAAGGAACTTCTGGAAA-3'
	Reverse 5'-ACTGTGCTTTCTGTGCTTGTGT-3'
IL17RE (interleukin 17 receptor E)	Forward 5'-GATCCAGAGACATGGGAAAGTC-3'
	Reverse 5'-CAGGGCAGAAGGAATTCATAAG-3'
IL22RA1 (interleukin 22 receptor, alpha 1)	Forward 5'-GCCTAAAGGTCAGCTTCAGAAA-3'
	Reverse 5'-GCCCTTTAGGTACTGTGGTGTC-3'
βActin	Forward 5'-GGAAATCGTGCGTGACATTA-3'
	Reverse 5'-AGGAAGGAAGGCTGGAAGAG-3'

241

Figure 1. Histological aspect of a skin biopsy of eschar from a patient suffering from Mediterranean spotted fever

Note vasculitis with intramural and perivascular infiltrates composed mainly of mononuclear cells (hematoxylin –eosin– saffron, original magnification X400). Courtesy Dr Hubert Lepidi .

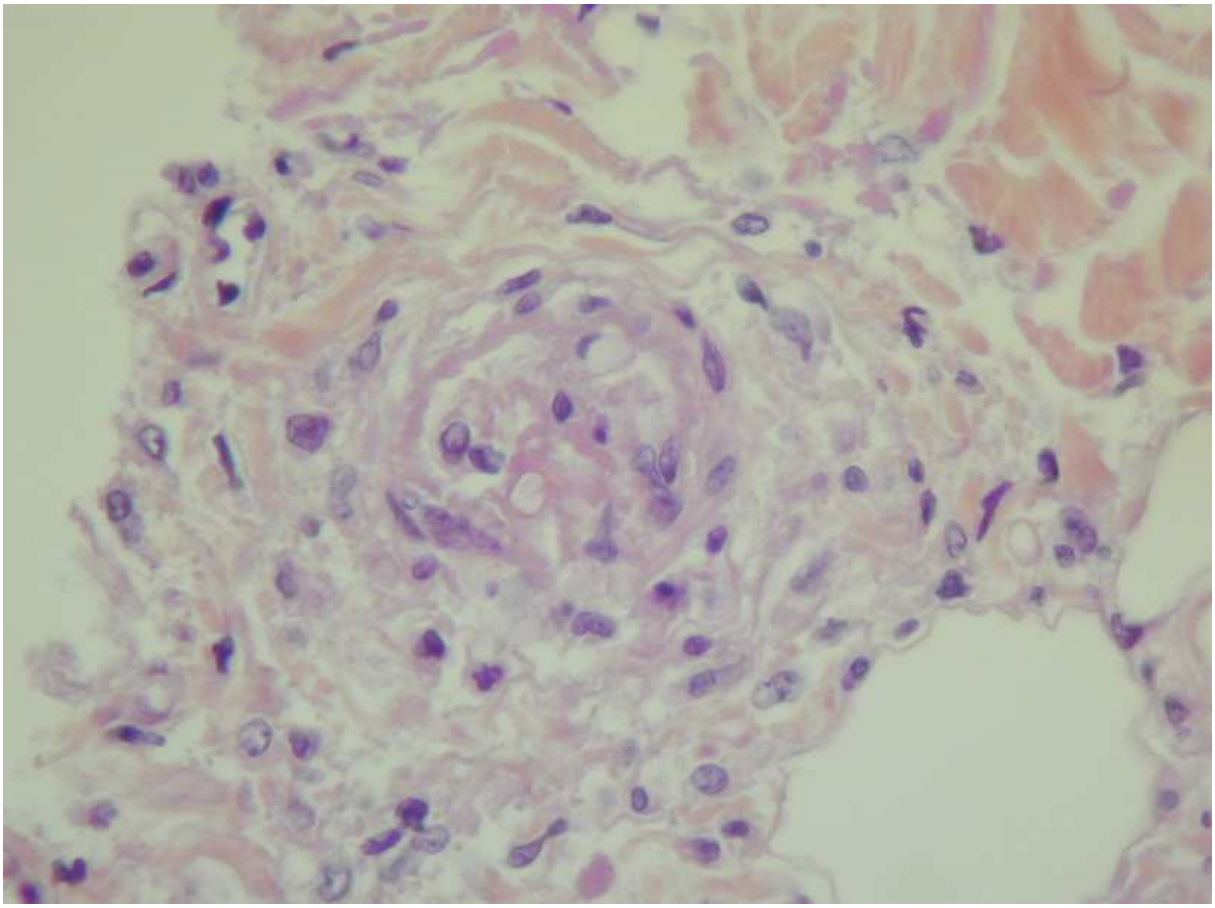


Figure 2. Principal Component Analysis: representation of all the eschars obtained from Mediterranean spotted fever patients using all the genes modulated.

C1 to C6 represented the controls (skin biopsies)

M1 to M12 and A1 to A7 represented eschars of patients suffering of MSF.

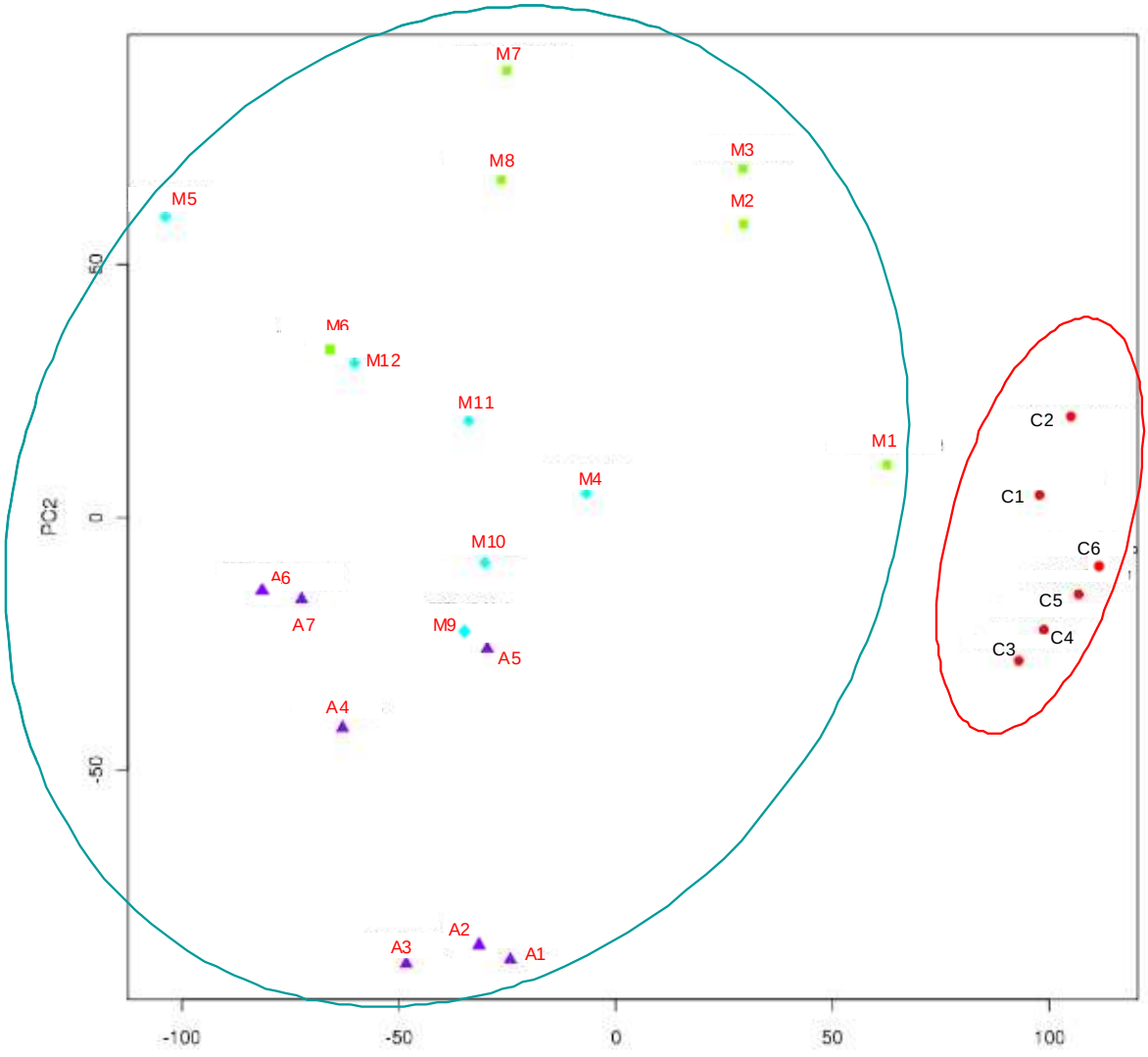
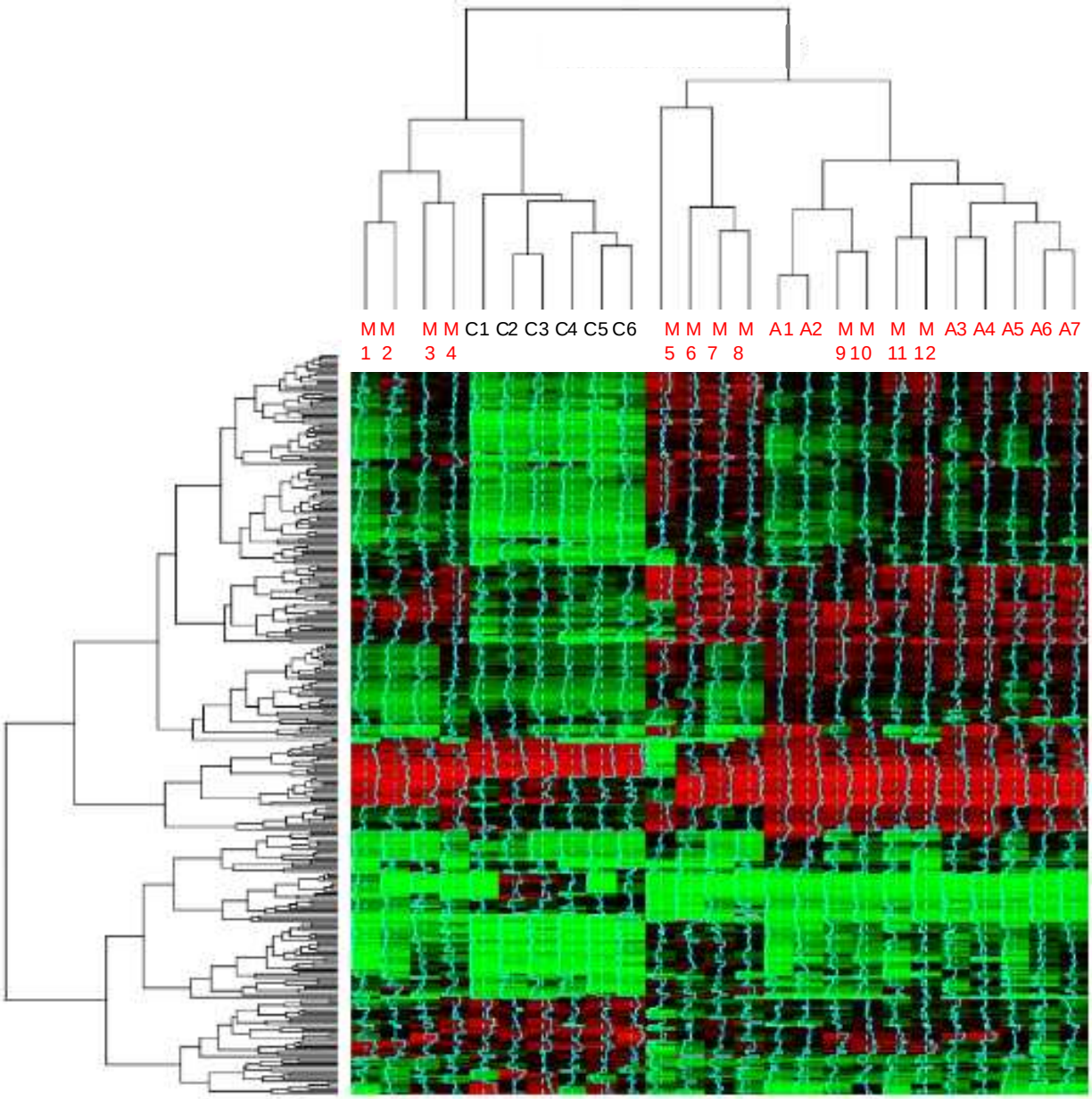


Figure 3. Hierarchical clustering analysis of transcriptional profiles of eschars and controls



This dendogram represents the 300 gene the most variables

C1 to C6 represented the controls

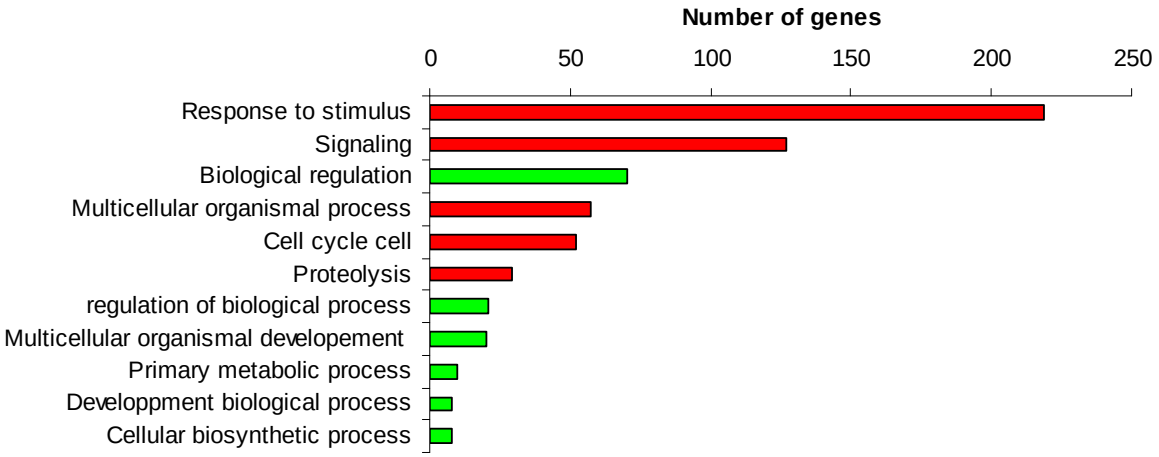
A1 to A7 represented eschars from Algeria

M1 to M12 represented eschars from Morocco

Red represented genes up-regulated, green represented genes down-regulated

277 **Figure 4:** GO annotation of the 698 differentially expressed genes.

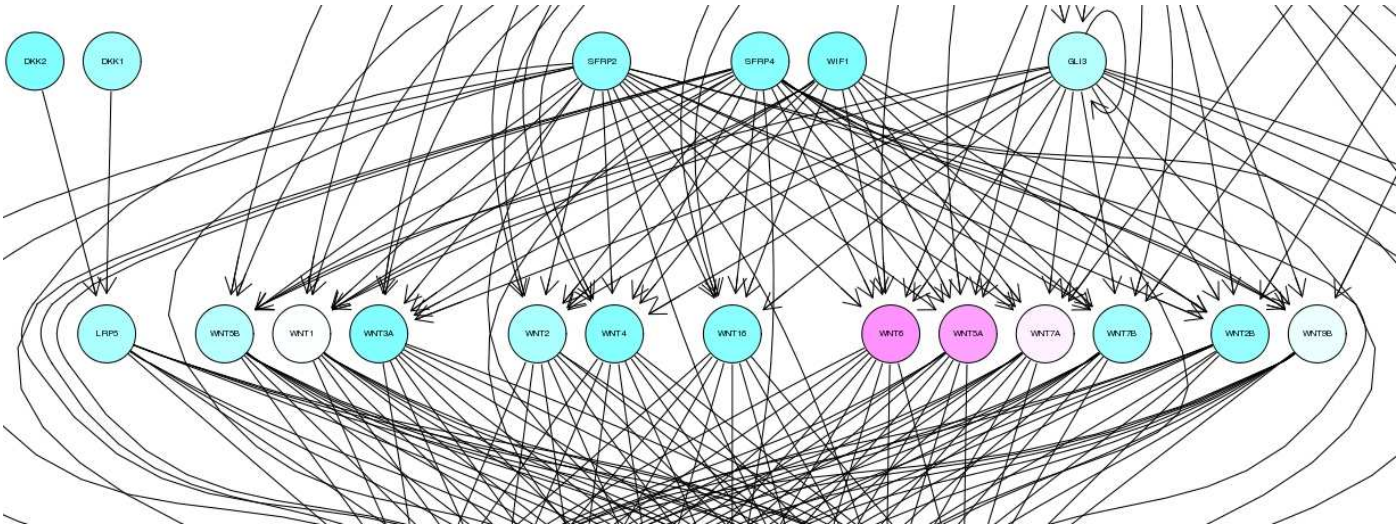
278 Differentially modulated genes in eschars were subjected to GO annotation to identify the
279 corresponding biological process modulated compares to controls. The major biological
280 processes are shown. Biological processes in green corresponded to involved down-regulated
281 genes and in red to genes up-regulated.



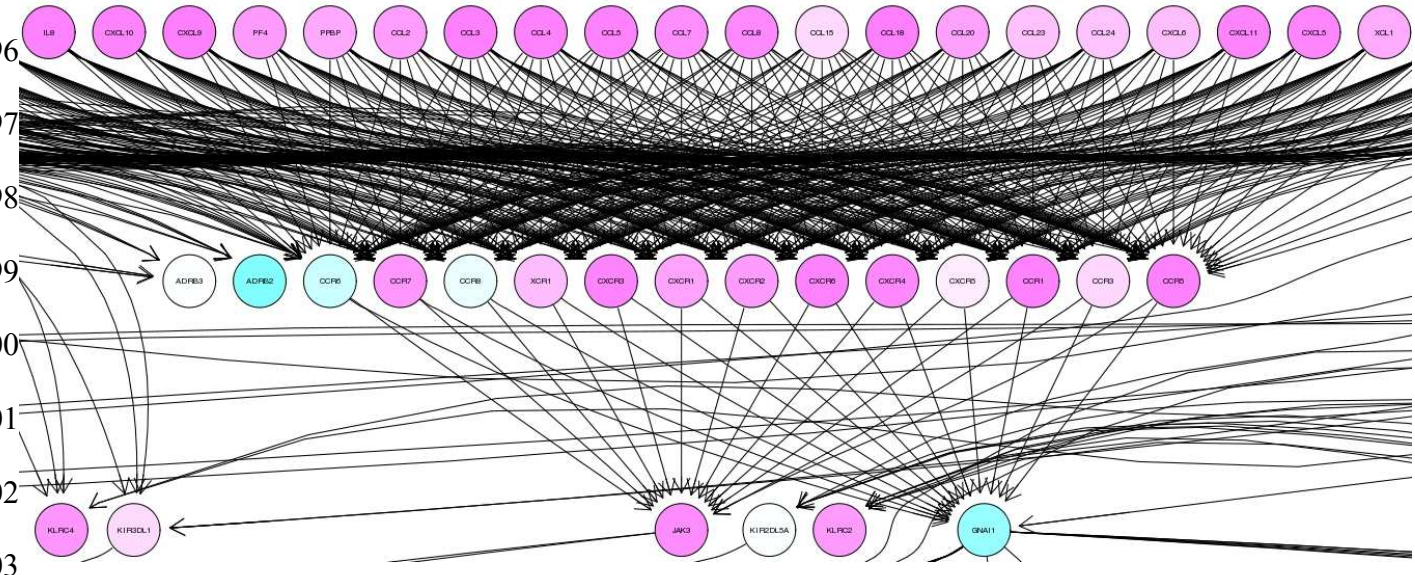
282

Figure 5. Main pathways involved in the transcriptomic profile of eschars

A. Wnt pathway



B. Chemokines and chemokine receptor around JAK3



The complete network was shown in supplementary figure 1

306 **Table 2**. Level of expression of some modulated genes

307 Values found with microarray and values of confirmation found with real time RT-PCR.

Genes	MICROARRAY	RT-PCR * All eschars vs controls	RT-PCR * Non severe eschars vs controls	RT-PCR * Severe eschars vs controls
CCL18	52,00	5,99	5,58	8,19
S100A9	52,00	14,83	16,06	8,28
FABP7	0,05	0,62	0,62	0,59
IL34	0,22	0,48	0,52	0,26
KRT27	0,03	0,18	0,19	0,13
IL6 [a]	9,00	1,27	1,34	0,91
ICAM1 [a]	3,70	3,13	3,35	1,94
FLG [a]	0,18	0,40	0,40	0,27
IL17RE [a]	0,40	0,54	0,58	0,34
IL22RA1 [a]	0,42	1,08	1,19	0,50

308

309 *Fold change with RT-PCR represented the mean of fold changes of the eschars

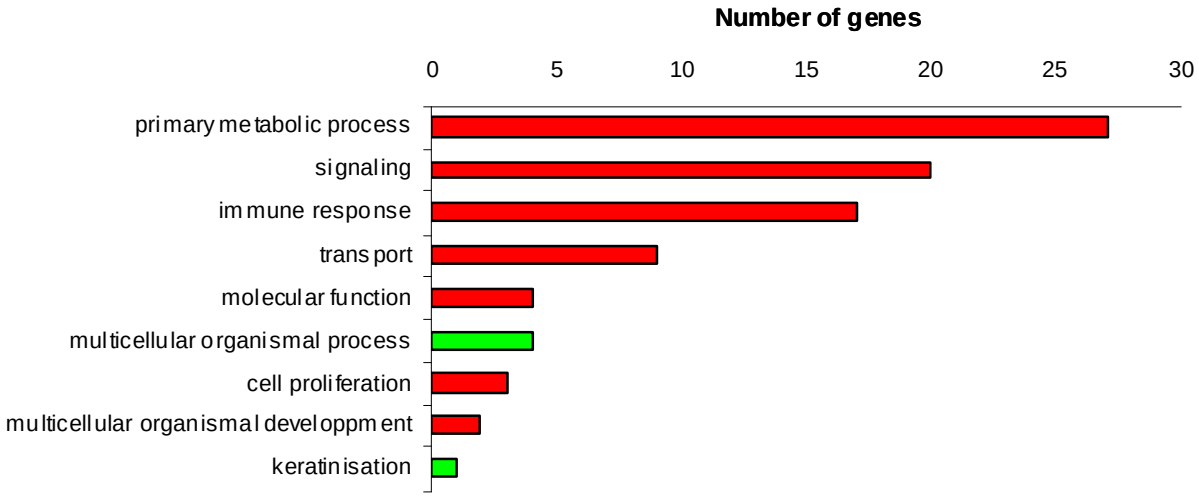
310 [a] Using microarray, these genes were found to be modulated only in eschars from severe cases

311 of MSF.

Figure 6: GO annotation of the 89 genes differentially expressed only in the group of eschars from severe cases of Mediterranean spotted fever compared to controls.

Green bars represented genes down-regulated (n=5)

Red bars represented genes up-regulated (n=84)



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CONCLUSIONS GENERALES

Au cours de cette thèse, nous avons étudié les facteurs de risques de sévérité en lien avec l'hôte et le traitement reçu au cours de la fièvre boutonneuse Méditerranéenne. Pour cela nous avons réalisé une étude clinique portant sur des patients présentant une FBM, diagnostiqués sur les 10 dernières années afin de voir si des facteurs de risques différents de ceux déjà rapportés pouvaient être observés. Au cours de cette étude portant sur des cas de FBM en lien avec *R. conorii* subsp *conorii,* nous avons appliqué une définition rigoureuse de ce qu'est la sévérité, commune à d'autres pathologies, à savoir la définition de syndrome de défaillance multiviscérale. Ceci nous a permis de définir les cas sévères et ainsi d'évaluer les facteurs en lien avec la sévérité.

Grâce à cette étude, nous avons observé de façon tout à fait inattendue que les fluoroquinolones, antibiotiques de plus en plus utilisés au cours de la FBM (article 1), étaient associées à une évolution vers la sévérité. A l'inverse, la doxycycline semble protéger les patients d'une évolution vers la sévérité, ce qui a déjà été observé au cours de la Rocky Mountain spotted fever (40). Il nous parait donc important de ne plus recommander les fluoroquinolones dans le traitement de la FBM.

Cette découverte fortuite nous a donc conduits à étudier l'effet *in vitro* de la ciprofloxacine sur *R. conorii* à partir d'un modèle cellulaire. Au cours de cette étude (article 2) nous avons confirmé que l'utilisation de ciprofloxacine à dose sub et supra-inhibitrice augmente significativement la taille de plages de lyse, confirmant ainsi un effet délétère de cette molécule sur la cellule hôte. Pour expliquer cette observation, nous avons voulu savoir si les fluoroquinolones pouvaient induire l'expression de la toxine de *R. conorii*. En effet, ces antibiotiques sont connus pour pouvoir induire des toxines dans d'autres modèles (31;32;34;35). Nous avons ainsi observé que la ciprofloxacine à dose supra-inhibitrice induisait une augmentation de la toxine et à un moindre degré de l'antitoxine. Cependant, il

reste à démontrer que la toxine de *R. conorii* (VapC1) agit par le biais d'une action RNAsique pour induire un effet délétère chez l'hôte, tel qu'il a été démontré au laboratoire pour la toxine de *R. felis* (37), avec passage intracytoplasmique.

En terme thérapeutique, nous avons également testé l'effet des statines sur l'infection à *R. conorii.* Ces molécules, aux fonctions très diverses, ayant une action anti-infectieuse, ont également une action bénéfique au cours de l'infection par *R. conorii* (article 3). Cette action semble être partagée par les 2 molécules testées (lovastatine et pravastatine) et être surtout active en prophylaxie. Cette action prophylactique supposée n'a malheureusement pas pu être vérifiée au cours de l'étude clinique du fait d'un trop faible effectif pour pouvoir conclure.

Enfin, nous avons étudié la réponse de l'hôte *in vivo*, au sein de l'escarre d'inoculation en utilisant le «microarray». Cette approche transcriptomique globale (article 4) a permis de mettre en évidence une signature escarre spécifique et de mettre en avant certains marqueurs inflammatoires à ce jour non étudiés au cours de la FBM tels que S100 A9 ou CCL-18. Nous avons aussi caractérisé les escarres issues de patients ayant des formes sévères de FBM, mettant ainsi notamment en évidence, des intensités d'expression différentes par rapport aux escarres issues de formes non sévères.

PERSPECTIVES

Ce travail ouvre des perspectives pour d'autres travaux à venir. Ainsi, l'effet délétère des fluoroquinolones au cours de la FBM est quelque chose de si surprenant, bien qu'indépendant et confirmé *in vitro*, qu'il demande à être confirmé par d'autres études. Il serait souhaitable d'étudier l'effet des fluoroquinolones dans d'autres séries de rickettsioses (typhus murin, «Israeli spotted fever»…) en prospectif voire rétrospectif pour des raisons éthiques, afin de détecter un effet délétère similaire. Idéalement, une étude clinique prospective sur la FBM, comparant le traitement par fluoroquinolones en double aveugle avec la doxycycline pourrait être évoquée. Cette étude ne semble toutefois pas être éthique au vue de notre article 1. Il est à noter cependant, que si il est dorénavant non conseillé d'utiliser les fluoroquinolones dans le traitement de la FBM, ce traitement est largement utilisé pour traiter en intraveineux (IV) les formes sévères de FBM dans de nombreux pays où la doxycycline IV n'est pas disponible. Il semble donc nécessaire de tester *in vivo* d'autres molécules antibiotiques efficaces et utilisables en IV. Un essai clinique prospectif devrait également permettre de conclure à un rôle bénéfique prophylactique ou non des statines sur les formes sévères de FBM.

En ce qui concerne la compréhension du mécanisme en cause expliquant l'effet délétère des fluoroquinolones, il est nécessaire d'explorer davantage le rôle de la toxine de *R. conorii*. Des travaux semblables à ceux réalisés avec *R. felis* pourrait être faits afin de prouver le rôle de la toxine VapC sur la mort des cellules hôtes. Des travaux de purifications de la toxine, afin de tester son action sur modèle cellulaire voire animal devraient être envisagés. Plus globalement, il pourrait être intéressant de tester l'effet des fluoroquinolones sur un modèle animal d'infection à *R. conorii.* Ceci pourrait permettre de voir précisément les effets délétères au niveau tissulaire. En utilisant le cobaye comme modèle expérimental (41), l'effet du traitement par fluoroquinolones pourrait être comparé à celui du traitement par

doxycycline sur la formation des escarres d'inoculation. Une approche transcriptomique pourrait être également utilisée dans ce sens. Les fluoroquinolones ont par ailleurs des propriétés immunomodulatrices qui pourraient également participer à l'effet délétère observé chez les patients souffrant de FBM et traités par fluoroquinolones. Cette action immunomodulatrice pourrait également être étudiée sur modèle animal, cellulaire ou encore *in vivo*. Par exemple, les fluoroquinolones inhibent le «tumour necrosis factor» (TNFα) qui est connu pour jouer un rôle crucial dans la réponse immune au cours de la FBM (38). Tester simplement le relargage du TNFα selon le traitement antibiotique utilisé pourrait être intéressant.

Enfin, les molécules d'intérêt différentiellement exprimées dans les escarres issues de formes sévères ou non sévères de FBM telles que CCL-18, S100A9 pourraient être testées *in vivo* dans la circulation systémique des patients souffrant de FBM pour confirmer leurs implications et pourraient servir de marqueur pronostique. S'il s'avérait qu'une de ces molécules est associée à l'apparition ou à la protection vis-à-vis de la forme sévère de la FBM, des stratégies thérapeutiques ciblant la molécule d'intérêt pourront être mise en place. Enfin, d'un point de vue physiopathologique, il serait également intéressant de comparer la réponse de l'hôte au sein de l'escarre à la réponse au sein de granulomes infectieux pour mieux caractériser la réponse inflammatoire spécifique au cours de la FBM.

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Ref Type: Unpublished Work

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REMERCIEMENTS

Je tiens tout d'abord à remercier, Monsieur le Professeur Didier Raoult, mon directeur de Thèse d'avoir dirigé ce travail de Thèse et de m'avoir accueillie dans votre laboratoire. Je vous remercie pour vos conseils, vos corrections attentives, et votre disponibilité.
Trouvez ici l'expression de toute ma reconnaissance pour m'avoir inspirée tout au long de mon cursus médical et scientifique votre passion pour la recherche. Votre énergie débordante est pour moi un exemple. Veuillez trouver en ce travail l'expression de mon plus profond respect.

***Je tiens également à remercier le Président de Jury et co-Directeur de Thèse, Monsieur le Professeur Jean Louis MEGE*.** Vous m'avez fait l'honneur de présider cette Thèse et d'encadrer une partie de ce travail de Thèse. Je vous adresse ici l'expression de mon plus grand respect et ma gratitude pour votre soutien et votre disponibilité.

***Je remercie Monsieur le Professeur Gilbert Greub*,** d'avoir accepté de faire partie de mon Jury en qualité de rapporteur et de bien vouloir juger ce travail.

Un merci particulier A Monsieur le Pr Frédéric Lucht. Je vous remercie d'avoir accepté de juger ce travail. Vous m'avez connue alors que j'étais étudiante en DCEM1 et c'est dans votre service que ma vocation pour les Maladies Infectieuses est née. Je vous remercie sincèrement pour l'intérêt que vous m'avez toujours porté, pour votre soutien et votre bienveillance.
Vous avez su me conseiller de partir pour mieux revenir.
J'espère que l'avenir nous réserve une collaboration fructueuse.

Je tiens aussi à remercier,

Monsieur le Professeur Philippe BROUQUI, vous m'avez accueillie dans votre service dès mon premier choix d'interne à Marseille et vous m'avez toujours enseigné avec enthousiasme les Maladies Infectieuses. Veuillez trouvez ici l'expression de ma gratitude pour m'avoir permis de me libérer afin de mener à bien cette Thèse. Je vous remercie également pour votre soutien. Soyez assuré de ma profonde estime et de mon admiration pour votre motivation et enthousiasme qui font que c'est un plaisir de travailler avec vous.

Monsieur Guy Vestris, tu m'as enseigné la culture cellulaire et la rigueur lors des expérimentations. Tu as toujours été là pour moi (et les autres). Ton soutien, ton humour, ta gentillesse vont de toi un vrai «papa poule», sans toi cette thèse n'aurait pas eu la même saveur.
Merci, vraiment

Tous les «seniors» de l'URMITE, avec qui j'été amenée à travailler de près ou de loin et qui ont toujours été disponible pour moi: les Professeurs Parola, Rolain, Fournier, Richet, Lépidi, Lascola, Drancourt, le docteur Fenollar

Tous les médecins hospitaliers et/ou universitaires qui m'ont aidé à devenir ce que je suis professionnellement.

Tous les techniciens du laboratoire, notamment du P3, pour leur aide ou les échanges sympathiques que j'ai eu avec eux.

Un merci à mes proches

Arnaud, cela fait 14 ans que tu es à mes cotés et ton soutien reste indéfectible, je t'en remercie
Tu es toujours là pour m'encourager et tu as toujours respecté mes choix et comprend mes impératifs professionnels.
Tu es ma force tranquille
Sans toi tout ceci serait impossible…
Tu m'as fait le plus beau des cadeaux, j'attends les prochains avec impatience,
Nous allons vers une nouvelle aventure…que je veux construire entièrement avec toi
Amo-te meu amor

Ma fille Alice, tu es ma plus belle réussite
Tout ce que je fais c'est pour toi
Ne m'en veut pas de beaucoup travailler mais saches que je serais toujours là pour Toi et que tu es ma priorité.
Je t'aime

Je dédie ce travail,

A mes Parents,
Vous êtes toujours là pour nous,
Votre soutien a toujours été sans faille et vous avez toujours respecté mes choix.
Vous m'avez transmis des valeurs simples telles que le respect d'autrui, l'humilité, l'honnêteté et la générosité, l'amour du travail bien fait et je vous en remercie.
Ces valeurs sont primordiales pour moi et j'essaye à mon tour de les transmettre à mes enfants.
Merci pour TOUT.

A ma Sœur,
Même loin tu es toujours proche
Tu es ma meilleure amie, ma deuxième maman, je sais que je peux compter sur toi à tout moment. Ne change rien à ce que tu es.
Merci de m'avoir donné un (beau)-frère, **Eduardo**, si gentil.
Merci de m'avoir donné un filleul, **Mickael**, si extraordinaire.
Merci de m'avoir donné une nièce, **Flavia**, si aimante et si espiègle.

A Jean-Marc, mon Beau-Père,
Tu nous as quitté il y a 3 ans mais je sais que tu serais fier de moi et de ce travail.
Notre complicité me manque…Merci pour la relation privilégiée que tu m'as permis d'avoir avec toi.

A Danielle, Gaetan, Lydie
Nous avons traversé des épreuves difficiles et nous avons su nous soutenir comme une «vraie» famille, merci à Danielle pour sa gentillesse, merci à Gaetan et Lydie pour les bons moments partagés avec Alice et Elia. Que l'avenir nous réserve de bons WE ensemble sur la mer ou la terre.

A toute ma famille,

Un grand merci à mes amis, les vrais qui sauront se reconnaitre et que j'espère garder très très longtemps où que je sois

Un énorme merci à ***mes «co-locataires» du bureau***, ceux du monde merveilleux des Stroumfs:, je veux bien sûr parler d'Aurélie, Marie, Yassina, Isabelle, JC, Samir, Stéphan (et Mickael de son temps). Merci pour nos fous rires, nos pauses kit-kat-chocolat-et-autres-sucreries-anti-baisse-de-moral, notre dérision permanente, notre complicité, notre entre-aide (spéciale mention à Yassina pour sa gentillesse, ses précieux conseils, sa patience et à Samir pour «=1/NB.SI($B$2:$B$5393;B2)). Un merci particulier à Aurélie et Marie mes 'lunchfriends' et pour tout ce qui va avec… Vous m'avez à votre façon tous soutenue pendant cette thèse pas toujours facile.

Merci aussi à tous les autres étudiants de l'URMITE avec qui j'ai eu l'occasion de discuter, de partager, je pense notamment à Nada, Amira, Quentin, Sophie, Vikram, Sudhir, Silpak…..j'en oublies forcement mais ne m'en tenez pas rigueur!!!!