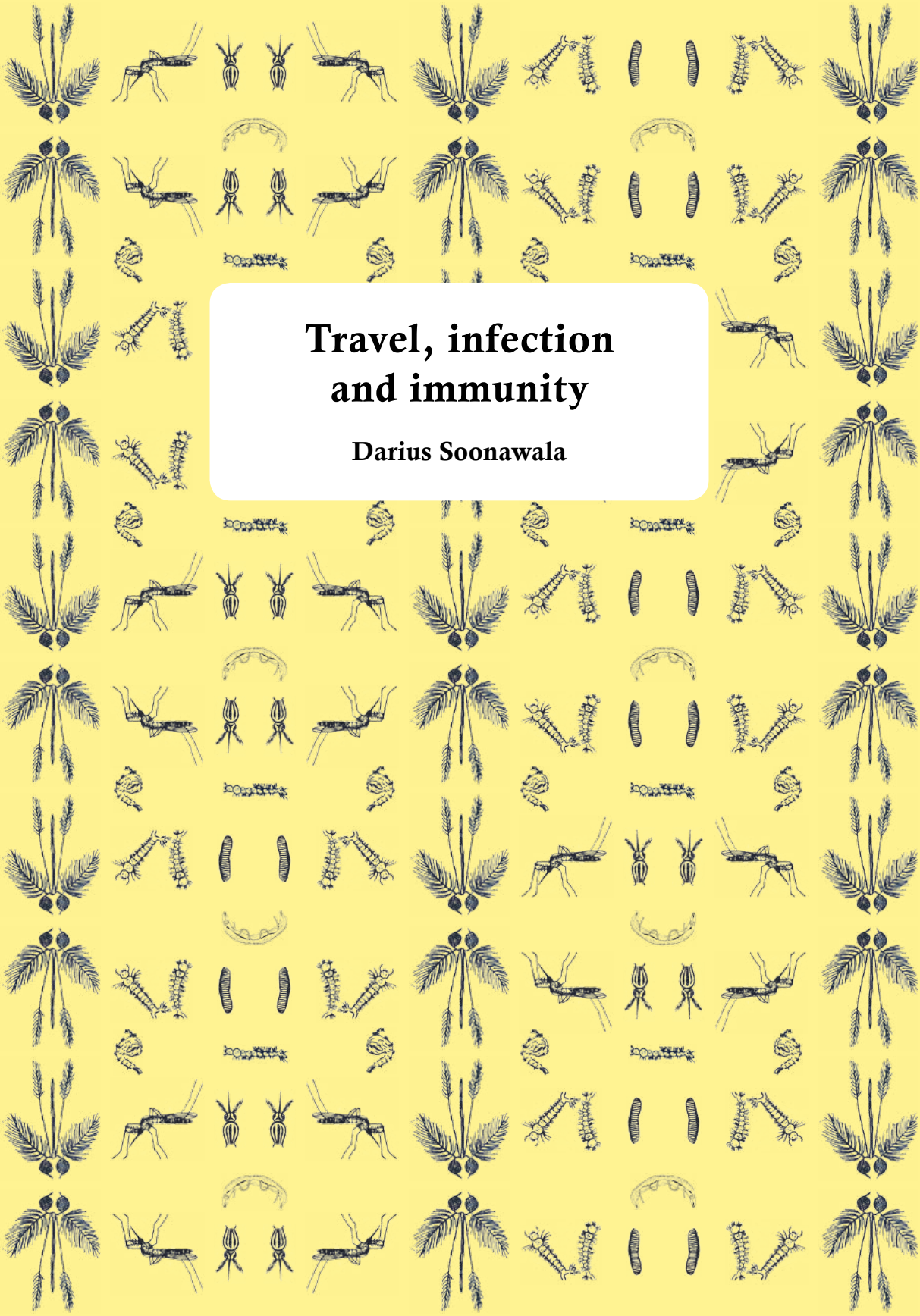

Travel, infection and immunity

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Travel, infection and immunity

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Car il est bien plus beau de savoir quelque chose de tout que de savoir tout d'une chose.
Blaise Pascal - Pensées

CONTENTS

PREFACE

The content of this thesis is based on research that was conducted at the travel and vaccination clinic at Leiden University Medical Centre (LUMC). This clinic provides pre-travel care to the general population, and to special groups of travellers, such as patients who use immunosuppressants or who have chronic diseases. The clinic is closely connected to the department of Infectious Diseases at LUMC. A team of specialized nurses provides pre-travel care. The setting of a travel clinic within an academic medical hospital, provides unique circumstances for medical research, like an experienced team of nurses, expertise regarding immunization, a constant flux of travellers and the knowledge and infrastructure that is required for research into microbiology, virology and parasitology. Examples of research that stem from this clinic are projects on immunization against malaria,[1,2] yellow fever,[3-6] travellers' diarrhea,[7,8] poliomyelitis[9] and hepatitis B,[10] vaccination of immunocompromised patients,[11-14] and projects on travel related acquisition of extended spectrum ß-lactamase producing Enterobacteriacae[15] and on the utility of post-travel screening of asymptomatic travellers for parasites.[16] We hope that the research that is conducted at our travel clinic, will be of benefit to travellers and to the population at large.

Introduction

INTRODUCTION

In 2014 more than one billion tourists travelled abroad, according to the World Tourism Barometer.[17] Over 500 million people travelled to an emerging economy.[1] The number is expected to grow between 3 and 4% in 2015, with the strongest growth in the Americas and in Asia and the Pacific. It is estimated that annually, two million Dutch tourists travel to low and middle income countries, one million to Turkey (800.000) and Egypt (200.000), 400.000 to countries in Asia, 400.000 to Latin America and 90.000 to Sub-Saharan Africa.[18,19] Travellers face specific health risks. Travel-related disease can be defined as diseases for which the chance of acquisition is increased due to increased exposure or increased susceptibility associated with temporary translocation.[20] Various measures can be used to numerically express the frequency of disease occurrence in a population, as is summarized in Box 1. In 1987 and in 2008 Steffen et al. have summarized the available data on the incidence proportions of travel-related morbidity (FIGURE 1 ▶ PAGE 14).[21,22] Cobelens has summarized the data for travel-related infectious diseases (FIGURE 2 ▶ PAGE 15).[20] The GeoSentinel Surveillance Network provides data on regional differences in *proportionate morbidity*, which is the number of travellers diagnosed with a certain disease divided by the total number of ill travellers reported.[23,24] GeoSentinel data cannot estimate travel-related incidences and are limited in estimating risk of disease, because only ill patients with a presumed travel-related illness are captured.[25] Diarrhoea is the main cause of travel-related morbidity.[22,26] Accidents are the main cause of mortality.[26-28] Over time, the incidence of travel-related morbidity has changed. The risk of diarrhoea has decreased. This is mainly due to improved levels of sanitation at many destinations, seeing that hygiene, is the main determinant of infections that are transmitted via the faecal-oral route.[19,29-31] The association between a determinant of disease and disease frequency can be numerically expressed, either as an *absolute difference* between groups that are being compared (e.g., exposed versus unexposed) or as relative differences.[32] An example of a determinant of travel-related morbidity is the difference in the risk of illnesses between tourists and those who travel to visit friends and relatives.[33,34]

[1] Classification based on the International Monetary Fund (IMF), see the Statistical Annex of the IMF World Economic Outlook of April 2012, page 177, at www.imf.org/external/pubs/ft/weo/2012/01.

BOX 1

Various measures can be used to numerically express the frequency of disease occurrence in a population. The *incidence rate* is defined as the number of new cases of disease in a specific population, divided by the person-time over the period.[35] This quantity is sometimes also called the *incidence density*.[35,36] If the incidence rate varies over time, this time-dependency can be traced by dividing time into small periods and calculating an incidence rate per time-period, as was demonstrated by William Far in 1838.[37-39] Alternatively, one can measure the *incidence proportion* per specified time period.[34] This quantity is sometimes also called the *cumulative incidence* or the *attack rate* and is defined as the proportion of a specified at-risk population that experiences an outcome over a given time period.[35,36]

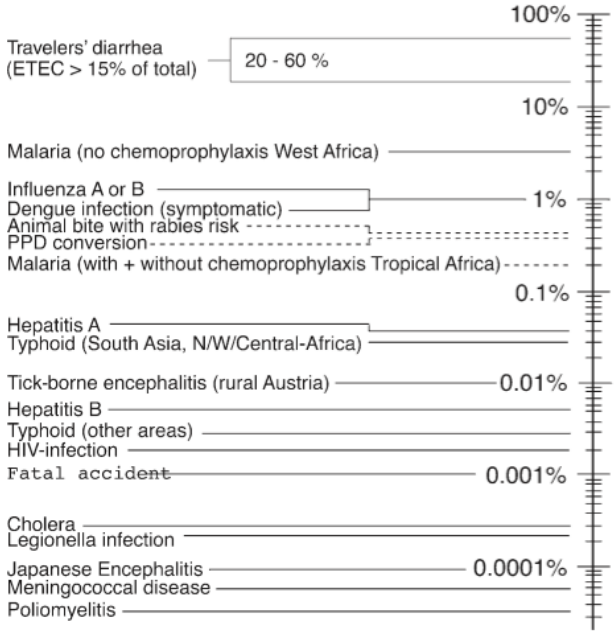

FIGURE 1

Cumulative incidence of health problems for a stay of one month in a developing country - 2008.[22] (Reproduced by permission from Journal of Travel Medicine 2008. Copyright International Society of Travel Medicine.)

FIGURE 2 (right)

Summary of risk estimates for selected travel-related infectious diseases by their preventability by vaccination.[20] (Reproduced by permission of F.G.J. Cobelens).

TABLE 1.6

SUMMARY OF RISK ESTIMATES FOR SELECTED TRAVEL-RELATED INFECTIOUS DISEASES BY THEIR PREVENTABILITY BY VACCINATION

Risk[1]	*Vaccine-preventable diseases*	*Other diseases*
> 1:10		traveller's diarrhoea (all)
		diarrhoea due to ETEC
		Schistosoma infection in Lake Malawi[5]
1:10		shigelloses in high-risk areas
		campylobacteriosis in high-risk areas
		schistosomiasis in Lake Malawi[5]
1:100	hepatitis A under high-risk conditions	falciparum malaria in sub-Saharan Africa[3]
1:1,000	hepatitis A in tourists	vivax malaria in India
	hepatitis B virus infection under high-risk conditions	cutaneous leishmaniasis in Suriname[6]
	meningococcosis during Haj	
1:10,000	acute clinical hepatitis B	paratyphoid in Nepal
	typhoid fever in high-risk areas	hepatitis E in expatriates in India
	meningococcosis in tourists during epidemic in Nepal	
1:100,000	typhoid fever in low-risk areas	falciparum malaria in Central America
	poliovirus infection[2]	cutaneous leishmaniasis in Belize-Peru
1:1,000,000	cholera	trichinosis in Africa
	untreated rabies in high-risk areas	
< 1:1,000,000	meningococcosis in tourists	
	Japanese encephalitis in tourists	
	paralytic poliomyelitis[2]	ascariasis
Unknown	yellow fever	brucellosis
	rabies	dengue and other arbovirus-infections[4]
	tuberculosis	filariases
	tetanus	rickettsioses and non-Lyme borrelioses
	diphtheria	visceral leishmaniasis
	measles	old world cutaneous leishmaniasis
		trypanosomiases
		leptospirosis
		hookworm infection
		strongyloidiasis

[1] *Assuming for short-term travel an average duration of 3 weeks.*
[2] *Proportion not at risk through naturally acquired or vaccine-induced immunity unknown.*
[3] *In travellers taking no chemoprophylaxis.*
[4] *Apart from yellow fever and Japanese encephalitis.*
[5] *On high-risk beaches at Monkey Bay.*
[6] *In areas with uncontrolled cutaneous leishmaniasis.*

Travellers are often categorized according to travel-purpose, such as: visiting family and friends, business, study or leisure. The last category, 'leisure' is a poorly defined category, that encompasses various reasons for people to travel. Although we do not routinely stop to reflect upon these reasons, philosophers have contemplated the act of travel. In his famous letters, the Greek philosopher Seneca writes: "*You need a change of soul rather than a change of climate. Though you may cross vast spaces of sea, and though, as our Vergil remarks you leave lands and cities behind you, your faults will follow you whithersoever you travel. Socrates made the same remark; to one who complained he said: 'Why do you wonder that travelling does not help you, seeing that you always take yourself with you? The reason which set you wandering is ever at your heels*."[40, 2], These words echo the modern day philosopher Alain de Botton: "*The pleasure we derive from journeys is perhaps dependent more on the mindset with which we travel than on the destination we travel to*."[41] However, in contrast to Seneca, de Botton argues that travel may be conducive to a change of soul: "*It is not necessarily at home that we best encounter our true selves. The furniture insists that we cannot change because it does not; the domestic setting keeps us tethered to the person we are in ordinary life, who may not be who we essentially are*." To paraphrase his words; a different surrounding with different people, and an encounter with new physical or mental challenges may provide circumstances that are more conducive to a change of soul and to personal growth than those at home.

Seeing that personal growth may be one of many benefits of working abroad, students are encouraged to pursue electives in other countries. Therefore it is deemed a positive development that more and more Dutch (bio)medical students venture abroad for study and work. Universities have a certain degree of responsibility for the health and well-being of students and a responsibility towards the medical staff and the hospitals abroad who facilitate the electives. Therefore students are made aware of the degree of responsibility that they can and may shoulder. For example, it should be clear beforehand whether a student is sufficiently experienced to participate in certain procedures, such as suturing or assisting in the operating theatre or delivery room. If new skills are to be acquired abroad, it should be specified beforehand whether the medical staff abroad has the time and facilities to supervise and teach new skills. Awareness of ethical aspects is also required. In clinics where resources and medical staff may be scare, it is important that students do not feel obliged to take on responsibility beyond their means and that they do not use scarce resources in an inefficient manner. **The first chapter** of this thesis describes a study which was designed to improve the quality of medical electives.

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[2] Animum debes mutare, non caelum. Licet vastum traieceris mare, licet, ut ait Vergilius noster, *terraeque urbesque recedant*, sequentur te quocumque perveneris vitia. Hoc idem querenti cuidam Sokrates ait, 'quid miraris nihil tibi peregrinationes prodesse, cum te circumferas? premit te eadem causa quae expulit'.

It describes the health risks and the quality and comprehensiveness of pre- and post-travel care for this group of travelers.[42] A second multi-centre study with a wider scope is under way.

For students on electives, as for most travellers to the tropics, diarrhoea is the most common health hazard. It can be a major nuisance but it is very seldom fatal. Although the world-wide incidence of travellers' diarrhoea has been well documented, there is limited data on the degree of inconvenience that travellers experience. **The second chapter** deals with this subject.[8] The data from this study provides an estimate of the burden of illness and of the size of the target population for preventive and curative measures.

Over the years, many pathogens that cause travellers' diarrhoea have been identified. This has led to dietary advice such as "boil it, cook it, peel it or forget it", and to algorithms for self-treatment with antidiarrhetic and antimicrobial agents. Some experts in the United States go so far as to advocate the use of a non-absorbable antimicrobial agent to prevent diarrhoea when traveling.[43] One of the arguments that is used to promote such practice, is that travellers' diarrhoea leads to a chronic condition known as post-infectious irritable bowel syndrome in a sizeable proportion of travelers.[44-49] Post-infectious IBS is characterized by relapsing and fluctuating gastrointestinal symptoms, including abdominal pain, discomfort, and changed bowel habits.[50] Until recently the incidence of post-infectious IBS in travellers remained obscure. A recent large and well-conducted questionnaire study by Pitzurra et al. shows that the incidence of new-onset IBS, as defined by the Rome III criteria, is 1.0% six months after travel.[51,52] The incidence is higher in those who had an episode of travellers' diarrhoea (3.0%) than in those who did not (0.7%). Of those who fulfilled the criteria for IBS, based on the answers to the questionnaire, one in three were diagnosed with a condition other than IBS by their physician. Pathogens, that are not detected with routine faecal culture, such as Enteroaggregative Escherichia coli (EAEC), can cause long-lasting diarrhea.[53,54] This reflects some of the difficulties in finding the cause and treating post-travel chronic gastro-intestinal complaints. Nowhere is this more challenging than in patients with inflammatory bowel disease (IBD), i.e. Crohn's disease and ulcerative colitis. An enteric infection is hard to distinguish from an exacerbation of IBD. Moreover, there are many anecdotal reports of exacerbations of inflammatory bowel disease after enteric infections and observational studies that found an association between enteric infection and the onset of IBD.[55] **The third chapter** describes a retrospective web-based questionnaire study on past travel experiences in which we investigated pre-travel preparation of Dutch IBD patients and the quality of pre-travel advice. We also surveyed health problems encountered during travel and investigated whether travel increased the risk of an exacerbation of IBD.[42]

One of the common causes of prolonged gastro-intestinal complaints after travel is infection with protozoa, in particular *Giardia lamblia*. However, up to a third of those who are infected with *G. lamblia* have no complaints at all. Although asymptomatic infection with *G. lamblia* is not known to have any long term sequelae, there are a number of protozoa and helminths that can cause morbidity long after a primary asymptomatic infection. Early detection and eradication would be beneficial. **Chapter four** describes a study in which we aimed to determine the utility of routine post-travel screening of asymptomatic long-term travellers to the (sub)tropics for intestinal parasites using molecular diagnostics and for schistosomiasis using serology.[16] **Chapter five** describes the long-term immune response to former *Schistosoma* infection in a group of travelers.[56]

Research on travellers' diarrhoea also has implications for people in low-income countries, where diarrhoea in infants is caused by many of the same pathogens as travellers' diarrhoea. It is well known that hygiene and better living conditions constitute the most important factors in reducing infant mortality due to communicable (enteric) diseases. This is illustrated by an elegant study in Indonesia, which shows that the presence of a bar of soap in the bathroom is an independent factor that protects against infection with *Salmonella typhi*.[57] The importance of hygiene is also illustrated by the fact that my grandfather, who was the chief medical officer in Bombay, dedicated a whole section of his book on preventive medicine, to environmental hygiene, going into considerable detail on purification and distribution of water, and collection and handling of sewage.[58] In low-income countries, infants carry the largest burden of diarrheal illness, because they are more vulnerable to dehydration and because early in life they lack immunity.[59] With time, and repeated exposure to enteric pathogens, a degree of (short-term) immunity develops.[60] This is also true for expatriates who reside in the tropics. Figure 3 shows how the incidence of diarrhoea decreases over time in expatriates living in Nepal.[61] The fact that people can acquire immunity to diarrheal illness offers potential for developing vaccines against enteric infection. Successful vaccines have been developed for preventing infection with *Vibrio cholerae*,[62,63] and Rotavirus.[64-68] Dukoral®, which protects against cholera, offers a degree of cross protection against the most common cause of travellers' diarrhoea, enterotoxigenic *Escherichia coli* (ETEC), because ETEC produces heat-labile toxins that are antigenically similar to the toxin produced by *Vibrio cholerae*. However, its efficacy to prevent all-cause travellers' diarrhoea is limited. This is due to i) the multitude of different causative pathogens of diarrhoea (i.e. different bacteria, viruses and protozoa); ii) simultaneous infection with more than one pathogen; and iii) the antigenic differences within groups of bacteria, such as diarrheagenic *E. coli*. **Chapter six** describes a randomized trial on the efficacy of a live attenuated oral cholera vaccine to prevent all-cause travellers' diarrhea.[7]

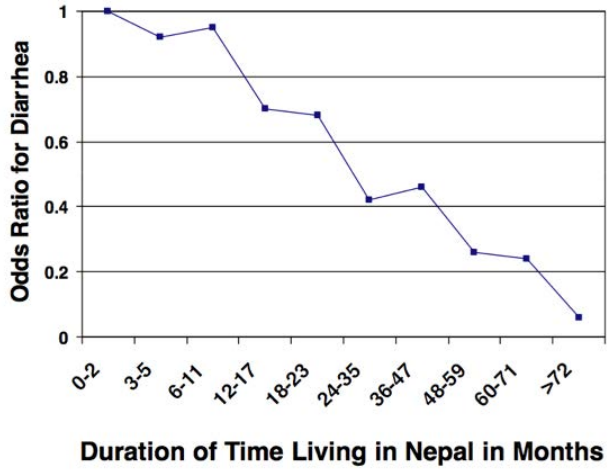

FIGURE 3
Incidence of diarrhoea decreases over time in expatriates living in Nepal. (Courtesy of Dr. David .R. Shlim, CIWEC Clinic Travel Medicine Centre, Kathmandu, Nepal).[61]

Innate and acquired mucosal immunity form the first line of defence against pathogens that invade the body through the respiratory- or intestinal mucosa. Some of these pathogens, such as enteropathogenic *Escherichia coli*, cause disease by invading and/or damaging the mucosa. Others, such as poliovirus cross the mucosa and cause disease at a distant site in the body. Poliovirus is transmitted through the oral-oral and fecal-oral route. Infection with wild type poliovirus induces mucosal and systemic immunity.[69] Live attenuated oral poliovirus vaccine (OPV) mimics infection with wild-type virus and induces a similar immune response.[70] Intramuscular immunization with inactivated poliovirus vaccine (IPV) however, does not induce a mucosal immune response.[70,71] Nevertheless, it is a very effective vaccine because it induces a reliable and robust systemic immune response and prevents poliovirus from travelling up the nervous system and causing morbidity. In general it is fair to state that eliciting a mucosal immune response is not always necessary to prevent illness from pathogens that are acquired through the mucosa but that eliciting a mucosal immune response is of paramount importance if the aim is to prevent diarrheal illness and reduce faecal shedding. In an indirect way this is exemplified by a study in which OPV is shown to be better than IPV in protecting people against re-infection with wild-type poliovirus and in which OPV is shown to shorten the time during which an infected person sheds poliovirus.[72,73] In this respect it is interesting that intradermal as opposed to intramuscular delivery of a vaccine may elicit both mucosal and systemic immunity.[74,75] Intradermal immunization may also have other advantages. It is thought to enhance the immunogenicity because of the abundance of antigen presenting cells in the papillary dermis.[76,77] This is relevant to the Global Polio Eradication Initiative of the World Health Organization. At some point in time, the use of OPV has to be

discontinued to prevent outbreaks due to circulating vaccine derived poliovirus, because OPV can sporadically mutate back to wild type virus.[78-80] IPV is a factor 20 more expensive than OPV. Therefore, one of the prerequisites for cessation of the use of OPV is to make IPV affordable and suitable for use in developing countries.[81] Using fractional-doses (reduced-doses) may impact affordability and optimize the utilization of the production capacity for IPV. Intradermal administration has the potential to lower the dose without reducing immunogenicity. A needle-free jet injector may be a reliable way to administer vaccines intradermally. **Chapter seven** describes a randomized controlled trial in which we compared the immunogenicity and tolerability of fractional-dose intradermal IPV booster vaccination administered with a jet injector to full-dose and fractional-dose intramuscular vaccination with a needle and syringe in healthy adult volunteers.[9]

Intradermal injection is best known for its application in the tuberculin skin test (TST). The TST is used to diagnose (latent) infection with *Mycobacterium tuberculosis* (MTb). Tuberculin is an extract from a culture of strains of MTb. Infection with MTb induces an immune response that is characterized by a T-helper 1 (Th1) cellular immune response, with secretion of IFN-γ, which activates bactericidal effector mechanisms in the macrophage.[82] In the majority of infected people, MTb remains dormant in macrophages, where it is kept in check by the immune system. If an individual's immunity is weakened due to poor nourishment, age, hypovitaminosis D, comorbidity such as Human Immunodeficiency Virus (HIV), or immunosuppressant medication, reactivation of latent MTb infection may occur. It is estimated that in 2012, 8.6 million people fell ill with tuberculosis and that 1.3 million died from tuberculosis.[83] About one-third of the world's population has latent infection with MTb.[83] Unfortunately, there is no vaccine that effectively protects against active tuberculosis, or against latent infection. The only available vaccine, BCG, which is a live attenuated vaccine derived from *Mycobacterium bovis* by Calmette and Guérin, offers limited protection.[84] Eliciting a stronger Th1 immune response to MTb may protect against infection and reactivation of MTb. Statens Serum Institute (SSI) has cloned and screened 250 antigens from *Mycobacterium tuberculosis*. Among these are the Early Secretory Antigenic Target (ESAT-6) and Antigen 85 (Ag85B). These antigens are strongly recognized by T-cells in the first phase of infection and they offer protective efficacy in animal models.[85,86] As compared to conventional vaccines, recombinant subunit vaccines are poorly immunogenic when administered alone, due to the high degree of purification. In order to improve their immunogenicity, these antigens must be administered with an adjuvant. Aluminum salts, which are used as adjuvants in conventional vaccines, promote the 'wrong' type of immune response, a Th2 antibody mediated response.[87] Therefore new adjuvants are needed, that promote a Th1 type response. **Chapter eight** describes the results of a clinical trials on the safety and immunogenicity of a recombinant subunit vaccine composed of the fusion protein

Ag85B-ESAT-6, combined with the novel adjuvant IC31(®), in subjects with a positive tuberculin skin test.[88] Chapter nine describes a phase I clinical trial on the safety and immunogenicity of the Ag85B-ESAT-6 subunit vaccine, combined with a new liposomal adjuvant system CAF01, in healthy adults.[89]

Reactivation of *Mycobacterium tuberculosis* is a peculiar phenomenon. Decades after the primary infection, age-related changes in the immune system may cause reactivation of latent tuberculosis. This is related to the process of immunosenescence. Immunosenescence can be studied in many ways, one of which is to compare the immune response to a standardized infection between the young and the elderly. Vaccination with a live attenuated vaccine offers an ideal opportunity to study such differences. Yellow fever vaccine is a well studied highly effective live-attenuated vaccine that replicates in the naïve host. In rare instances it may cause serious illness; either yellow fever-associated neurotropic disease, which manifests as encephalitis, or the viscerotropic variant, which mimics wild type infection.[90,91] Neurotropic disease occurs mainly in young infants and in the elderly. The risk of viscerotropic disease increases with age, with an estimated risk ratio of 4 for vaccinees who are 60-69 years of age and a risk ratio of 13 for vaccinees older than 70 when compared with young adults.[92] Therefore advanced age is regarded to be a relative contra-indication for vaccination against yellow fever. **Chapter 10** describes the results of a controlled study on age-related differences in the immune response, after administering yellow fever vaccine.[6]

Live-attenuated vaccines are contra-indicated for immunocompromised patients, such as those who are infected with HIV and who have low CD4+ T-cell counts. These patients are more likely to develop vaccine-derived disease, due to their decreased ability to mount an adequate immune response and inhibit viral replication. This is less so for memory immune responses and more so for responses to antigens that have never been encountered before, so called neo-antigens. In 2009 an antigenically distinct influenza virus emerged, to which most people lacked immunity, namely 2009 pandemic influenza A(H1N1) (pH1N1). It spread rapidly over all parts of the world and was deemed a major health risk, particularly for immunocompromised patients. This led to the swift development and use of vaccines, to prevent severe morbidity. Although immune responses to influenza vaccines in HIV-infected patients has been studied quite extensively, the circumstances of the 2009 vaccination campaign were unique for a number of reasons: i) the antigen (hemagglutinin of strain A/California/7/2009) was considered to be a neo-antigen; ii) the vaccine was administered twice instead of once; and iii) most people had received standard trivalent inactivated influenza vaccine (TIV) the month before. In 2009 we quickly initiated an observational controlled study in which we measured the humoral immune response to pH1N1 vaccine in HIV-infected patients and in healthy controls.

In addition we tested whether recent vaccination with seasonal trivalent inactivated vaccine (TIV) induced cross-reactive antibodies to pH1N1 and whether a second vaccination induced a typical booster immune response. **Chapter 11** describes the results of this study. We were surprised to find that seasonal TIV, induced cross-reactive antibodies to pH1N1 in a sizeable proportion of volunteers.[14] The specificity of these antibodies for pH1N1 was further studied with a virus neutralization assay, as is described in the **Chapter 12**.

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I. Travel-related morbidity

Chapter 1 Health risks encountered by Dutch medical students during an elective in the tropics and the quality and comprehensiveness of pre-and post-travel care. *BMC Med Educ. 2010 Dec 2;10:89.*

Chapter 2 Inconvenience due to travellers' diarrhoea: a prospective follow-up study. *BMC Infect Dis. 2011 Nov 20;11:322.*

Chapter 3 Pre-travel preparation and travel-related morbidity in patients with inflammatory bowel disease. *Inflamm Bowel Dis. 2012 Nov;18(11):2079-85.*

Chapter 4 Post-travel screening of long-term travellers to the tropics for intestinal parasites using molecular diagnostics. *Am J Trop Med Hyg. 2014 May;90(5):835-9.*

Chapter 5 The immune response to schistosome antigens in formerly infected travellers. *Am J Trop Med Hyg. 2011 Jan;84(1):43-7.*

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RESEARCH ARTICLE Open Access

Health risks encountered by Dutch medical students during an elective in the tropics and the quality and comprehensiveness of pre-and post-travel care

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Abstract

Background: Clinical and research electives abroad offer medical students many unique experiences. However, participating in an unfamiliar health-care setting combined with limited medical experience may place students at risk of illness. To improve pre-and post-travel care, we assessed the health risks and the quality and comprehensiveness of pre-and post-travel care in a cohort of Dutch medical students returning form an elective abroad.

Methods: All medical students who had performed an elective in the tropics between July 2006 and December 2008 were sent an informative email asking them to complete a web-based questionnaire.

Results: 180 of 242 (74%) students completed the questionnaire. Regarding the risk of bloodborne viral infection: 67% of all students and 32% of junior students engaged in procedures that constitute a risk of exposure to bloodborne viral infection, often in countries with high HIV prevalence rates. None of nine students who experienced possible or certain mucosal or percutaneous exposure to potentially infectious body fluids reported the exposure at the time it occurred and none used PEP. Regarding other health risks: 8 of 40 (20%) students stopped using mefloquine due to adverse effects. This left a sizeable proportion unprotected in countries that are hyperendemic for malaria. Post-travel screening for schistosomiasis, tuberculosis (tuberculin skin test) and carriage of methicillin-resistant Staphylococcus aureus (MRSA) encompassed approximately half of all students who should have been screened.

Conclusions: Based on the results of this study we have adopted an integral set of measures to reduce the health risks associated with an elective abroad. The pre and post-travel consult has been centralized and standardized as well as the distribution of PEP. In addition we have developed a mandatory module on Global Health for all medical students planning an elective abroad.

Background

Clinical and research electives abroad offer medical students many unique experiences. Shouldering responsibility in a different health care system and working with underserved patients broadens the personal and medical horizon. This may even influence future career choice as international medical experience is associated with an increase in the choice for a primary care specialty [1]. A number of studies have surveyed the health risks facing students during an elective abroad and the pre-travel advice [2-9]. Particular regard has been given to the risk of bloodborne viral infection. For example, it is worrying that 75% of students fail to report exposures to potentially infectious body fluids [4].

Each year approximately 300 students enroll in the medical program at Leiden University Medical Center (LUMC) in The Netherlands. Approximately half of them perform one or more electives abroad. Unlike

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other medical schools, ours allows students to go on electives in countries where infection with Human Immunodeficiency Virus (HIV) is endemic and does not restrict senior students who have completed the fourth college year from performing surgical or obstetric practice in such countries. To receive study credits it is mandatory that the students obtain permission from the student registrar before departure. If study credits are obtained, it is also mandatory for students to seek a Dutch supervisor who assesses the quality of the planned elective and who judges the students' written report at the end of the elective. The registrar's office provides general information on preparation for an elective abroad and advises students to obtain pre-travel counseling and immunization. Although the university occupational health department provides such counseling and immunizations, the students are free to visit any other travel clinic including the LUMC in-hospital travel clinic or their general practitioner. As part of the travel advice, and depending on the destination and intended elective, the health department or travel clinic may refer the student to an infectious disease consultant for counseling on the need of carrying post-exposure prophylaxis for HIV (PEP) with them and on its use. Upon return home, no standard post-travel counseling is offered.

To improve pre-and post-travel care, we performed a questionnaire study of students returning from an elective abroad. We assessed the health risks and the quality and comprehensiveness of pre-and post-travel care. This led to improvements that are described in the discussion.

Methods

All medical students who had performed an elective abroad between July 2006 and December 2008, who had visited countries where hepatitis A is endemic, and who had notified the student registrar to obtain study credits, were sent an informative email asking them to complete a web-based questionnaire. This study was designed in 2008. Students who had returned home prior to December 2007 were sent an email in May 2008. Students who returned between December 2007 and November 2008, which is during the conduct of this study, were sent an email in November 2008. Non-responders were sent a reminder two weeks after the first email. The questionnaire was designed to seek information on pre-travel preparation including vaccinations, on characteristics of the elective, on health risks (in particular the exposure to and protection against bloodborne viruses), on adherence to advice regarding anti-malarial measures and on illness while abroad and upon returning home. In addition the rate of routine screening for tuberculosis using one pre-and one post travel Tuberculin Skin Test (TST) was surveyed. We also surveyed the rate and result of screening for methicillin-resistant Staphylococcus aureus (MRSA) as students visiting foreign hospitals may import MRSA to Dutch hospitals. Finally we surveyed the rate and result of screening for schistosomiasis. The questionnaire was piloted among acquaintances and among staff of the department of Clinical Epidemiology at the Leiden University Medical Center. The protocol of this study (protocol 08/37B) was studied by the Medical Ethics Committee of Leiden University Medical Center in The Netherlands. The Medical Ethics Committee did not object to the conduct of this study.

Results

The mean number of days between having completed the elective and completing the questionnaire was 235 days (interquartile range 121 to 325 days, range 2 to 638 days). The characteristics of the responders and of the electives are described in Table 1; 242 students were sent a questionnaire. Of the 180 (74%) who completed it the majority (78%) was female; 77% had planned a holiday before or after the elective, and the mean duration of the time spent abroad was 74 days (median 69 days, range 10 to 224 days). The majority went for the purpose of a clinical (47%) or pre-clinical elective (16%) as opposed to research or volunteer work (37%). Surinam was visited by 31%, making it the most popular destination. Obstetrics and gynecology (42%) was the most popular rotation. Before departure 90% consulted a center specialized in travel medicine; 4% sought advice from their general practitioner and 6% did not obtain advice from a qualified source.

Risk of infection with bloodborne viruses

All 180 students had been vaccinated against hepatitis B. The vaccine response is checked by the university occupational health department. For privacy reasons we did not have access to the response data; 120 students (67%) performed at least one type of procedure that is associated with an increased risk of exposure to bloodborne viral infection (i.e. surgical or obstetric practice, suturing, phlebotomy) (Table 2). In general, before completing the fourth college year, students have not yet been trained to perform many of these procedures. Therefore it is surprising that of the 58 junior students, 18 (32%) did take part in such activities. Procedures associated with an increased risk of exposure to bloodborne viral infection were also performed in countries with high HIV prevalence rates (Table 3). Some students received medical care while on elective which increases the risk of exposure to bloodborne viruses. Two students received dental care and ten received an intramuscular or intravenous injection.

Depending on type of elective, the destination and the on-site availability of antiretroviral drugs students were

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Table 1 Baseline characteristics of 180 Dutch medical students returning from an elective abroad.

Parameter		
Mean age *years* (range)	23	(19-38)
Female (%)	141	(78)
College year *n* (%)		
Second	18	(10)
Third	39	(22)
Fourth	25	(14)
Fifth	27	(15)
Sixth	71	(39)
Type of elective *n* (%)		
Pre-clinical elective	29	(16)
Clinical elective	85	(47)
Research elective	60	(33)
Volunteer work	6	(3)
Mean duration of stay *days* (range)	74	(10-224)
Travel destination *n* (%)		
Africa	75	(42)
sub-Saharan Africa	54	
Malawi	16	
Cameroon	9	
Ghana	5	
Kenia	5	
Tanzania	4	
Uganda	4	
Other	11	
South-Africa	21	
Latin America	67	(37)
Surinam	56	
Other	11	
Asia	32	(18)
Nepal	11	
Indonesia	10	
China	6	
Other	5	
Middle-East	5	(3)
Eastern Europe	1	(1)
Holiday at the end of the elective *n* (%)	139	(77)

advised to take post-exposure prophylaxis with them; 31 students (17%) carried their own supply of PEP but 12 of these students need not have done so as they did not perform procedures that put them at risk of exposure to HIV. Of the 120 students who did perform such procedures, 66 (55%) either had onsite access to PEP or carried a personal supply; 51 (43%) did not know whether the hospital where they performed their elective had PEP and three students (2%) knew that they did not have onsite access to PEP.

Four students experienced mucosal or percutaneous exposure to potentially infectious body fluids while on elective (two in Surinam, one in South Africa and one in Malawi). Five students were unsure whether the event they had experienced qualified as such. None of the students had reported the exposure at the time it occurred and none had used PEP even though all except one either had onsite access to PEP or carried a personal supply. As a result of their response to the questionnaire these nine students were offered screening for HIV and hepatitis C. For reasons of confidentiality we could not find out whether these students opted to be screened.

Other health risks

Nearly all students (98%) filled out the optional questions regarding sexual contact during the time abroad. Eight female students (6%) and three male students (8%) reported having had sex with a new partner; in seven instances with a partner native to the country where the elective was performed. We did not ask whether a condom was used.

Schistosomiasis may be acquired through fresh water contact; 76 students had swum or waded in fresh water in countries where schistosomiasis is prevalent. Of these students 22 had swum in highly endemic countries in sub-Saharan Africa. Eleven of these 22 students had consulted a physician upon return and had mentioned the fresh water contact, 10 were screened of which two showed seroconversion for antischistosomal antibodies.

One student reported a bite by an unidentified animal in the forest in Surinam. He was not vaccinated for rabies. Overall 28 students had been vaccinated against rabies prior to departure.

Table 2 Number and percentage of 180 Dutch medical students who performed procedures associated with an increased risk of exposure to bloodborne viral infection during an elective abroad.

College year completed	2nd (*n* = 18)	3rd (*n* = 39)	4th (*n* = 25)	5th (*n* = 27)	6th (*n* = 71)	All (*n* = 180)
Activity *n* (%)						
Obstetric practice	2 (11)	6 (15)	6 (24)	6 (22)	50 (70)	70 (39)
Surgical practice	1 (6)	13 (33)	10 (40)	16 (59)	58 (82)	98 (54)
Suturing	0	5 (13)	2 (8)	8 (30)	50 (70)	65 (36)
Phlebotomy	1 (6)	1 (3)	6 (24)	8 (30)	38 (53)	54 (30)
Any of the above	*3 (17)*	*15 (39)*	*15 (60)*	*19 (70)*	*68 (96)*	*120 (67)*
None of the above	*15 (83)*	*24 (62)*	*10 (40)*	*8 (30)*	*3 (4)*	*60 (33)*

Results stratified by college year.

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Table 3 Number and percentage of 180 Dutch medical students who performed procedures associated with an increased risk of exposure to bloodborne viral infection during an elective abroad.

HIV prevalence rate	1-5% (*n* = 77)	5-15% (*n* = 23)	> 15% (*n* = 25)
Activity *n* (%)			
Obstetric practice	48 (62)	11 (48)	3 (12)
Surgical practice	57 (74)	9 (39)	8 (32)
Suturing	42 (55)	5 (22)	9 (36)
Phlebotomy	33 (43)	5 (22)	11 (44)
Any of the above	*66 (86)*	*14 (61)*	*15 (60)*
None of the above	*11 (14)*	*9 (39)*	*10 (40)*

Results stratified by adult HIV prevalence rates in the country where the elective was carried out.

Malaria chemoprophylaxis

The majority of students (83%) who visited areas that are endemic for malaria used a bed net. Of the 129 students who visited such areas nearly all were prescribed an adequate chemoprophylaxis (75 atovaquone-proguanil, 43 mefloquine, two proguanil, one primaquine and one doxycycline). One student had been prescribed chloroquine by a relative and six students did not remember which prophylaxis had been prescribed. Many students visited countries where malaria prophylaxis is only indicated for selective parts of the country. Of this group 17 did not start prophylaxis. In total 112 students started malaria chemoprophylaxis.

Of the 40 students who used mefloquine 18 (33%) reported an adverse effect: mainly sleep or mood disorder. One student returned prematurely due to neuropsychological adverse effects. Of the 62 students on atovaquone-proguanil 12 (19%) experienced an adverse effect: mainly gastro-intestinal complaints. Eight students who used mefloquine (20%) stopped the drug prematurely as did ten students on atovaquone-proguanil (16%) and the student on doxycycline. Only two of these students switched to another prophylaxis. One did so after having had malaria. All students who stopped using mefloquine did so due to adverse effects. Shortage of tablets or simply forgetting to take the prophylaxis constituted the main reasons for stopping the use of atovaquone/proguanil. Premature stopping of prophylaxis left eight students (15%) unprotected during part of their elective in hyperendemic regions in sub-Saharan Africa.

One student in Benin and one in Kenia were diagnosed with malaria. Both had used mefloquine, but the latter was one of those who had stopped the use due to side effects.

Health problems

Diarrhea was the most common illness and was reported by 117 of 180 students (65%). The incidence was even higher (93%) among 40 students who did not have running water at their lodgings. Most cases were self-limiting and did not last beyond a week. However, 25 of 117 students (21%) had diarrhea accompanied by either bloody stools or fever, and in 29 of 117 students (25%) diarrheal illness caused a temporary interruption of the elective for a mean duration of 2.5 days (median 2 days, range 1 to 7 days). Thirteen of 117 students (11%) consulted a physician for diarrheal illness, three were admitted to hospital, and five received intramuscular or intravenous treatment.

Other common health problems were: constipation (33%), skin infections and wounds (29%) and upper respiratory tract infection (11%). Two students were involved in a traffic accident.

Twenty eight students used an antimicrobial agent; thirteen for enteritis, seven for a urinary tract infection and four each to treat a skin infection and respiratory tract infection.

Post-travel

Seven students (4%) reported having had a fever shortly after returning home. Two of these students consulted a physician and one was diagnosed with Dengue. Travel-related illness after having returned home caused five of 180 students to interrupt their medical course for a period of 7 to 28 days; one due to Dengue, one due to neuropsychological problems attributed to the use of mefloquine, one due to an upper respiratory tract infection and two because they were identified as carriers of MRSA. Dutch hospitals have a low MRSA infection rate and adopt a strict policy to prevent spread of this bacterium [10]. Screening for MRSA using pharyngeal and nasal swabs is mandatory for hospital employees with recent employment abroad. Upon return, 79 of 180 students (44%) were screened of which two were found to be MRSA carriers (3%). The main focus of screening should be aimed at senior year students involved in clinical work; 70 of 121 senior year students (58%) had been screened for MRSA. Screening was mainly done at the instigation of hospital occupational health departments.

Depending on the destination and the duration of the elective, students are advised to have themselves tested for tuberculosis before departure and 8 weeks after returning home; 84 of 173 students (49%) had a TST performed after returning home. Two students (2%) had a positive reaction which had been negative before the elective abroad. Both had been on a clinical elective; one in Benin and one in Nepal. Both were referred to the municipal health service for counseling.

Discussion

We assessed the health risks that face medical students on an elective abroad to improve the quality and

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comprehensiveness of pre-and post-travel care. A number of results are related to the risk of bloodborne viral infection. Firstly, we found that regardless of the study year the students were in, none took action following mucosal or percutaneous exposure to potentially infectious body fluids. This result is similar to that of a survey among British medical students [4]. Secondly, junior students on pre-clinical electives often took part in procedures that pose a risk for bloodborne viral infection. We were not informed about the individual capabilities of the students. Junior students may have had extra-curricular training to perform certain procedures before starting the elective, or they may have been supervised adequately during the elective while learning new procedures. Nevertheless, junior students have not yet received the standard curricular training and in general have limited clinical experience. This puts them at a greater risk of mucosal or percutaneous exposure to potentially infectious body fluids while performing procedures. They may also be less well informed how to act in case of such exposure. Thirdly, we found that allocation of PEP starter kits was inadequate. Kits were commonly handed out to students who turned out not to be at risk of coming in contact with potentially infected body fluids and were not handed out to a sizeable group of students who may have been at risk of such exposure. Due to the difficulty in predicting what students will do while on the elective, improving the pre-travel assessment of who should carry a PEP starter kit is not straightforward. Lastly, systematic education on safe sex should be stressed, as 6% of the students reported that they had sex with a new partner while abroad.

This survey also detected other health risks. One in five students stopped using mefloquine due to adverse effects, which means that a sizeable proportion was left unprotected against malaria. Diarrheal illness was very common as is to be expected. Importantly, a small proportion needed to be hospitalized or required intramuscular or intravenous treatment for diarrheal illness. We also found that medical care following return from the elective can be improved upon. Screening for schistosomiasis, tuberculosis and MRSA did not encompass all who should have been screened.

This study has a number of strengths and limitations. It was restricted to students who had applied for study credits, and we expect this group to constitute the majority of students who perform an elective abroad. For a web-based questionnaire, the response rate was relatively high and none of the questionnaires was incomplete. There are two limitations. The survey was not completely anonymous as we asked the age, gender, study year and e-mail address of the participants. This may have prompted socially desirable answers. The time between having completed the elective and filling out the questionnaire was not standardized and was sometimes quite long which may have reinforced recall bias. To reduce the chance of such bias, we mainly surveyed events that are unlikely to be forgotten, such as needle-stick injury, malaria and diarrheal illness.

Measures that are intended to limit the health risks associated with an elective abroad

Based on the results of this study a number of measures have been adopted to reduce the health risks associated with an elective abroad. Firstly, it has been made mandatory that all medical students planning an elective abroad follow a module on Global Health prior to departure [11-13]. The aim of this module is to enhance student safety and student learning, and to highlight the ethical dimension of an elective abroad. Secondly, at the visit to the administrative department all students are now strongly advised to visit the university occupational health department instead of opting to visit another travel clinic or the general practitioner. By centralizing pre-travel advice, as has been suggested by Tilzey and Banatvala [14] we expect to achieve a number of improvements. The risk of bloodborne viral infection and the on-site availability of PEP are systematically assessed. This assessment has been standardized. We now ask students to fill out a form describing which procedures they plan to perform. This form is signed by the Dutch supervisor, who judges whether the student is competent to perform the planned procedures and who judges whether the student will be adequately supervised during the elective in case he/she is to learn a new procedure. Based on this signed form, an assessment can be made during the pre-travel consult whether PEP needs to be provided. Whereas students first had to pay for their PEP kit, it is now provided at no cost by the university. To reduce the threshold for reporting and acting on an exposure to potentially infectious body fluids, the written information has been adapted. It now contains a checklist that specifies which steps to take in case of exposure.

If a traveler is to experience adverse effects when using mefloquine, such effects often manifest in the first few weeks of usage. Therefore it is common policy to prescribe mefloquine on trial prior to departure. By centralizing pre-travel advice we aim to increase the proportion of students that receive mefloquine on trial. We have also adapted the written information. In case of adverse effects which seem attributable to mefloquine, students are advised to use half the dosage twice weekly instead of the standard full dosage once a week in order to lower the peak plasma concentration [15]. Furthermore, students are urged to contact the on call infectious disease consultant in our hospital if they are considering stopping chemoprophylaxis.

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To improve post-travel care, upon return all students must now fill out a standard short web-based checklist which assesses certain health risks (exposure to potentially infected body fluids, the risk of schistosomiasis and the need for screening for tuberculosis and MRSA). This results in a computer generated recommendation which states whether the student needs to contact the occupational health department or another care provider for a post-travel consult.

Conclusion

Many of the health risks that were detected in this survey are probably not unique to Dutch medical students. We believe that adopting a standardized pre-and post-travel consult will reduce these health risks by reinforcing knowledge regarding prevention of bloodborne viral infection, by maintaining a clear-cut policy on provision of PEP, by addressing the problem of treatment limiting adverse events with regard to malaria prophylaxis, by reducing the chance of (latent) tuberculosis and chronic schistosomiasis and by preventing spread of MRSA. In a future survey we intend to see whether the new policy is indeed effective in protecting our medical students by limiting health risks.

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List of abbreviations

(LUMC): Leiden university Medical Center; (HIV): Human Immunodeficiency Virus; (PEP): post-exposure prophylaxis for HIV; (TST): Tuberculin Skin Test; (MRSA): methicillin-resistant Staphylococcus aureus

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Authors' contributions

ES participated in the preparation of the protocol and in data acquisition and in revising the manuscript. DS participated in the data analysis and interpretation of the data and in writing the manuscript. JPV participated in the preparation of the protocol and in interpretation of the data and in revising the manuscript. EH participated in the preparation of the protocol and in data acquisition and in revising the manuscript. LGV conceived the study, participated in the preparation of the protocol, interpretation of the data and in revising the manuscript. All authors read and approved the final manuscript."

Competing interests

The authors declare that they have no competing interests.

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RESEARCH ARTICLE Open Access

Inconvenience due to travelers' diarrhea: a prospective follow-up study

Darius Soonawala*, Jessica A Vlot and Leo G Visser

Abstract

Background: Limited data exist documenting the degree to which travelers are inconvenienced by travelers' diarrhea (TD). We performed a prospective follow-up study at the travel clinic of Leiden University Medical Center in The Netherlands to determine the degree of inconvenience and to determine how experiencing TD affects travelers' perception.

Methods: Healthy adults who intended to travel to the (sub)tropics for less than two months were invited to take part. Participants filled out a web-based questionnaire before departure and after returning home. TD was defined as three or more unformed stools during a 24-hour period.

Results: 390 of 776 Eligible travelers completed both questionnaires. Participants' median age was 31 years and mean travel duration 23 days. Of 160 travelers who contracted TD (incidence proportion 41%, median duration of TD episode 2.5 days) the majority (107/160, 67%) could conduct their activity program as planned despite having diarrhea. However, 21% (33/160) were forced to alter their program and an additional 13% (20/160) were confined to their accommodation for one or more daylight days; 53 travelers (33%) used loperamide and 14 (9%) an antibiotic. Eight travelers (5%) consulted a physician for the diarrheal illness. When asked about the degree of inconvenience brought on by the diarrheal illness, 39% categorized it as minor or none at all, 34% as moderate and 27% as large or severe. In those who regarded the episode of TD a major inconvenience, severity of symptoms was greater and use of treatment and necessity to alter the activity program were more common. Travelers who contracted travelers' diarrhea considered it less of a problem in retrospect than they had thought it would be before departure.

Conclusion: Conventional definitions of TD encompass many mild cases of TD (in our study at least a third of all cases) for which treatment is unlikely to provide a significant health benefit. By measuring the degree of inconvenience brought on by TD, researchers and policy makers may be able to better distinguish 'significant TD' from mild TD, thus allowing for a more precise estimation of the size of the target population for vaccination or stand-by antibiotic prescription and of the benefit of such measures.

Background

Travelers' diarrhea (TD) affects 20-50% of travelers from industrialized regions to developing countries [1-3]. Many travel medicine experts recommend loperamide for mild TD and self-administered antibiotic treatment in case of moderate or severe TD [4-6]. Compared with placebo, antibiotics shorten the duration of diarrhea by 0.7-1.5 days and reduce the number of unformed stools per 24 hour time interval by 1.6 on the first day of treatment, 2.1 on the second day, and 1.4 on the third day [7]. No studies exist that have assessed to what extent early antibiotic treatment significantly impacts the subjective and objective (i.e. incapacitation) degree of inconvenience due to TD. The benefit of prescribing all travelers with antibiotics for self-treatment in case of TD should be weighed against the drawbacks. Although side-effects are seldom serious, use of an antibiotic makes a person more susceptible to colonization by drug resistant *Enterobacteriaceae* [8,9]. Furthermore, large-scale use and disposal of antibiotics in the environment induces resistance among pathogens. For these reasons, there are pro- and opponents regarding routine pre-travel prescription of stand-by antibiotics for travelers [10]. An argument favoring routine prescription

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is that there is an increasing concern about purchasing antibiotics abroad, many being false. A central argument for those who advocate wide-spread use of antibiotics for TD is that it can cause considerable inconvenience, ruin holidays and cause financial loss and that it may cause chronic gastro-intestinal complaints [6,11,12]. A number of studies describe the impact of TD on quality of life and incapacitation [2,3,13-15]. Of those with TD, 20-45% is unable to pursue planned activities for 1 day and the quality of life is affected, mostly with regard to the ability to participate in leisure activities, sexual activity, and the feeling of general well-being [13]. The present prospective follow-up study was designed to determine the degree of subjective and objective inconvenience that Dutch travelers experience when they contract diarrhea during travel to the (sub)tropics. In addition we determined how an episode of TD affects travelers' perception of TD and we explored risk factors.

Methods

Design and study population

This was a single-center prospective cohort study at the travel clinic of Leiden University Medical Center in The Netherlands. It was conducted from March until November 2010. Healthy adults who visited the travel clinic and intended to travel to the (sub)tropics were invited to take part by way of an informative letter. The letter was attached to a standard intake form that clients fill out before their appointment at the travel clinic. All who read the letter were asked to fill out an accompanying answer card that provided three options: (i) "yes, I want to participate", (ii) "no, I do not want to participate", (iii) "I am not eligible to participate". Exclusion criteria were: travel duration of more than two months, use of systemic immunosuppressive medication, history of inflammatory bowel disease or insulin-dependent diabetes mellitus. Participants were sent two web-based questionnaires via e-mail, the first before departure, and the second a week after returning home. In The Netherlands no formal approval by a medical ethics committee is required for this kind of questionnaire study.

The pre-travel consult was not different for participants than for other travelers. All received a brochure about preventive measures and self-treatment with loperamide and oral rehydration solution in case of TD. In The Netherlands, pre-travel supply of antibiotics for self-treatment in case of TD is restricted to high-risk travelers who are at increased risk of severe infection or dehydration, and to those who travel to remote areas with limited access to health care facilities [16].

Definition of travelers' diarrhea

In order to avoid misinterpretation we used a straightforward definition of TD. In the questionnaires TD was defined as: the passage of three or more unformed stools during a 24-hour period with or without additional symptoms [14,17]. In the analyses, 'classic TD' was defined separately as: the passage of three or more unformed stools during a 24-hour period with one or more symptoms of enteric disease such as nausea, abdominal cramps, vomiting, fever or fecal urgency [18,19].

Questionnaires

The first questionnaire (Q1) consisted of questions on past travel to the tropics, past experience with TD and past inconvenience due to TD. In addition, we surveyed the incidence of diarrhea among participants during a two-month period in The Netherlands and during past travel to the tropics. The second questionnaire (Q2) was sent within a week after returning home and dealt with travel characteristics, the incidence of TD and accompanying symptoms, the use of anti- diarrheal medication, the incidence of other health problems, the incidence of TD among travel companions, health-care use for TD and subjective and objective inconvenience due to TD. The *objective* degree to which TD inconvenienced travelers was measured by asking: "To what extent were you inconvenienced by your episode of diarrhea?". Participants could choose one of the following answers: (i) "I interrupted my journey and returned home due to the diarrhea and abdominal complaints", (ii) "I was ill, I altered my activity program and stayed indoors for one or more days due to the diarrhea and abdominal complaints", (iii) "I altered my activity program due to the diarrhea and abdominal complaints", or (iv) "despite the episode of diarrhea, I could take part in all planned activities". Some travelers may have had more than one episode of TD. All questions concerning symptoms of TD and the degree of inconvenience due to TD pertained to the most severe episode. The *subjective* degree of inconvenience due to TD was measured by asking: "To what degree did you experience inconvenience due to the episode of diarrhea?". Participants could choose from the following answers: (i) "no inconvenience", (ii) "a minor degree of inconvenience", (iii) "a moderate degree of inconvenience", (iv) "a large degree of inconvenience", or (v) "a severe degree of inconvenience". In addition, we explored how an episode of TD during travel changed travelers' own perception of TD. This was done as follows. Before departure we asked: "If you were to contract travelers' diarrhea with fecal urgency and abdominal cramps for three days, how large a problem would you consider this to be?". (i) "no problem", (ii) "a small problem", (iii) "neither a small nor a large problem", (iv) "a large problem", (v) "a very large problem". After returning home all travelers were presented a similar scenario pertaining to a hypothetical future travel. We thought that the answer to this question would change in

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travelers who had contracted TD and would remain the same in those who had not. The overall direction in which the answer changes, reflects how experiencing an episode of TD influences the perception of TD. We piloted the questionnaire among travelers, acquaintances and staff of the department of Clinical Epidemiology at Leiden University Medical Center.

Data editing

Travel destination was categorized according to the United Nations (UN) International Migrant Stock [20]. Travel destination was also categorized according to the UN Human Development Index (HDI) value (0 to 1) and UN HDI category (high, medium, low) [21]. The HDI is based upon indicators of life expectancy, education and living standards. If a participant visited more than one country, the HDI value of the country with the lowest HDI was used. In regression analyses, continuous variables that were not linearly associated with the dependent variable were categorized based on exploratory analyses of the continuous data in small categories to see at which values of the continuous variable the regression coefficient changed.

Sample size

The sample size was based on the rule of thumb that 10 cases are needed for each covariate that is introduced in a logistic regression model [22]. Based on an assumed incidence proportion (i.e. the incident number of cases in relation to the size of the population) for TD of 25% we estimated that 400 travelers were needed to be able to introduce a maximum of 10 separate covariates in a logistic regression analysis.

Regression analyses

In a prediction model we explored which variables significantly increased the odds of contracting classic TD. Categorical variables were analyzed with χ^2-tests and continuous variables with t-tests. Variables with $p < 0.2$ were entered in a multiple logistic regression model based on maximum likelihood estimation. Interaction terms were not entered in the model to prevent overfitting and because interaction was deemed unlikely. Cook's distance values, leverage values and standardized residuals were examined to detect cases that might be influencing the model disproportionately. Variance inflation factors were examined to test whether any covariates were highly collinear. The relative strength of each covariate in the final regression model was determined by computing the delta in Nagelkerke R^2 when one covariate was deleted and by dividing delta by the final model's Nagelkerke R^2. In another logistic regression analysis restricted to travelers who had TD, we explored which person- and travel characteristics predicted incapacitation due to TD. In a third model we explored which symptoms predicted incapacitation due to TD. All analyses were done using PASW Statistics, version 18.0, IBM®. Statistical significance was defined as a p-value < 0.05.

Results

Study population and travel characteristics

At our travel clinic 776 of 1,000 travelers fulfilled the inclusion criteria of which 406 provided informed consent (response rate 52%). Of the 224 people who were not eligible to participate, travel duration in excess of two months was the most common exclusion criterion. Three hundred and ninety travelers completed both the pre- and post-travel questionnaire (follow-up rate 96%) (Figure 1). The median age was 31 years (IQR 24-50 years). The majority was female (65%), and had completed higher education (62%) (i.e. a Bachelor degree). Person- and travel characteristics are described in Table 1 and 2. Tourism was the main reason for travel and South-eastern Asia was visited most frequently (31%). The mean travel duration was 23 days (range 4-57 days). At the pre-travel consult, 27 travelers (7%) received a stand-by antibiotic prescription (ciprofloxacin or azitromycin). In total 335 travelers (86%) carried treatment for TD in their travel-kit, mainly loperamide (282/390, 72%), oral rehydration solution (229/390, 59%) or activated carbon (83/390, 21%).

Travelers' diarrhea: incidence, symptoms, treatment and risk factors

One hundred and sixty travelers (160/390, 41%) (26% per any two weeks of stay) contracted TD. Of these 160 travelers with TD, 16 did not have any accompanying symptom of enteric disease such as nausea, abdominal cramps, vomiting, fever or fecal urgency, making the incidence proportion of classic TD 37% (144/390). The overall TD Incidence Rate (IR) was 1.78 cases per 100 person days of travel (pdt). IRs were highest for travelers to Northern-Africa (3.95/100 pdt) and South-central Asia (2.55/100 pdt) (Table 2). Most affected travelers had typical symptoms: watery stools (138/160, 86%), fecal urgency (114/160, 71%) and abdominal discomfort (123/160, 77%) (Table 3). The diarrheal episode lasted a median of 2.5 days (IQR 1-2.5 days). Sixty-five of 160 travelers with TD (41%) started treatment with an anti-motility agent or an antibiotic: 26% (41/160) used loperamide only, 4% (6/160) activated carbon only, 3% (4/160) used both loperamide and activated carbon and 9% (14/160) used an antibiotic, of whom most (9/14, 64%) used the antibiotic in combination with an anti-motility agent. Five travelers who used an antibiotic (5/14, 36%) had been prescribed the antibiotic at the pre-travel consult. Loperamide was started a median of 1 day (IQR 0-2 days) after onset of symptoms. Antibiotics were started later (median 3 days after onset of symptoms; IQR 2-5 days). In total eight travelers (8/160,

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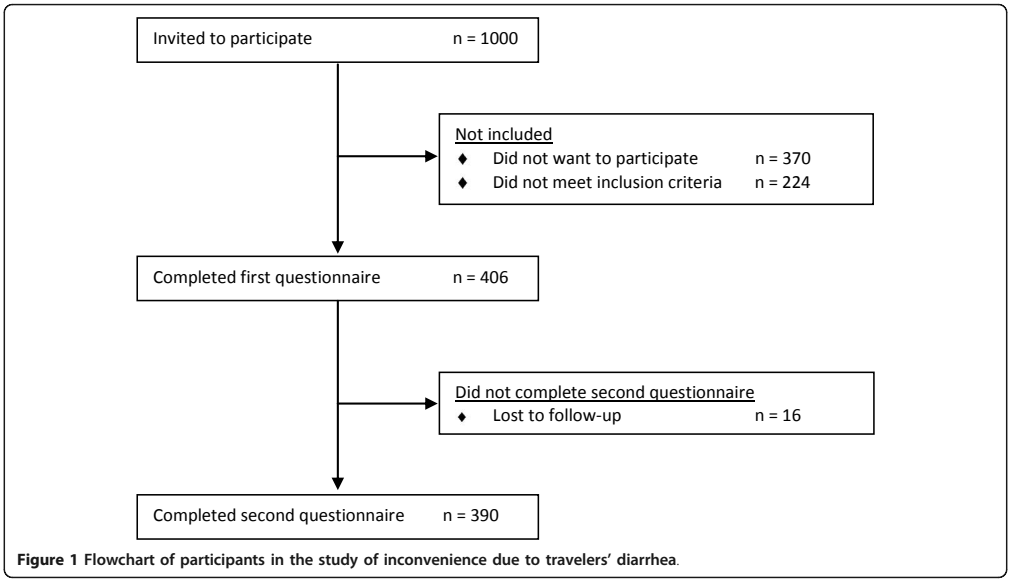

Figure 1 Flowchart of participants in the study of inconvenience due to travelers' diarrhea.

5%) consulted a physician for the diarrheal illness of whom two (2/160; 1%) were admitted to hospital in Africa with fever, diarrhea, vomiting and dehydration. One hundred and two travelers (102/390, 26%) reported non-travelers' diarrhea related health problems: 13 vomiting without diarrhea, 11 abdominal discomfort or loose stools that did not fit the definition of TD, 6 constipation, 24 a respiratory tract infection, 19 a skin or eye infection, 3 a urinary tract infections, 2 fever (1 unknown cause, 1 malaria), 12 headache or tiredness, and 12 some other health problem.

The following variables independently increased the odds of contracting TD: younger age, use of an antacid, longer travel duration, lower Human Development Index of the country that was visited, backpacking as type of travel and staying in luxury hotels. Travelers whose main travel purpose was to visit friends/relatives or who traveled for business/professional reasons had reduced odds for contracting TD. Nagelkerke's R^2 was 0.22, which means that the model accounted for 22% of the variance in TD (Table 1).

Inconvenience due to travelers' diarrhea

Although most travelers (107/160; 67%) could conduct their activity program as planned despite having diarrhea, 21% (33/160) were forced to alter their program and an additional 13% (20/160) were confined to their accommodation for one or more daylight days (median 1 day; IQR 1-2 days). When asked about the degree of inconvenience brought on by the diarrheal illness, 39% (63/160) categorized it as minor or none at all, 34% (54/160) as moderate and 27% (43/160) as large or severe. Severity of symptoms was greater and use of treatment and necessity to alter the activity program were more common in those who were incapacitated due to TD (Table 3). In a logistic regression model, restricted to travelers who contracted TD, none of the person- or travel-characteristics were significantly ($p < 0.05$) associated with incapacitation due to TD. The following symptoms independently increased the odds of incapacitation due to TD: stool frequency, nausea and fever (Table 4).

Before departure, we surveyed the incidence of diarrhea among participants during a two-month period in The Netherlands; 22% answered that they had an episode of diarrhea according to our definition of (travelers') diarrhea. The normal stool pattern of these participants may come close to fulfilling the definition of TD, making these participants more likely to report TD during travel without significant inconvenience. Therefore, we performed a sensitivity analysis in which we excluded these participants. This did not cause a major change in the results. In the remaining subset 62% could conduct their activity program as planned, 27% were forced to alter their program and 10% were confined to their accommodation. In another sensitivity analysis restricted to 144 participants with classic TD, 65% could conduct their activity program as planned, 22% were forced to alter their program and 14% were confined to their accommodation.

Table 1 Travel characteristics and risk factors for travelers' diarrhea in a cohort of 390 Dutch travelers.

Characteristic	All n = 390	Classic TD n = 144	No TD n = 246	Univariate OR [95% CI]	*p*-value	Multivariate OR [95% CI]	Relative contribution of each characteristic to the model's R^2 (%)
Gender, female[1]	253 (65)	98 (68)	155 (63)	1.25 [0.81-1.93]	0.31		
Age					< 0.001		18.6
18-34 years	217 (56)	102 (71)	115 (47)	1.0		1.0	
≥ 35 years	173 (44)	42 (29)	131 (53)	0.36 [0.23-0.56]		0.38 [0.23-0.65]	
Use of an antacid[2]	24 (6)	14 (10)	10 (4)	2.54 [1.10-5.88]	0.03	2.95 [1.14-7.60]	6.8
Born in the tropics[3]	19 (5)	5 (3)	14 (6)	0.60 [0.21-1.69]	0.33		
Traveled to the tropics in the preceding 5 years[4]	251 (64)	91 (63)	160 (65)	0.92 [0.60-1.42]	0.71		
Mean travel duration - *days* (SE)	22.9 (0.65)	25.6 (1.2)	21.4 (0.7)	1.03 [1.01-1.04]	0.002	1.02 [0.99-1.04]	2.7
Travel destination, Human Development Index					0.02		10.6
High	79 (20)	18 (13)	61 (25)	1.0		1.0	
Medium	219 (56)	90 (63)	129 (54)	2.36 [1.31-4.27]	0.004	2.29 [1.22-4.33]	
Low	92 (24)	36 (25)	56 (23)	2.18 [1.11-4.27]	0.02	2.51 [1.19-5.33]	
Main travel purpose[5]							
Holiday	236 (61)	89 (62)	147 (60)	1.09 [0.72-1.66]	0.69		
Visit friends/relatives	55 (14)	13 (9)	42 (17)	0.48 [0.25-0.93]	0.03	0.56 [0.27-1.19]	3.3
Business/professional	32 (8)	5 (4)	27 (11)	0.30 [0.11-0.81]	0.02	0.31 [0.11-0.88]	7.6
Study	51 (13)	29 (20)	22 (9)	2.57 [1.41-4.67]	0.002	1.16 [0.56-2.40]	0.2
Volunteer work	16 (4)	8 (6)	8 (3)	1.75 [0.64-4.77]	0.27		
Type of travel[6]							
Self-arranged, not backpacking	170 (44)	56 (39)	114 (46)	0.74 [0.49-1.12]	0.15	1.17 [0.68-2.01]	0.4
Backpacking	85 (22)	44 (31)	41 (17)	2.20 [1.35-3.58]	0.002	1.89 [0.96-3.70]	4.5
Organized group travel	108 (28)	37 (26)	71 (29)	0.85 [0.54-1.36]	0.50		
Other	27 (7)	7 (5)	20 (8)	0.58 [0.24-1.40]	0.23		
Type of accommodation[7]							
Luxury hotel only	98 (25)	43 (30)	55 (22)	1.48 [0.93-2.36]	0.10	2.94 [1.64-5.29]	18.2
Budget hotel only	95 (24)	34 (24)	61 (25)	0.94 [0.58-1.52]	0.79		
Camping (tent/camper)	26 (7)	9 (6)	17 (7)	0.90 [0.39-2.07]	0.80		
Holiday home	15 (4)	5 (4)	10 (4)	0.85 [0.28-2.53]	0.77		
Stayed with friends or relatives	12 (3)	3 (2)	9 (4)	0.56 [0.15-2.10]	0.39		
Stayed with locals	13 (3)	8 (6)	5 (2)	2.84 [0.91-8.84]	0.07	2.91 [0.80-10.55]	3.4
Combination of the above[†]	131 (34)	42 (29)	89 (36)	0.73 [0.47-1.13]	0.16		
Diarrheal episode 2 months prior to departure[*]					0.003		
No	195/251 (78)	61/91 (67)	134/160 (84)	1.0			

Table 1 Travel characteristics and risk factors for travelers ' diarrhea in a cohort of 390 Dutch travelers. *(Continued)*

Yes	56/251 (22)	30/91 (33)	26/160 (16)	2.54 [1.38-4.65]	
Subjective susceptibility for travelers' diarrhea*					0.12
Never	104/251 (41)	33/91 (36)	71/160 (44)	1.0	
Sometimes	121/251 (48)	44/91 (48)	77/160 (48)	1.23 [0.71-2.14]	0.47
Often/Always	26/251 (10)	14/91 (15)	12/160 (8)	2.51 [1.05-6.02]	0.04

Data are presented as n (%), unless otherwise stated. OR: odds ratio; SE: standard error of the mean; CI: confidence interval. *P*-values based on χ^2-tests for categorical variables and t-tests for continuous variables. Variables with $p < 0.2$ were included in the multivariate logistic regression model. Reference category: [1]male gender, [2]no use of an antacid, [3]born in The Netherlands, [4]not having traveled to the tropics in the preceding 5 years, [5]not the specified travel purpose, [6]not the specified type of travel, [7]not having stayed in the specified type of accommodation. [†]Not included in the multivariate model because it is not a uniform category. *Not included in the multivariate logistic regression model because subjects who had not traveled to the tropics in the past 5 years had missing values for these variables. Model: constant = 0.19, Nagelkerke's $R^2 = 0.22$, Hosmer and Lemeshow test for goodness of fit $p = 0.4$.

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Table 2 Travelers' diarrhea, incidence proportions and incidence rates for 390 Dutch travelers.

Travel destination	Travelers - *n*	TD cases - *n*	TD incidence proportion - % (SE)	Mean travel duration - days	TD Incidence rate - per 100 pdt (SE)
Northern Africa	17	7	41 (12.3)	10.4	3.95 (1.47)
South-central Asia	31	16	52 (9.1)	20.2	2.55 (0.63)
Central America and Caribbean	24	11	46 (10.4)	18.9	2.42 (0.72)
South-eastern Asia	121	61	50 (4.6)	22.5	2.25 (0.28)
Eastern Africa	57	25	44 (6.6)	23.4	1.88 (0.37)
Central Africa	7	3	43 (20.2)	23.4	1.83 (1.05)
Central and Western Asia	32	8	25 (7.8)	14.3	1.75 (0.61)
Western Africa	15	7	47 (13.3)	28.6	1.63 (0.61)
Southern Africa	15	4	27 (11.8)	23.1	1.16 (0.58)
Eastern Asia	36	11	31 (7.8)	29.4	1.04 (0.31)
South America	46	7	15 (5.4)	26.4	0.58 (0.22)
All travelers	401[†]	160	41 (2.4)	22.4	1.78 (0.14)

pdt: person days of travel; SE: standard error. [†]11 participants travelled to more than one destination. NOTE: Incidence rates were not corrected for the time to first episode of TD or for the number of episodes of TD.

Table 3 Characteristics of the episode of travelers' diarrhea for 160 Dutch travelers, stratified by the objective degree of inconvenience.

Objective degree of inconvenience - *n* (%)	Conducted program as planned 107/160 (67%)	Forced to alter program 33/160 (21%)	Confined to accommodation 20/160 (13%)	Total 160 (100%)
Stool frequency - *n* (%)				
3 stools/day	64 (60)	11 (33)	1 (5)	76 (48)
4-5 stools/day	35 (33)	15 (46)	8 (40)	58 (36)
6-10 stools/day	7 (7)	6 (18)	9 (45)	22 (14)
> 10 stools/day	1 (1)	1 (3)	2 (10)	4 (3)
Watery stools, duration - *n* (%)				
No watery stools	20 (19)	1 (3)	1 (5)	22 (14)
1 day	37 (35)	11 (33)	4 (20)	52 (32)
2-3 days	31 (29)	14 (42)	8 (40)	53 (33)
4-7 days	10 (9)	5 (15)	5 (25)	20 (13)
> 7 days	9 (8)	2 (6)	2 (10)	13 (8)
Fecal urgency, duration - *n* (%)				
No fecal urgency	38 (36)	8 (24)	-	46 (29)
1 day	30 (28)	10 (30)	7 (35)	47 (29)
2-3 days	22 (21)	10 (30)	6 (30)	38 (24)
4-7 days	11 (10)	3 (9)	1 (5)	15 (9)
> 7 days	6 (6)	2 (6)	6 (30)	14 (9)
Abdominal cramps, duration - *n* (%)[†]				
No abdominal cramps	32 (30)	3 (9)	2 (10)	37 (23)
1 day	32 (30)	11 (33)	4 (20)	47 (29)
2-3 days	30 (28)	12 (36)	6 (30)	48 (30)
4-7 days	9 (8)	5 (15)	4 (20)	18 (11)
> 7 days	4 (4)	2 (6)	4 (20)	10 (6)
Nausea, duration - *n* (%)				
No nausea	82 (77)	13 (39)	4 (20)	99 (62)
1 day	17 (16)	9 (27)	6 (30)	32 (20)
2-3 days	6 (6)	8 (24)	6 (30)	20 (13)
4-7 days	1 (1)	2 (6)	3 (15)	6 (4)
> 7 days	1 (1)	1 (3)	1 (5)	3 (2)

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Table 3 Characteristics of the episode of travelers ' diarrhea for 160 Dutch travelers, stratified by the objective degree of inconvenience. *(Continued)*

Vomiting - *n* (%)*	13 (12)	12 (36)	7 (35)	32 (20)
Fever - *n* (%)	6 (6)	8 (7)	11 (55)	17 (11)
Treatment - *n* (%)				
Loperamide	29 (27)	11 (33)	14 (70)	54 (34)
Activated carbon	3 (3)	6 (18)	2 (10)	11 (7)
Antimicrobial agent	3 (3)	6 (18)	5 (25)	14 (9)
Subjective degree of inconvenience - *n* (%)				
None/Minor	58 (54)	5 (15)	-	63 (39)
Moderate	33 (31)	13 (39)	8 (40)	54 (34)
Large/Severe	16 (15)	15 (46)	12 (60)	43 (27)

*13 additional travelers who did not have diarrhea reported vomiting; †10 additional travelers who did not have travelers' diarrhea according to the definition, reported abdominal cramps.

Before departure all travelers were asked the following question: "If you were to contract travelers' diarrhea with fecal urgency and abdominal cramps for three days, how large a problem would you consider this to be?". After returning home all travelers were presented a similar scenario. Table 5 shows that the distribution of participants' answers did not shift in those who did not contract TD (p = 0.6, Wilcoxon signed rank test for two-related samples, comparison of the distribution of two variables). However, those who did contract TD tended to consider TD a smaller problem when asked the question upon return than they had thought it would be prior to departure (p < 0.001). Surprisingly, even the participants who were forced to alter their planned activities (p = 0.01) and the participants who were forced to stay indoors (p = 0.03) tended to consider TD less of a problem when asked the question upon return than they had thought it would be before departure.

Discussion

This study was specifically designed to measure the degree of inconvenience brought on by TD. We found that approximately one-third of travelers who contracted TD were forced to change their activity program or stay indoors, which is in line with other reports [2,3,13-15]. Two travelers were even admitted to hospital. Two-thirds did not need to change their activity program and a sizeable proportion (39%) said that the episode of TD caused only minor inconvenience. Those who reported minor inconvenience seldom used an anti-diarrheic agent meaning that the reported degree of inconvenience in this subgroup was not significantly influenced by treatment. As it

Table 4 Logistic regression model evaluating which symptoms best predicted incapacitation due to travelers' diarrhea.

Characteristic	All with TD n = 160	Conducted program as planned n = 107	Incapacitated n = 53	Univariate OR [95% CI]	*p*-value	Multivariate OR [95% CI]
Stool frequency					< 0.001	
3 stools/day	76 (48)	64 (60)	12 (23)	1.0		1.0
4-5 stools/day	58 (36)	35 (33)	23 (43)	3.51 [1.56-7.88]		2.05 [0.77-5.43]
> 5 stools/day	26 (16)	8 (8)	18 (34)	12.0 [4.26-33.8]		4.84 [1.40-16.8]
Abdominal cramps					0.005	
No abdominal cramps	37 (23)	32 (30)	5 (9)	1.0		1.0
1 -3 days	95 (59)	62 (58)	33 (62)	3.41 [1.21-9.57]		1.86 [0.55-6.34]
> 3 days	28 (18)	13 (12)	15 (28)	7.39 [2.22-24.5]		2.64 [0.62-11.3]
Fecal urgency[1]	114 (71)	69 (65)	45 (85)	3.10 [1.32-7.25]	0.009	0.93 [0.32-2.70]
Nausea[2]	61 (38)	25 (23)	36 (68)	6.95 [3.35-14.4]	< 0.001	4.38 [1.70-11.3]
Vomiting[3]	32 (20)	13 (12)	19 (36)	4.04 [1.80-9.06]	0.001	0.96 [0.32-2.91]
Fever[4]	25 (16)	6 (6)	19 (36)	9.41 [3.47-25.5]	< 0.001	5.65 [1.80-17.7]

TD: travelers' diarrhea; OR: odds ratio; CI: confidence interval. *P*-values based on χ^2-tests for categorical variables. Variables with $p < 0.2$ were included in the multivariate logistic regression model. Reference category: [1]no fecal urgency, [2]no nausea, [3]no vomiting, [4]no fever. Model: constant = 0.06, Nagelkerke's $R^2 = 0.42$, Hosmer and Lemeshow test for goodness of fit $p = 0.7$.

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Table 5 How did an episode of travelers' diarrhea (TD) influence travelers' perception of TD? The expected amount of subjective inconvenience due to travelers'' diarrhea before and after travel is stratified by whether travelers had TD.*

	Travelers who had TD n = 160		Travelers who did not have TD n = 230	
	Before departure	After returning	Before departure	After returning
No problem - *n* (%)	1 (1)	11 (7)	1 (0.4)	3 (1)
A small problem - *n* (%)	22 (14)	42 (26)	50 (22)	53 (23)
Neither a small nor a large problem - *n* (%)	51 (32)	56 (35)	61 (27)	57 (25)
A large problem - *n* (%)	69 (43)	49 (31)	99 (43)	99 (43)
A very large problem - *n* (%)	17 (11)	2 (1)	19 (8)	18 (8)

*Participants were presented the following scenarios: *Before departure:* If you were to contract travelers' diarrhea during the coming journey, with a duration of three days accompanied by urgency and abdominal cramps, how large a problem do you think this would be for you? *After returning*: If you were to make the exact same journey in the future and you were to contract travelers' diarrhea with a duration of three days accompanied by urgency and abdominal cramps, how large a problem do you think this would be for you?.

is to be expected, the severity of symptoms was greater in those who regarded the episode of TD a major inconvenience. The travelers' perception of TD changed based on the current experience. Travelers who contracted TD considered it less of a problem in retrospect than they had thought it would be before departure. Surprisingly, this was even true for those who were forced to change their plans and for those who had to stay indoors. Although this finding may simply mean that travelers are less apprehensive about problems they have faced before, it suggests that TD is less of a nuisance than travelers expect beforehand.

Most risk factors for contracting TD were in line with recent reports. Unexpectedly, we found that staying in luxury hotels increased the odds for contracting TD. Travel duration for participants who stayed in luxury hotels was shorter and they were more likely to have traveled to high risk destinations, such as Indonesia and Egypt (data not shown). Residual confounding due to incomplete adjustment for destination and for the time to the first episode of TD may account for (part of) the unexpected association between accommodation in luxury hotels and TD. Alternatively, staying in luxury hotels may be associated with consumption of more elaborate food which bears more risks [13].

This study has a number of strengths. First, participants were recruited before departure. This way we aimed to limit the chance of preferentially selecting travelers with more severe TD who may be more inclined to respond to a questionnaire taken after the facts. Secondly, surveying travelers both before- and after travel, enabled analysis of how travelers' perception of TD changed depending on whether or not TD was contracted during travel. Thirdly, nearly all participants completed both questionnaires, further limiting the chance of bias. Lastly, we measured both the objective and subjective degree of inconvenience. Participants' reporting of both kinds of inconvenience was consistent, which shows that the data are robust. The study also has limitations. First, although we piloted the questionnaire among travelers, acquaintances and epidemiologists, questions could have been misinterpreted. To limit the chance of misinterpretation, participants could contact us by e-mail in case of any ambiguity. We also provided ample opportunity for participants to further specify answers. For example, those who reported that they had to change their activity program or remain indoors due to TD were requested to describe which activities were cancelled. Secondly, the normal stool pattern of some participants may come close to fulfilling the definition of TD, making these participants more likely to report TD without significant inconvenience. This may have led to an underestimation of the inconvenience associated with 'real TD'. However, two sensitivity analyses in which such participants were excluded did not yield different results. Therefore it is unlikely that we underestimated the inconvenience associated with TD during the stay abroad. Thirdly, many travelers used an anti-motility agent or an antibiotic to treat TD. It stands to reason that the degree of inconvenience would have been larger if nobody had used treatment and would have been smaller if all had used treatment. Lastly, TD incidence rates were not corrected for the time to the first episode of TD or for the number of episodes. This may have inflated incidence rates for destinations for which travel duration was longer than average and deflated incidence rates for destinations for which it was shorter than average.

Most cases of TD in this study fitted the classic definition of TD. Overall incidence rates and risk factors were in line with recent reports [1,3,13,14,23]. These aspects increase the generalizability of this study. Some aspects limit the generalizability. Firstly, the study population consisted mainly of Dutch born nationals. Dutch people may be more inclined to await the natural course of a self-limiting illness than travelers from other countries [24]. This could influence the way in which they perceive TD as a problem. However, such cultural differences would probably not impact the objective degree of inconvenience. Secondly, participants were recruited at our travel clinic. The results may not be representative of travelers who do not seek health-related travel advice before

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travel. Furthermore, the response rate was 50%. The demographic features of those who refused to participate may be different. Lastly, although the majority of visitors to our hospital based travel clinic can be classified as 'general travelers', relatively more hospital employees and (bio)medical students visit our travel clinic compared with other out-of-hospital based travel clinics.

Conclusion

This study shows that conventional definitions of TD encompass many cases of mild TD (in our study at least a third of all cases) for which vaccination or antibiotic treatment is unlikely to provide a significant health benefit. By measuring the degree of inconvenience brought on by TD, researchers and policy makers may be able to better distinguish 'significant TD' from mild TD, thus allowing for a more precise estimation of the size of the target population for vaccination or stand-by antibiotic prescription and of the benefit of such measures. We suggest that a future study should investigate to what extent routine stand-by antibiotic prescription impacts on the subjective and objective degree of inconvenience due to TD as well as the incidence of chronic gastrointestinal complaints. This could be done by randomizing a similar group of travelers at the pre-travel consult, either to receive a stand-by antibiotic prescription or not.

List of abbreviations

TD: Travelers' diarrhea; UN: United Nations; HDI: Human Development Index.

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Authors' contributions

DS and JV were involved in the study design, in data collection, in the analysis and interpretation of the data and in drafting the article. LV was involved in the study design, in the interpretation of the data and in drafting the article. All authors gave final approval to the manuscript.

Competing interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Pretravel Preparation and Travel-related Morbidity in Patients with Inflammatory Bowel Disease

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Background: There are no published data on health preparation and travel-related morbidity in patients with inflammatory bowel disease (IBD).

Methods: A retrospective web-based questionnaire study on past travel experiences with more detailed questions concerning the most recent journey. Participants were recruited from the IBD outpatient clinic and via the website of the Dutch patient organization.

Results: In all, 277 patients who had traveled abroad during the past 5 years (172 Crohn's disease, 105 ulcerative colitis) filled out the questionnaire. The majority (62%) answered that IBD limited their choice of travel destinations. Forty-three percent traveled to resource-limited destinations and 76% thereof obtained pretravel advice. Only 48% were prescribed an antibiotic for self-treatment in case of infectious diarrhea, and 23% were not protected against hepatitis A. Fecal urgency and incontinence were the main IBD-related inconveniences. Thirty-two percent reported a new episode of diarrhea and 28% thereof attributed it to an enteric infection. In total, 15/277 (5%) consulted a foreign physician, of whom five were admitted to hospital. Fifty-four (19%) had a self-reported exacerbation of IBD within 2 months following travel and 24% thereof attributed it to the recent travel. The Mantel–Haenszel odds ratio for an exacerbation within a 2-month period after travel was 1.1 (95% confidence interval [CI] 0.7–1.8) when the number of self-reported exacerbations in a 5-year period was used as reference and 1.5 (95% CI 0.9–2.6) when the year 2008 was used as reference.

Conclusions: Pretravel advice for IBD patients was often deficient. There was a considerable amount of travel-related morbidity and inconvenience.

(Inflamm Bowel Dis 2012;000:000–000)

Key Words: inflammatory bowel disease, travel, vaccination

Travelers with inflammatory bowel disease (IBD) are at a greater risk of travel-related morbidity. First, use of immunosuppressive therapy increases susceptibility to and severity of infections,[1–6] attenuates the immune response to vaccination,[4,7,8] and increases the chance of morbidity after vaccination with live attenuated vaccines such as yellow fever vaccine.[9,10] Second, in long-standing IBD functional asplenia may occur, which increases the chance of fulminant infections with polysaccharide encapsulated bacteria and *Plasmodium falciparum*.[11,12] Third, an episode of gastroenteritis, which is the most common travel-related illness, is regarded as a risk factor for an exacerbation of IBD and may influence drug absorption and elimination.[4,13] Use of inactivated carbon to treat diarrhea also influences drug absorption.[14] It stands to reason that pretravel preparation reduces the risk of morbidity.[15] The European Crohn's and Colitis Organization and the Dutch national guideline for pretravel advice offer specific recommendations for (immunocompromised) travelers with IBD[4,16]: 1) An antibiotic should be prescribed for self use in case of gastroenteritis (Evidence Level (EL) 5, Recommendation Grade (RG) D).[17] 2) Acquisition of immunity should be monitored after administering certain vaccines (EL 2a, RG B). 3) Travel to areas where yellow fever is endemic is discouraged if vaccination with live attenuated vaccines is contraindicated (EL 5, RG D). Of note, an inactivated yellow fever vaccine is being developed.[27] 4) Individuals with functional asplenia should be informed of the extra risk associated with malaria and should be vaccinated for *Streptococcus pneumoniae, Haemophilus influenzae*, and *Neisseria meningitides* and should carry an antibiotic for self use in case of fever (EL 5, RG D). 5) As immunosuppressants and in particular tumor necrosis factor alpha (TNF-α) inhibitors increase the risk of tuberculosis, certain high-risk travelers should be screened for tuberculosis before and after travel (EL5, RG D). The rate of *Mycobacterium tuberculosis* infection is of similar magnitude to that of the local population.[18]

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No prior studies have reported on health preparation and travel-related morbidity in patients with IBD. In order to evaluate and improve the quality of pretravel advice we performed a web-based questionnaire study on past travel experience and investigated pretravel preparation of Dutch IBD patients and the quality of pretravel advice. We also surveyed health problems encountered during travel and investigated whether travel increased the risk of an exacerbation of IBD.

MATERIALS AND METHODS

Design and Study Population

Between April 2009 and April 2010 we performed a retrospective web-based questionnaire study on travel experiences in the past 5 years with more detailed questions regarding the most recent journey abroad. Dutch-speaking IBD patients at the outpatient clinic of the Leiden University Medical Center (LUMC) in the Netherlands received an informative letter in the waiting room inviting them to participate in a survey. Patients who provided informed consent were sent a web-based questionnaire via e-mail. LUMC is a tertiary referral center for IBD. To obtain a representative sample of IBD patients' travel experiences we also recruited participants by posting information and a link to the questionnaire on the website of the Dutch IBD patient organization.[19] The study protocol was approved by the Medical Ethics Committee at LUMC.

Definitions

Travel destination was categorized into two groups: 1) countries where hepatitis A is endemic, meaning that vaccination is recommended according to the national guideline (VAC+ countries), and 2) countries where hepatitis A is not endemic (VAC− countries).[16] Drug use was divided into four categories: i) none, or loperamide or mebeverine only; ii) 5-aminosalicylate or topical steroids only; iii) glucocorticoids or immunomodulators (azathioprine, 6-mercaptopurine, methotrexate); iv) TNF-α inhibitors. As specified in the guideline,[16] we defined those who should have been screened for tuberculosis as those who had traveled to high-risk countries for at least 8 weeks, and those who traveled to high-risk countries for less than 8 weeks, but more than 2 weeks and whose travel was characterized by at least two of the following factors: very frequent use of public transportation in resource-limited countries, lodging with local population under resource-limited circumstances or intense (medical) professional contact with the local population.

Questionnaire

All participants filled out questions on disease characteristics. Those who had only traveled to VAC− countries in the past 5 years reported the number of journeys abroad in the past 5 years and answered questions regarding the most recent travel: questions regarding the bowel disease, pretravel preparation, health-related issues while abroad, and travel-related illness upon return. Participants who had traveled to a VAC+ country in the past 5 years answered additional questions on pretravel vaccination for hepatitis A and yellow fever and questions designed to estimate the risk of latent tuberculosis. We piloted the questionnaire among IBD patients and staff of the department of Clinical Epidemiology at our center. A question on the nature of inconvenience due to IBD during travel was added at a later stage and was completed by half of all respondents.

Statistical Analysis

We investigated whether travel abroad was associated with an increased risk of an exacerbation of IBD. For each traveler the expected risk of an exacerbation within any 2-month period was compared with the observed risk of an exacerbation within the first 2 months after returning home from travel. The expected risk of an exacerbation within any 2-month period was calculated by dividing the number of self-reported exacerbations in the past 5 years by 30 (5 years multiplied by 12 months, divided by 2 months). If this fraction exceeded 1, the risk was defined as 1. The observed risk of an exacerbation within 2 months following travel was either 1 or 0, depending on whether such an exacerbation had or had not occurred. Using the observed and expected risk of an exacerbation per traveler we calculated the Mantel–Haenszel overall odds ratio (MH-OR) for the risk of an exacerbation within a 2-month period after travel. Because the number of self-reported exacerbations in the past 5 years may not reflect the current rate of exacerbations per year, a sensitivity analysis was performed which was limited to those who had traveled after 2008 and in which the number of exacerbations in 2008 was used as reference.

All participants were asked whether IBD did or did not influence their choice of travel destinations. In a prediction model we explored which factors influenced respondents' answers to this question. The following variables were analyzed with χ^2-tests for categorical variables and *t*-tests for continuous variables: "gender," "age," "type of bowel disease (Crohn's disease or ulcerative colitis)," "Montreal classification of disease activity and extent,"[20] "time since diagnosis," "past bowel surgery," "type of medication," "work disability due to IBD," and "the average number of exacerbations of IBD over the past 5 years in categories (0 exacerbations per year, up to 1 exacerbation per year, 1 to 2 exacerbations per year, more than 2 exacerbations per year)." Variables with *P*-values < 0.2 were entered in a multiple logistic regression model based on maximum likelihood estimation. Interaction terms were not entered in the model to prevent overfitting. Variables with *P*-values < 0.2 in the final model were reported. Cook's distance values, leverage values, and standardized residuals were examined to detect cases that might be influencing the model disproportionately. Variance inflation factors were examined to test whether any covariates were highly collinear.

TABLE 1. Demographic and Disease Characteristics of 277 Dutch Patients with Inflammatory Bowel Disease (IBD) Who Filled Out a Web-based Questionnaire on Pretravel Preparation and Travel-related Morbidity

Characteristic	Crohn's disease $n = 172$	Ulcerative colitis $n = 105$	All $n = 277$
Mean age, years (SE)	42 (1.0)	43 (1.2)	43 (0.8)
Gender, female *n* (%)	127 (74)	71 (68)	198 (71)
Median time since diagnosis, years (IQR)	13 (8–20)	12 (6–17)	13 (7–19)
Location of disease activity *n* (%)[a]			
Ileum only	45 (26)	—	—
Colon only	48 (28)	—	—
Ileocolon	69 (40)		
Any localization and jejunum or stomach	10 (6)	—	—
Fistulas	66 (38)	—	—
Strictures	74 (43)	—	—
No strictures or fistulas	53 (31)	—	—
Proctitis only	—	11 (10)	—
Left hemicolon only	—	24 (23)	—
Pancolonic	—	60 (57)	—
Unknown	—	10 (10)	—
Past bowel surgery *n* (%)[b]	90 (52)	22 (21)	112 (40)
Colostoma	6 (4)	1 (1)	7 (3)
Ileostoma	14 (8)	8 (8)	22 (8)
Ileorectal anastomosis	4 (2)	1 (1)	5 (2)
Pouch	2 (1)	10 (10)	12 (4)
Current medication for IBD *n* (%)			
None / loperamide / mebeverine only	28 (16)	17 (16)	45 (16)
5-aminosalicylate / topical steroid only	31 (18)	41 (39)	72 (26)
Systemic immunosuppressant	78 (45)	43 (41)	121 (44)
TNF-α inhibitor	60 (35)	13 (12)	73 (26)
Work-disabled (complete or in part) *n* (%)	65 (38)	31 (30)	96 (35)
Comorbidity requiring medication *n* (%)[c]	33/87 (38)	22/60 (37)	55/147 (37)
Admitted to hospital for an exacerbation of IBD in the past 5 years *n* (%)	76 (44)	35 (33)	111 (40)

SE: standard error of the mean; IQR: interquartile range.
[a]Montreal classification of IBD.[20]
[b]i.e., part of the bowel was removed.
[c]Denominator is different due to missing data.

RESULTS

Study Population

In all, 277 IBD patients who had traveled abroad during the past 5 years (172 Crohn's disease, 105 ulcerative colitis) filled out the questionnaire. The response rate at the outpatient clinic was 70%. Respondents' mean age was 43 years, median duration of IBD 13 years, and 40% underwent bowel surgery in the past. At the time of the survey 44% used a glucocorticoid or an immunomodulator, mostly azathioprine, and 26% a TNF-α inhibitor (10% infliximab, 16% adalimumab) (Table 1). None of the participants had been diagnosed with functional asplenia. Fifty-five percent (153/277) had been recruited at the outpatient clinic at LUMC. Use of glucocorticoids or an immunomodulator (73/153, 48%) and of a TNF-α inhibitor (49/153, 32%) was more common in this group than in participants who had been recruited via the website of the IBD patient organization (48/124, 39%, and 24/124, 19%, respectively), reflecting the fact that LUMC is a tertiary referral center for IBD.

Factors Influencing the Choice of Travel Destinations

The majority (171/277, 62%) answered that IBD limited their choice of a travel destination. This was significantly more so for those on a TNF-α inhibitor (odds ratio [OR] 2.2, 95% confidence interval [CI] 1.2–4.3), for those

TABLE 2. Travel Characteristics of 277 Dutch Patients with Inflammatory Bowel Disease Regarding the Most Recent Travel Abroad

Characteristic	All $n = 277$
Destination, *n* (%)	
Countries not endemic for hepatitis A, *n* (%)*	157/277 (57)
Countries endemic for hepatitis A, *n* (%)	120/277 (43)
Eastern Europe	9
Central America	21
South America	28
North Africa	31
South Africa	6
Africa remaining	14
Middle-East	20
Central and Eastern Asia	14
Indian subcontinent	2
South-East Asia	24
Median travel duration, weeks (IQR)	2 (1–3)
Main travel purpose, *n* (%)	
Tourism	216/277 (78)
Visit friends/relatives	39/277 (14)
Business/professional/study/volunteer work	22/277 (8)
Medication for IBD at the time of travel, *n* (%)	
None / loperamide / mebeverine only	38/277 (14)
5-aminosalicylate / topical steroid only	97/277 (35)
Systemic immunosuppressant	123/277 (44)
TNF-α inhibitor	45/277 (16)

IQR: interquartile range;
*Defined as countries for which vaccination against hepatitis A is recommended according to the national guideline for travel medicine.

who underwent bowel surgery in the past (OR 1.5, 95% CI 0.9–2.6), for those who were work-disabled due to IBD (OR 2.2, 95% CI 1.2–3.9), and for those who reported a larger average number of exacerbations of IBD over the past 5 years (reference category 0 exacerbations OR 1.0, up to 1 exacerbation per year OR 2.9 (95% CI 1.3–6.4), 1 to 2 exacerbations per year OR 2.2 (95% CI 0.8–5.6), more than 2 exacerbations per year OR 4.8 (95% CI 1.7–13).

Pretravel Advice and Vaccination

Forty-three percent (120/277) had traveled to countries where hepatitis A is endemic (VAC+), often while on a glucocorticoid or an immunomodulator (58/120, 48%) or a TNF-α inhibitor (20/120, 17%) (Table 2). Of those who traveled to VAC+ countries 76% (91/120) obtained pretravel advice from a qualified source. Fewer men (22/37, 59%) than women (69/83, 83%) obtained pretravel advice. Forty-eight percent (44/91) received a prescription for an antibiotic for self-treatment in case of infectious diarrhea. Travelers to VAC+ countries who underwent bowel surgery in the past may be at a greater risk of dehydration. Therefore, this group in particular should carry an antibiotic to treat infectious diarrhea. Only 53% (17/32) were advised to do so. At the time of travel 23% (27/120) were not protected against hepatitis A (i.e., they had never been vaccinated for hepatitis A and had never been infected with hepatitis A). Guidelines stipulate that the antibody response to inactivated hepatitis A vaccine should be checked in vaccinees on immunosuppressant drugs and that nonresponders should receive passive immunization. Antibody titers were checked in less than half (10/26; 38%) of those in whom the response should have been checked. Regarding vaccination for yellow fever, three of 11 participants (27%) who received the live attenuated vaccine should not have been vaccinated, as two used azathioprine and one a TNF-α inhibitor. All three were vaccinated at specialized travel clinics. Three travelers who visited countries for which yellow fever vaccination is required had never been vaccinated. Two of these travelers visited countries where no cases of yellow fever have been reported in recent years (Kenya and Tanzania), and for which travelers with a contraindication for vaccination are routinely exempted. The other traveled to Uganda and used prednisolone and azathioprine.

Travel-related Morbidity

Fecal urgency and incontinence were mentioned most frequently as the main IBD-related inconveniences during travel (36%). Abdominal discomfort (10%) and fatigue or joint or muscle pain (9%) were also common hindrances. Onset of a new episode of diarrhea was reported by 32% (90/277) and more so by those with Crohn's disease than by those with ulcerative colitis. Past bowel surgery, Montreal Classification of disease activity, and extent and type of medication were not associated with the onset of a new episode of diarrhea (data not shown). Twenty-eight percent (25/90) thought that the diarrheal episode was due to an enteric infection, of which 56% (14/25) used an antimicrobial agent. In total 15/277 (5%) consulted a foreign physician, of whom five were admitted to hospital. Three of those who were admitted had undergone bowel surgery in the past, one of whom had a pouch. Details are specified in Table 3.

Posttravel Screening and Posttravel Morbidity

According to the national guideline, 11 travelers should have been screened for tuberculosis after travel by way of a Tuberculin Skin Test (TST). The guideline is based on travel destination, duration, and intensity of contact with the local population. Nine of these 11 travelers used an immunosuppressant at the time of travel, but none used a TNF-α inhibitor. Three travelers were actually

TABLE 3. Travel-related Morbidity For 277 Dutch Patients with Inflammatory Bowel Disease

Characteristic	Crohn's Disease $n = 172$		Colitis Ulcerosa $n = 105$		All $n = 277$
	VAC− $n = 99$	VAC+ $n = 73$	VAC− $n = 58$	VAC+ $n = 47$	
Any illness while abroad, *n* (%)	55/99 (56)	39/73 (53)	22/58 (38)	18/47 (38)	134 (48)
Onset of a new episode of diarrhea, *n* (%)	37/99 (37)	31/73 (43)	7/58 (13)	14/47 (30)	89 (32)
Duration, *n* (%)					
1-3 days	20/37 (54)	17/31 (55)	5/7 (71)	7/14 (50)	49/89 (55)
3-7 days	12/37 (32)	8/31 (26)	1/7 (14)	4/14 (29)	25/89 (28)
1-3 weeks	2/37 (5)	2/31 (6)	—	2/14 (14)	6/89 (7)
More than 3 weeks	3/37 (8)	2/31 (6)	1/7 (14)	1/14 (7)	7/89 (8)
Does not remember	—	2/31 (6)	—	—	2/89 (1)
Symptoms, *n* (%)					
Vomiting	1/37 (3)	—	1/7 (14)	3/14 (21)	5/89 (6)
Fever	3/37 (8)	—	1/7 (14)	2/14 (14)	6/89 (7)
Treatment, *n* (%)					
None	20/37 (54)	10/31 (32)	5/7 (71)	4/14 (29)	39/89 (44)
Loperamide / activated carbon	13/37 (35)	12/31(39)	1/7 (14)	6/14 (43)	32/89 (13)
Antimicrobial agent	1/37 (3)	7/31 (23)	2/7 (29)	4/14 (29)	14/89 (16)
Oral rehydration solution	—	4/31 (13)	1/7 (14)	2/14 (14)	7/89 (8)
Extra prednisolone	2/37 (5)	1/31 (3)	1/7 (14)	—	4/89 (1)
5-aminosalicylate	1/37 (3)	—	—	—	1/89 (1)
Cause of diarrheal episode (participants' opinion), *n* (%)					
IBD	32/37 (86)	11/31 (35)	3/7 (43)	6/14 (43)	52/89 (58)
Infection	3/37 (8)	12/31 (39)	3/7 (43)	7/14 (50)	25/89 (28)
Does not know	2/37 (5)	8/31 (26)	1/7 (14)	1/14 (7)	12/89 (13)
Consulted a physician while abroad, *n* (%)	5/99 (5)	6/73 (8)	3/58 (5)	1/47 (2)	15 (5)[a]
Admitted to hospital	1/99 (1)	2/73 (3)	1/58 (2)	1/47 (2)	5 (2)[b]
Journey interrupted, returned home	1/99 (1)	—	—	—	1 (0.4)

VAC-: traveled to countries that are not endemic for hepatitis A; VAC+: traveled to countries that are endemic for hepatitis A.
[a]Reasons for consulting a physician while abroad: gastrointestinal complaints/dehydration ($n = 6$, of whom 4 had a history of bowel surgery), joint/muscle pain or trauma ($n = 3$), upper airway tract infection ($n = 2$), uveitis ($n = 1$), altitude sickness ($n = 1$), itch ($n = 1$), hot flushes ($n = 1$).
[b]Details for those who were admitted to hospital: cholangitis/dehydration, South Africa; gastro-enteritis with dehydration due to *Salmonella enteritidis*, admitted in The Netherlands upon return from Tunesia; vomiting/dehydration, Europe; infectious diarrhea/exacerbation colitis ulcerosa, Europe; enteritis, central Asia.

screened before and 8 weeks after travel (3/11, 27%); eight were not, of whom six used an immunosuppressant.

Nineteen percent (54/277) reported an exacerbation of IBD within 2 months following travel; 24% (13/54) attributed the onset to the recent travel. The OR for an exacerbation within a 2-month period after travel, using the number of self-reported exacerbations in a 5-year period as reference, was not increased (MH-OR 1.1, 95% CI 0.7–1.8). Using 2008 as reference, the MH-OR was higher (1.5, 95% CI 0.9–2.6).

DISCUSSION

In this study on past travel experiences among 277 Dutch patients with IBD, we found that more than half of the patients traveled while using systemic immunosuppressive therapy, that 40% traveled to resource-limited destinations, and that one in four failed to obtain pretravel advice and vaccinations for these medically more hazardous destinations. The pretravel consult was deficient in some respects. There was considerable travel-related morbidity and IBD caused much inconvenience and limited the choice of travel destinations. The risk of an exacerbation of IBD after travel was increased, but the increase was not statistically significant.

This is the first survey on pretravel preparation and travel-related morbidity in patients with IBD. Apart from a number of useful websites with advice for travelers with IBD[21–23] and apart from guidelines, review articles, and expert opinion,[4,11,16,24,25] we know of only one cohort study that examined morbidity in travelers with IBD. In

that study few of the 71 IBD patients used systemic immunosuppressive therapy and travel-related morbidity was not significantly higher in patients than in controls.[26] In another study among Dutch renal transplant recipients, a comparable proportion of transplant recipients failed to obtain pretravel advice and a comparable proportion was not protected against hepatitis A. Hospitalization for travel-related morbidity was higher in transplant patients.[13]

Regarding the deficiencies in the pretravel consult, one should take into account that guidelines on travel medicine are based on consensus and that there may be valid reasons not to follow a guideline. First, although IBD patients with a shorter bowel are at increased risk of dehydration and although immunosuppressive drugs increase the risk of severe salmonellosis[6] there are no compelling reasons for all travelers with IBD to carry an antibiotic for self-treatment in case of diarrhea. Opponents may argue that distinguishing infectious diarrhea from IBD activity is very difficult and that unnecessary use of antibiotics may aggravate IBD. Second, our finding that many travelers were not screened for latent tuberculosis can be commented upon. We did not have detailed information on the circumstances under which respondents came in contact with the local population. Therefore, based on our definition, we may have over- or underestimated the "true" proportion that should have been screened for tuberculosis. Furthermore, a TST has reduced sensitivity in IBD patients in general and in those on systemic immunosuppressive therapy in particular.[28,29] An interferon gamma release assay (IGRA) increases sensitivity of screening for tuberculosis. Because IGRA is costly, TST is still used in most travel clinics.

Although we did not find that travel increased the risk of an exacerbation of IBD within a 2-month period after travel, one should realize that there may be inaccuracies in patients' self-reported number of exacerbations and that patients may be more inclined to travel when their disease is in a stable phase. Therefore, the individual's self-reported number of exacerbations over the past 5 years may not be a valid marker for the expected incidence of an exacerbation following travel. In this respect it is interesting that the MH-OR was higher in the sensitivity analysis in which the number of exacerbations in 2008 was used as reference, which may be a better estimate of the expected incidence of an exacerbation after travel. It should be noted that we inquired about exacerbations of IBD within a 2-month period after travel and did not account for the incubation period of travel-related infectious diarrhea. Therefore, it is possible that some self-reported exacerbations were actually episodes of travel-related infectious diarrhea. Such bias may have increased the MH-OR.

This study has a number of limitations. First, it was designed as a questionnaire study taken after the fact. This allowed us to survey a larger number of patients in a shorter time-frame than a study in which participants are recruited before travel. The downside is an increase in the chance of recall bias among respondents and an increase in the chance of preferentially selecting travelers with more severe travel-related morbidity who may be more inclined to respond to a questionnaire taken after the fact. Second, we could not determine the response rate for those who were recruited via the website of the IBD patient organization. Third, besides enteric symptoms the survey only contained a general question on any additional travel-related morbidity. Morbidity that travelers deemed less relevant may not have been mentioned. Last, although we piloted the questionnaire among patients and epidemiologists, questions could have been misinterpreted.

Based on our study, we make the following recommendations: 1) The physician caring for patients with IBD is best positioned to raise awareness of the risks associated with travel and to refer patients to a travel medicine clinic. 2) Travel clinics should check serology after hepatitis A vaccination in those who use systemic immunosuppressants. Even if seroprotection is not attained after one dose, a second dose is often effective, as has been shown in organ transplant recipients.[30] 3) Continued vigilance is needed when prescribing live attenuated vaccines such as yellow fever vaccine. 4) Fecal urgency and incontinence are major sources of inconvenience and should be addressed by IBD physicians and healthcare workers at travel clinics.

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Post-Travel Screening of Asymptomatic Long-Term Travelers to the Tropics for Intestinal Parasites Using Molecular Diagnostics

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Abstract. The incidence of asymptomatic travel-related parasitic infection is uncertain. Previous studies did not distinguish new incident infections, from past infections. Regardless of symptoms, we performed multiplex real-time polymerase chain reaction on pre- and post-travel stool samples of Dutch long-term travelers to the (sub)tropics. Serological screening for *Schistosoma* spp. was only performed in travelers to sub-Saharan Africa. In total, 679 travelers were included in the study. The follow-up rate was 82% (556 of 679). Participants' median travel duration was 12 weeks. There was one incident infection with *Strongyloides stercoralis*; there were none with *Entamoeba histolytica*, 4 with *Cryptosporidium* spp. (1%), and 22 with *Giardia lamblia* (4%). Nine of 146 travelers (6%) seroconverted for *Schistosoma* spp. Routine screening of stool samples for parasitic infection is not indicated for asymptomatic people, who travel to the (sub)tropics for up to 3 months. Screening for *Schistosoma* spp. should be offered to travelers with fresh-water contact in endemic regions.

INTRODUCTION

Asymptomatic infection with protozoa or helminths can cause morbidity long after the primary infection. Travelers to the tropics are at risk of such infections. Asymptomatic infection, in particular with parasites such as *Entamoeba histolytica*, *Strongyloides stercoralis*, or *Schistosoma* spp. can cause devastating morbidity later on in life. Early detection and eradication is beneficial. Some travel clinics offer post-travel screening, which includes screening for parasites. However, the prevalence of asymptomatic parasitic infections after travel remains uncertain. Two sizeable studies have been performed, in which people from highly industrialized countries were screened for parasitic infections, after having stayed in the tropics. Whitty and others[1] determined the use of post-travel screening in the United Kingdom. They studied two cohorts, totaling over 1,000 symptomatic and asymptomatic travelers and expatriates who stayed in the tropics or subtropics for at least 3 months. The majority had lived in the tropics for well over a year. A stool specimen was screened with stool concentrate microscopy. Gut helminths were detected in 3% of samples, as was *Giardia lamblia*. The study did not distinguish between *E. histolytica* and *Entamoeba dispar* cysts, which were detected in 9% of samples. Schistosomal serology was positive in 13% and was limited to those who had stayed in Africa. These results are not generalizable to most long-term non-expatriate travelers, whose travel duration is considerably shorter than 1 year. Furthermore, the sensitivity of traditional microscopic methods to detect parasitic infections is limited, even in experienced well-equipped laboratories. In addition, the available serological tests for *S. stercoralis* either have a limited sensitivity during the early stage of infection, or have a low specificity, and therefore are less useful for screening travelers.[2] A study by Ten Hove and others[3] screened over 2,500 samples from symptomatic and asymptomatic people who visited a large travel clinic in Belgium for a variety of reasons. The majority was born in Europe. The multiplex real-time polymerase chain reaction (PCR) analysis that they used was clearly more sensitive to detect infection with *E. histolytica* (prevalence 0.5%), *S. stercoralis* (0.8%), *G. lamblia* (6%), and *Cryptosporidium* spp. (1.3%) than the more traditional methods. This cross-sectional study could not distinguish recently contracted incident infections from infections that may have been contracted earlier, during past travel or during residency in the tropics.

We aimed to determine the use of routine post-travel screening for asymptomatic parasitic infections. The screening was restricted to parasites that may cause asymptomatic infection and late-onset morbidity. Therefore, we studied the incidence of infection with *E. histolytica*, *G. lamblia*, *Cryptosporidium* spp., *S. stercorali*, and *Schistosoma* spp. in a cohort of Dutch long-term travelers who visited the tropics.

MATERIALS AND METHODS

Design and study population. This prospective cohort study was conducted in The Netherlands from July 2007 until November 2009. Healthy adults who visited the travel clinics at Leiden University Medical Center (LUMC) and Wageningen University and Research center (WUR) and who intended to travel to the (sub)tropics for more than 1 month were invited to take part. The majority of those who visit the travel clinic at LUMC are "general travelers." However, relatively more hospital employees and (bio)medical students visit the travel clinic at LUMC compared with other out-of-hospital based travel clinics. The WUR focuses on the field of agriculture and the living environment and only university students and staff visit the travel clinic at WUR. All participants submitted stool samples and filled out web-based questionnaires before departure and 2 and 12 weeks after returning home. Pre- and post-travel serum samples were only obtained from those who visited sub-Saharan Africa. Stool specimens were sent by regular mail. Pre-travel samples were stored and analyzed later in case of a positive post-travel sample. Post-travel samples (i.e., the samples that were collected 2 weeks and 12 weeks after returning

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home) were processed directly upon arrival. Participants were notified of their result. In case of an infection, the participants' general practitioner was also notified. The study protocol was approved by the Medical Ethics Committee at LUMC and participants provided written informed consent.

Questionnaires. The first questionnaire (Q1) was sent before participants' departure and consisted of questions on past travel to the tropics, current illness, past and current bowel complaints, use of medication, and past infection or treatment of enteric parasites. The second questionnaire (Q2) was sent 2 weeks after participants had returned home and consisted of questions on travel characteristics and (treatment of) intestinal infection during travel. The third questionnaire (Q3) was sent 12 weeks after participants had returned home and dealt with persisting complaints and use of medication after travel. Travelers' diarrhea was defined as the passage of three or more unformed stools during a 24-hour period with or without additional symptoms.[4]

Laboratory methods. *Stool samples.* Participants were asked to fill an empty tube with stool and send it by regular mail to the diagnostic laboratory of LUMC. The DNA isolation and multiplex real-time PCR amplification were performed upon arrival of each post-travel sample. Pre-travel samples were stored at –20°C and analyzed later in case of a positive post-travel sample. The DNA isolation and multiplex real-time PCR were performed as described previously.[3,5,6] For DNA isolation, 200 µL of feces suspension (~0.5 g/mL feces in phosphate buffered saline [PBS] containing 2% polyvinylpolypyrrole [PVPP; Sigma, Steinheim, Germany]) was heated for 10 min at 100°C. After sodium-dodecyl-sulphate-proteinase K treatment (overnight at 55°C), DNA was isolated with the MagNA Pure LC 2.0 instrument using the MagNA Pure LC DNA isolation kit III (Roche, Almere, Nederland). In each sample, a fixed amount of phocin herpes virus 1 (PhHV-1) was added within the isolation lysis buffer, to serve as an internal control for the isolation procedure and to monitor inhibition of the multiplex real-time PCR assays. *Entamoeba histolytica*, *G. lamblia*, and *C. hominis*/*C. parvum* DNA amplification was performed in a multiplex real-time PCR including the PhHV internal control (HGC PCR).[5] *Strongyloides stercoralis* DNA amplification was performed in a separate assay, also including PhHV-1 as an internal control.[6] Amplification, detection and data analysis was performed on the CFX96 real-time detection system (BioRad, Veenendaal, The Netherlands).

Serum samples. Before travel and 12 weeks after travel a serum sample was collected from participants who had visited sub-Saharan Africa. Pre-travel samples were stored at –20°C and analyzed later in case of a positive post-travel sample. Antibodies to *Schistosoma mansoni*-derived somatic antigens (Adult Worm Antigen, AWA) were assessed by an indirect immunofluorescence assay for the detection of immunoglobulin G (IgM) antibodies, using paraffin sections of adult male *S. mansoni* with Rossmann fixative. The IgG antibodies to egg antigens (Soluble Egg Antigens, SEA) were assessed by

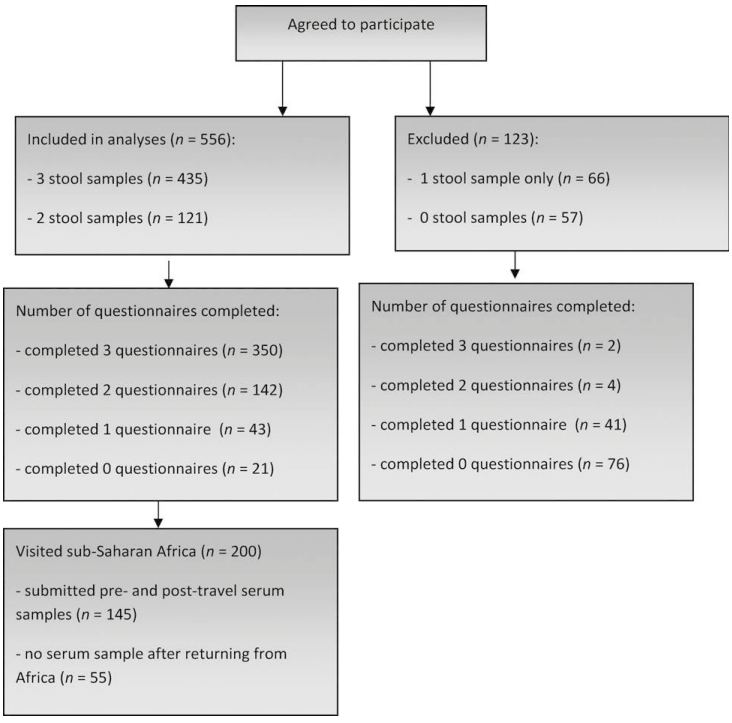

FIGURE 1. Flowchart of participants in the study of post-travel screening for intestinal parasites.

enzyme-linked immunosorbent assay.[7,8] Stool samples of travelers who seroconverted were analyzed for *Schistosoma* spp. using PCR as has been described previously.[9] No urine samples were collected.

Data editing. Travel destination was categorized according to the United Nations (UN) International Migrant Stock.[10]

Sample size. This was a descriptive study. We deemed an incidence rate of at least 1% for each separate parasitic infection to be a significant finding. Based on this assumption, we chose to screen 500 travelers.

RESULTS

Study population, travel characteristics, and travelers' diarrhea. Six hundred and seventy-nine travelers (84% LUMC, 16% WUR) provided informed consent of who 123 were excluded from the analyses because they submitted less than two stool samples (follow-up rate 556 of 679, 82%) (Figure 1); 200 participants visited sub-Saharan Africa of whom 54 did not submit a post-travel serum sample (follow-up rate 146 of 200, 73%). Participants' median age was 25 years (interquartile range [IQR] 23–30) and the median travel duration was 12 weeks (IQR 6–20 weeks). Among the participants from Leiden, South-Eastern Asia was the most popular travel destination. Among the participants from Wageningen, Western and Eastern Africa were the most popular destinations. The main travel purpose was study (55%) and the main mode of travel was qualified as backpacking (47%) (Table 1). The incidence proportion of travelers' diarrhea was very high (74%). The majority reported more than one episode, although it needs to be mentioned that a formal definition of what constituted an episode was not included in the questionnaire (Table 2).

Table 1
Demographic and travel-related characteristics of 556 Dutch travelers*

Characteristic	Leiden N = 467	Wageningen N = 89	All N = 556
Median age, years (IQR)	25 (23–32)	25 (23–26)	25 (23–30)
Gender, female, n (%)	323 (69)	52 (58)	375 (67)
Median travel duration, weeks (IQR)	13 (7–20)	17 (13–22)	12 (6–20)
Travel destination, n (%)†			
Latin America	111 (24)	23 (26)	134 (24)
Central America and Caribbean	38 (8)	6 (7)	44 (8)
South America	85 (18)	19 (21)	104 (19)
Africa	160 (34)	46 (52)	206 (37)
Northern Africa	4 (1)	1 (1)	5 (1)
Western Africa	39 (8)	17 (19)	56 (10)
Middle Africa	14 (3)	3 (3)	17 (3)
Eastern Africa	82 (18)	24 (27)	106 (19)
Southern Africa	42 (9)	4 (5)	46 (8)
Asia	184 (39)	18 (20)	202 (36)
Central and Western Asia	11 (2)	1 (1)	12 (2)
Eastern Asia	34 (7)	2 (2)	36 (7)
South-Eastern Asia	108 (23)	12 (14)	120 (22)
Southern Asia	67 (14)	6 (7)	73 (13)
Unknown	16 (3)	2 (2)	18 (3)
Main travel purpose, n (%)			
Study	170 (36)	75 (84)	307 (55)
Tourism	197 (42)	5 (6)	202 (36)
Volunteer work	58 (12)	4 (5)	62 (11)
Professional	15 (3)	2 (2)	17 (3)
Visit friends/relatives	5 (1)	1 (1)	6 (1)
Unknown	22 (5)	2 (2)	24 (4)
Type of travel, n (%)			
Backpacking	204 (44)	48 (54)	252 (45)
Self-arranged, not backpacking	184 (39)	37 (42)	221 (40)
Organized group travel	57 (12)	2 (2)	59 (11)
Type of accommodation, n (%)			
Guesthouse/small budget hotel	309 (66)	43 (48)	352 (63)
With locals in rural area	43 (9)	23 (26)	66 (12)
With locals in city	43 (9)	19 (21)	62 (11)
Large hotel	32 (7)	–	32 (6)
Camping/boat	17 (4)	3 (3)	20 (4)
Unknown	22 (5)	2 (2)	24 (4)

*IQR = interquartile range.
†Four participants traveled to more than one continent.

Table 2
Travelers' diarrhea, incidence proportions for 496 Dutch travelers

Characteristic	Leiden N = 416	Wageningen N = 80	All* N = 496
Travelers' diarrhea, n (%)*	309/416 (74)	59/80 (74)	368/496 (74)
1 Episode	84/309 (27)	15/59 (25)	99/368 (27)
2 Episodes	82/309 (27)	20/59 (34)	102/368 (28)
More than 2 episodes	143/309 (46)	24/59 (41)	167/368 (45)
Accompanying symptoms, n (%)			
Abdominal cramps	178/309 (58)	26/59 (44)	204/368 (55)
Nausea	89/309 (29)	14/59 (24)	103/368 (28)
Vomiting	78/309 (25)	10/59 (17)	88/368 (24)
Fever	54/309 (17)	9/59 (15)	63/368 (17)
Blood in stool	10/309 (3)	3/59 (5)	13/368 (4)
Travelers' diarrhea by continent, n (%)			
Latin America	73/111 (66)	15/23 (65)	88/134 (66)
Africa	116/160 (73)	32/46 (70)	148/206 (72)
Asia	122/184 (66)	12/18 (67)	134/202 (66)

*Data is missing for 60 travelers who did not return the questionnaire after travel. Diarrhea was defined as the passage of three or more unformed stools during a 24-hour period.

Post-travel screening results: fecal parasites. The results of real-time PCR for all fecal parasites are summarized in Table 3. None of the 542 stool samples obtained 2 weeks after participants' return was positive for *E. histolytica*. Twelve weeks after travel only 1 of 437 samples (0.2%) was positive for *S. stercoralis* (Ct-value 27.5). This participant was asymptomatic and had traveled to Indonesia for 14 weeks of field work involving water management. No rhabditiform larvae were detected with microscopy of a direct smear of the same sample, possibly because the sample was no longer fresh. *Strongyloides* serology was positive. His pre-travel stool sample was negative, as was his sample taken 2 weeks after return (i.e., no specific amplification). He was treated with ivermectin. *Cryptosporidium* spp.-specific amplification was detected in four samples (1%). The pre-travel samples of these participants were negative for *Cryptosporidium*. With microscopy

Table 3
Results of routine post-travel screening of stool samples of long-term travelers to the tropics using multiplex real-time PCR and serological screening*

Characteristic	Leiden	Wageningen	All
Entamoeba histolytica, n (%)	0/456 (0)	0/86 (0)	0/542 (0)
Giardia lamblia, n (%)	19/456 (4)	10/86 (12)	29/542 (5)*
Cryptosporidium spp., n (%)	3/456 (1)	1/86 (1)	4/542 (1)
Strongyloides stercoralis, n (%)	0/369 (0)	1/78 (1)	1/437 (0.2)
Schistosoma spp. Seroconversion, n (%)	7/113 (6)	2/33 (6)	9/146 (6)

*Twenty-three of 542 (4%) were newly infected with *G. lamblia* after travel and 6 of 29 were already infected, but asymptomatic, before departure. Half of the travelers who were infected with *G. lamblia* after travel reported gastrointestinal complaints (i.e. abdominal discomfort and/or diarrhea), for which the majority did not consult a physician.

using carbol-fuchsine-staining *Cryptosporidium* oocysts were seen in 3 of 4 PCR-positive samples (75%). *Giardia lamblia*-specific amplification was detected in 29 individuals (5%). Six of these participants were also positive for *G. lamblia* before departure. These six participants did not have abdominal complaints before departure, nor did they develop complaints after returning home. With microscopy, *G. lamblia* cysts and/or trophozoites were seen in 23 of 26 (88%) PCR-positive samples. In the PCR, Ct-values ranged from 20.2 to 35.3 (median 26.7). As expected, negative microscopy was associated with higher Ct values (*P* value 0.002, Kruskal-Wallis test).

***Giardia lamblia* and *Cryptosporidium* spp., symptoms, risk factors, and treatment.** The four cases of cryptosporidium infection were detected in travelers returning from Western Africa (two cases) and Eastern Africa or Southern Asia (one case each) (Table 4). All four had experienced travelers' diarrhea and three had abdominal complaints in the first 2 weeks after having returned home. None were treated. Twelve weeks after travel *Cryptosporidium* spp. were no longer detected. The incidence proportion of travelers' diarrhea and travel duration was not higher in those who were newly infected with *G. lamblia*, compared with all other travelers. Half of all travelers infected with *G. lamblia* were asymptomatic at the time of diagnosis. Ct values for *G. lamblia* were not higher in symptomatic compared with asymptomatic participants. Gastrointestinal complaints (i.e., abdominal discomfort and/or diarrhea) were more common in those infected with *G. lamblia* (48%) than in their non-infected counterparts (31%) (relative risk [RR] 1.6, 95% confidence interval [CI] 1.0–2.4). Of note, two symptomatic participants who had contracted *G. lamblia* in Ghana did not respond to treatment with metronidazole.

***Giardia lamblia* and *Cryptosporidium* spp., household contacts.** We screened 27 household contacts of 11 *G. lamblia* infected travelers and eight household contacts of two *Cryptosporidium*-infected travelers. *Giardia lamblia* was detected in one household contact of a traveler who had contracted *G. lamblia* during travel, and in one household contact of a traveler whose pre- and post-travel sample was positive for *G. lamblia*. *Cryptosporidium* spp. was not detected.

Post-travel screening results: schistosomiasis. Twelve weeks after travel, nine of 146 travelers (6%) seroconverted for *Schistosoma* spp. All had been swimming in Lake Malawi or Lake Victoria. Seven were asymptomatic and two had Katayama syndrome. Three had antibodies to both SEA and AWA. Six only had antibodies to AWA. The stool samples of these nine travelers were analyzed for *Schistosoma* spp. using PCR. Two, both with antibodies to AWA only, were positive. Treatment and follow-up was done by the travelers' general practitioners.

TABLE 4

Incidence proportion of infection with *Giardia lamblia* and *Schistosoma* spp. according to travel destination

Characteristic	*Giardia lamblia*, n (%)	*Schistosoma* spp. Seroconversion, n (%)
Latin America*	7/133 (5)	–
Central America and Caribbean	2/44 (5)	–
South America	6/104 (6)	–
Africa	10/205 (5)	9/146 (6)
Northern Africa	0/5 (0)	–
Western Africa	7/56 (13)	0/37 (0)
Middle Africa	1/17 (6)	0/11 (0)
Eastern Africa	2/106 (2)	9/78 (12)
Southern Africa	0/46 (0)	0/27 (0)
Asia*	12/202 (6)	–
Central and Western Asia	1/12 (8)	–
Eastern Asia	2/36 (6)	–
South-Eastern Asia	3/120 (3)	–
Southern Asia	9/73 (12)	–

*A few participants traveled to more than one region or continent.

DISCUSSION

The incidence of asymptomatic infection with *E. histolytic*a and *S. stercoralis* after travel was low. Only one infection with *S. stercoralis* was found in over 400 travelers and no infection with *E. histolytic*a in over 500 travelers. Therefore, routine screening of asymptomatic travelers is not indicated for those who travel to the (sub)tropics for up to 3 months. The incidence of infection with *Schistosoma* spp. was higher. However, each case was associated with exposure to highly endemic lakes in Malawi and Tanzania. Therefore, screening for *Schistosoma* spp. can be limited to travelers with a history of exposure to fresh water exposure in endemic regions. In the study by Whitty and others,[1] freshwater exposure did not correlate with schistosomiasis. However, their result may be influenced by a considerable amount of recall bias, considering the fact that most participants had lived in the tropics for well over a year.

Infection with *G. lamblia* was seen in 5% and was associated with gastrointestinal complaints in 48%, meaning that half of the infected participants were asymptomatic. Participants, who already had *G. lamblia* in their stool before departure, did not have abdominal complaints. Infection is usually self-limiting and does not have long-term repercussions. Furthermore, the prevalence of asymptomatic *G. lamblia* infection in The Netherlands is comparable to the post-travel incidence in this study[11]; therefore, routine screening of asymptomatic travelers for *G. lamblia* is not warranted.

This study has a number of strengths. First, the participants were recruited prospectively and samples were collected before and after travel. This allowed us to differentiate post-travel incident cases from pre-existent infections. Second, we used a well-validated multiplex real-time PCR to screen stool samples. This method is more sensitive than traditional methods to detect infection.[3,12,13] Furthermore, it can differentiate pathogenic *E. histolytica* from apathogenic *E. dispar*. Finally, although 18% were lost to follow-up, there is no reason to assume that these participants had higher rates of symptomatic or asymptomatic infection. This study also has limitations. The results may not be representative of all travelers. First, the results pertain to travelers who seek health-related travel advice before travel and who traveled for ~3 months. Post-travel screening of specific groups of asymptomatic travelers, such as migrants, expatriates, or aid workers may yield higher infection rates. Second, half of the participants in this study were students. Although most students combined their stay abroad with travel for touristic purposes, this may limit the generalizability of this study.

To conclude, based on the low incidence of infection, routine screening for *E. histolytica*, *S. stercoralis*, *Cryptosporidium* spp., and *G. lamblia* is not indicated for asymptomatic people, who travel to the (sub)tropics for up to 3 months.

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The Immune Response to Schistosome Antigens in Formerly Infected Travelers

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Abstract. We investigated the type and strength of the immune response to schistosome antigens in a group of 20 Dutch travelers who had been infected with *Schistosoma* spp. during a group visit to Mali in 1991 and 8 non-infected controls. At the time, 9 had Katayama syndrome (KS), and 11 remained asymptomatic. All had been treated with praziquantel. Eight years later, serology remained positive in all 20 formerly infected travelers. The lymphocyte proliferative responses and cytokine responses (interleukin 13 [IL-13], IL-10, and interferon [IFN-γ] responses to soluble egg antigens and the IL-13, IL-10, and IL-5 response to adult worm antigen) were stronger in the travelers than in the controls and tended to be stronger in those with KS compared with those who had remained asymptomatic. In conclusion, *Schistosoma* infection induced a memory immune response, and people who experienced KS tended to have a stronger immune response to schistosome antigens than their asymptomatic counterparts.

INTRODUCTION

Most of the information on the immunopathology of schistosomiasis is derived from murine models.[1–6] Studies in humans have mainly focused on chronic infections seen in endemic areas.[7] The acute response known as Katayama syndrome is thought to occur in non-immune hosts only.[8] Prior exposure to antigens *in utero*[9–12] or infection early as opposed to late in life is believed to account for this difference in symptoms between persons living in an endemic area and non-immune hosts.

Several studies have analyzed the acute response after a primary *Schistosoma* infection.[13–16] The symptoms in non-immune hosts vary widely.[17,18] Some non-immune subjects develop Katayama syndrome, whereas others remain (virtually) asymptomatic The reason for this difference remains unknown.[19–21] Immunologically, eosinophilia and circulating immune complexes have been associated with acute schistosomiasis,[14,17,19] and it has been suggested that the cause of Katayama syndrome is a systemic hyperreactive immune response to migrating schistosomula.[21]

Schistosomiasis in travelers can be considered an experiment of nature with a defined exposure in time, a non-immune host, low infection intensity, and lack of coinfection or reinfection. The aim of the present study was to investigate the type and strength of the cellular immune response to schistosome antigens in a defined group of previously treated travelers. The secondary aim was to analyze the difference in the immune response between those who had and those who had not experienced Katayama syndrome.

MATERIALS AND METHODS

Subjects. Subjects were recruited from an single episode of schistosomiasis that occurred among 28 Dutch travelers who had been infected during a swim in fresh water pools in the Dogon area in Mali in 1991.[17] At the time, 15 had developed Katayama syndrome, which was defined as occurrence of two or more of the following symptoms: fever, sweating, abdominal pain, myalgia, arthralgia, diarrhea, dry cough, weight loss, hepatomegaly, splenomegaly, urticaria, or swollen eyelids.

Treatment with praziquantel had resulted in parasitological cure in all travelers. In 1999, when this current study was performed, 21 of the initial 28 subjects could be contacted for collection of venous blood. To exclude actual *Schistosoma* infection, stool and urine samples of all 21 subjects were screened for schistosome eggs by sedimentation selective filtration methods.[22] In short, washed stool samples were sifted first through a sieve with 106-μm pores and then through a sieve with 53-μm pores. Five wet smears of each sample were searched for schistosome eggs. Urine samples were centrifuged for 10 minutes at 2,500 rpm, and the entire sediment was examined. Stool and urine tests were performed two times on separate occasions before considered negative. As controls, eight Dutch individuals who had never traveled to *Schistosoma*-endemic regions provided venous blood.

Serology. Antibodies to *S. mansoni*-derived somatic antigens (adult worm antigen [AWA]) were assessed by an indirect immunofluorescence assay (IFA) for the detection of immunoglobulin M (IgM) antibodies using paraffin sections of adult male *Schistosoma* mansoni with Rossmann fixative. IgG antibodies to egg antigens (soluble egg antigens [SEA]) were assessed by enzyme-linked immunosorbent assay (ELISA).[23,24]

Antigens. AWA and SEA were prepared from 1.5 to 2 g *S. mansoni* adult worms and eggs, respectively. After homogenizing in an all-glass homogenizer in a 0.035 M phosphate buffered saline (PBS), pH 7.8, at 0°C, the homogenate was transferred to a glass tube and sonicated for 3 minutes at level 7 in a sonicator (Branson Sonic Power Company, Sonicator B-12 power supply and converter, Danbury CT) at 0°C. Next, the homogenate was centrifuged for 20 minutes at 25,000 rpm at 4°C, and the supernatant was collected. The pellet was homogenized again, and the supernatant was collected for a second time. The first and second collected supernatants were pooled together and dialyzed against distilled water at 4°C. During this procedure, the water was changed two times. The dialyzed supernatant was lyophilized and stored at 4°C. The protein content of the antigen fractions in the dialyzed supernatants was determined by a bichronic acid method (BCA; Pierce III, Rockford, IL) against standard series from solution of bovine serum albumin. Finally, the antigens were dissolved in Iscoves medium at a protein concentration of 20 μg/mL.

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Purified protein derivative (PPD) of *Mycobacterium tuberculosis* (Statens Serum Institute, Copenhagen, Denmark) was diluted in Iscoves medium (Gibco, Pailsey, Scotland) to a concentration of 20 µL derivative per 1 mL. Tetanus toxoid (TT; RIVM, Bilthoven, The Netherlands) was diluted to a concentration of 1.5 Lf (flocculation units) per 1 mL of Iscoves medium. Phytohaemaglutine (PHA; Murex Biotech Ltd., United Kingdom) was diluted to a concentration of 4 µg per 1 mL of Iscoves medium.

Cellular stimulation assay. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. Cells were frozen in Roswell Park Memorial Institute medium (RPMI; Gibco) supplemented with 2 mM/L glutamine, 1 mM/L pyruvate, 20% (vol/vol) pooled human serum, and 10% (vol/vol) dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany). Viability after thawing was determined by trypan blue dye exclusion. Only cell suspensions with at least 90% viability were used. For the proliferation assay, PBMC (10^5 cells per well) were incubated in flat-bottomed microtiter wells (NUNC maxisorb; Life Technologies, Breda, The Netherlands) in 100 µL of Iscoves medium (Gibco) supplemented with 10% (vol/vol) pooled human serum, 2 mM/L glutamine, 1 mM/L pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin in triplicate at 37°C in humidified air containing 7.5% $C0_2$ in the presence or absence of antigen.

For the determination of cytokine production, PBMC (10^6 cells per well) were incubated in round-bottomed microtiter wells (NUNC maxisorb) in the presence or absence of antigen in 100 µL of Iscoves medium (Gibco) supplemented with 5% (vol/vol) fetal calf serum, 2 mM/L glutamine, 1 mM/L pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin in triplicate at 37°C in humidified air containing 7.5% $C0_2$. After the indicated time, the supernatants were collected, and they were immediately frozen and stored at –20°C for subsequent determination of cytokine production. The final concentrations of antigens used were: AWA, 10 µg/mL; SEA, 10 µg/mL; PHA, 2 µg/mL; PPD, 10 µg/mL; TT, 0.75 Lf.

Proliferation assay. The lymphoproliferative responses to antigen stimulation (AWA and SEA) and stimulation with the mitogenic stimulus PHA were determined by adding 1 µCu of [^{3}H]-thymidine at day 5. After 15 hours of incubation with [^{3}H]-thymidine, uptake was measured by a scintillation counter. Values were expressed as stimulation index (SI). SI equals the geometric mean of (mean counts per minute [cpm] of the stimulated culture)/(mean cpm of the unstimulated cultures).

Cytokine production. Supernatants were collected for determination of interleukin 10 (IL-10) and IL-13 on day 3 and interferon (IFN-γ) and IL-5 responses on day 5. Cytokines were measured by use of ELISA using specific capture and detection monoclonal antibodies (IFN-γ, IL-13, and IL-10, Pelikine Compact ELISA kit; Central Laboratory of Bloodtransfusion, Leiden, The Netherlands and IL-5; BD; Pharmingen; Franklin Lakes, NJ). The detection limits of the assays were 3 pg of IFN-γ/mL, 3 pg of IL-10/mL, 3 pg of IL-5/mL, and 3 pg of IL-13/mL. The upper limit was 30.000 pg/mL, and any value above was defined as 30.000 pg/mL. Detectable values in unstimulated cultures were subtracted from the value in stimulated cultures. When this difference was negative, the value of produced cytokine after stimulation was defined as 1.5 pg/mL. Cytokine responses could not be determined in all subjects because of technical problems with the assay and the limited amount of blood.

Statistical analysis. Differences between responses were tested with the non-parametric Mann–Whitney test. Statistical significance was defined as a P value < 0.05. No correction was made for multiple testing.

RESULTS

Study subjects' infection and clinical status. Twenty-one subjects who had been treated for schistosomiasis in 1991 volunteered to participate in the current study. At the time of diagnosis in 1991, all subjects had positive schistosome serology, and 15 subjects (71%) had eggs in the feces and/or urine. In 1999, renewed microscopic examination of stool and urine was performed two times on separate occasions in all subjects. Schistosome eggs were found in the stool of only 1 traveler. This patient was treated with praziquantel and excluded from further analysis. Twenty travelers were included in the present analysis, 9 who had suffered Katayama syndrome in 1991 and 11 who were asymptomatic. Of these 20 subjects, 14 (70%) had eggs in the feces and/or urine in 1991. Twelve of these subjects (12/14; 86%) had been infected with *S. mansoni*, often as part of a mixed infection with *S. haematobium* (Table 1).

Serologic response to AWA and SEA. Serum antibodies to AWA and SEA were determined in all travelers at 12.6 (±2.5) weeks after fresh water exposure in 1991 and 55.7 (±15) weeks and 8 years after treatment. Eight years after treatment, none of the travelers had reverted to negative serology for both AWA and SEA, although IgM anti-AWA titers had decreased. Median IgM anti-AWA titers were: 1:1,024 (IQR = 1:1,024–1:2,048) at 12.6 weeks, 1:1,024 (IQR = 1:512–1:1,024) at 55.7 weeks, and 1:362 (IQR = 1:128–1:861) at 8 years. Median IgG anti-SEA titers were 1:128 (IQR = 1:64–1:256) at 12.6 weeks, 1:256 (IQR = 1:76–1:256) at 55.7 weeks, and 1:128 (IQR = 1:76–1:256) at 8 years. At all three time points, median antibody levels did not differ significantly between the group with Katayama syndrome and the group that had remained asymptomatic.

Lymphocyte proliferative response. The lymphoproliferative response could be determined for 18 of 20 travelers. A lymphoproliferative response was seen to both AWA and SEA in the 18 formerly infected travelers but not in 8 non-infected controls: the median SI in travelers in response to AWA was

TABLE 1
Patient characteristics

	Katayama syndrome (N = 9)	No Katayama syndrome (N = 11)	Controls (no infection; N = 8)
Male/female	4/5	4/7	5/3
Mean age in years	52.0	52.1	22
Number of subjects positive for *Schistosoma* spp. eggs in stool or urine in 1991	7 (78%)	7 (64%)	–
S. mansoni	3 (33%)	1 (9%)	–
S. intercalatum	1 (11%)	0	–
S. haematobium	0	1 (9%)	–
S. mansoni and *S. intercalatum*	2 (22%)	2 (18%)	–
S. mansoni and *S. haematobium*	0	2 (18%)	–
S. mansoni, *S. intercalatum*, and *S. haematobium*	1 (11%)	1 (9%)	–
No eggs found	2 (22%)	4 (36%)	–
Number of subjects positive for *Schistosoma* spp. eggs in stool or urine in 1999	0	0	–

TABLE 2
Stimulation index to AWA, SEA, and PHA 8 years after treatment in 20 Dutch travelers*

	Stimulation index median (mean ± standard error)		
Antigen	Katayama syndrome ($N = 8$)	No Katayama syndrome ($N = 10$)	P value†
AWA	30 (33 ± 10)	9 (18 ± 7)	0.17
SEA	15 (13 ± 2)	7 (10 ± 5)	0.08
PHA	408	568	0.48

SEA = soluble egg antigen; AWA = adult worm antigen; PHA = phytohaemaglutinin.
*The stimulation index was not determined for 1 person in each group, because their sample had less than 90% viable peripheral blood mononuclear cells after thawing.
† Mann–Whitney test.

15 (mean = 25, standard error = 6) and in response to SEA was 10 (mean = 12, standard error = 3); the median SI in controls in response to both AWA and SEA was 1 (mean = 1, standard error = 1). Although the median responses were stronger in those who had experienced Katayama syndrome in the past (SI for AWA = 30, SI for SEA = 15) compared with those who had remained asymptomatic (SI for AWA = 9, SI for SEA = 7), these differences did not reach statistical significance (P values for the differences = 0.17 and 0.08, respectively) (Table 2).

Cytokine responses. In comparison to non-infected controls, travelers had higher levels of IL-5, IL-10, and IL-13 in response to AWA. In response to SEA, travelers showed higher production of IFN-γ, IL-10, and IL-13 (Figure 1). Travelers who had experienced Katayama syndrome in the past showed higher production of IL-13 ($P = 0.03$) in response to AWA and higher production of IL-13 ($P = 0.009$), IFN-γ ($P = 0.004$), and IL-5 ($P = 0.06$) in response to SEA compared with those who had been infected but remained asymptomatic (Figure 1). The IL-10 responses were similar in those with and without Katayama syndrome. Ten samples were taken at random and stimulated with TT and PPD. No differences in production of IL-5, IL-10, IL-13, and IFN-γ were seen between four travelers who had Katayama syndrome and six travelers who did not.

DISCUSSION

Eight years after treatment of schistosomiasis, positive serology persisted in all 20 travelers. There was also a specific lymphoproliferative response to schistosome antigens, which indicates that an acute schistosome infection in a naïve subject induces a memory response that lasts for at least 8 years. Long-lasting positive serology after treatment of schistosomiasis is consistent with previous reports.[25,26] This may be caused by persisting egg antigens, providing a stimulus to the immune system even after worms are eliminated. However, worms have been known to survive in the human host for up to 31 years,[27] and treatment has been known to fail. Egg secretion was the only method to establish whether an active infection was still present. Although we performed microscopic analysis two times, examining five wet smears per stool sample and the entire urine sediment on each occasion, we cannot exclude the possibility of persisting low-grade infection as a cause of long-lasting positive serology. Furthermore, the antibodies can be cross-reactive to antigens from other sources such as certain carbohydrates.[28] Therefore, even without infection,

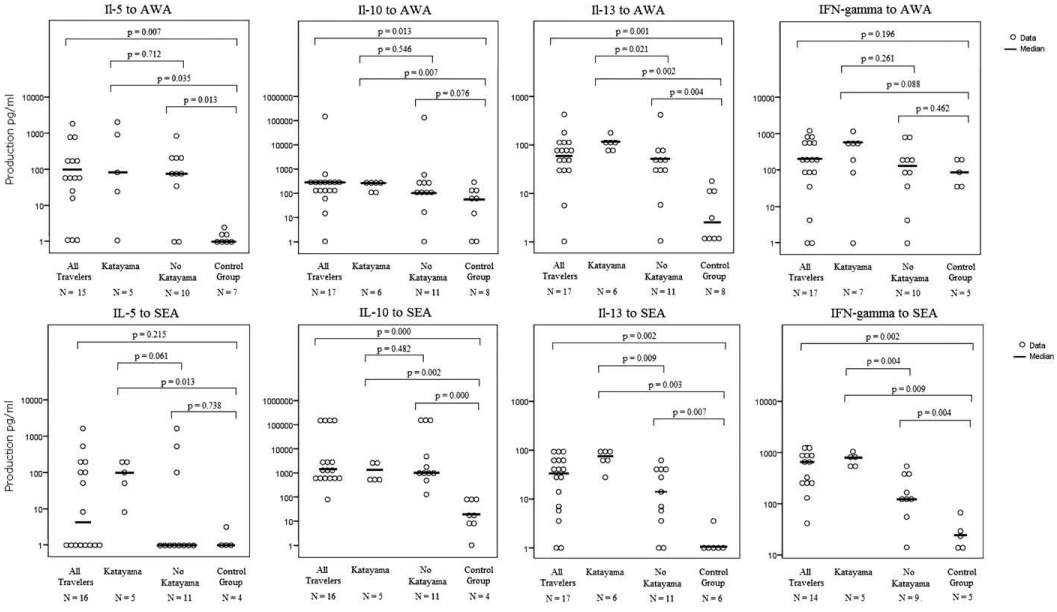

FIGURE 1. *In vitro* cytokine production in response to AWA and SEA 8 years after treatment of schistosomiasis; 20 formerly infected travelers, of which 9 had experienced Katayama syndrome and 11 had not, were compared with 8 non-infected controls. Cytokine responses could not be determined for all subjects because of technical problems with the assay and the limited amount of blood. Differences were tested using the non-parametric Mann–Whitney test.

stimulation of antibody production may occur from time to time.

It is surprising that AWA induced IFN-γ production in the controls but no lymphocyte proliferation in the controls. High IFN-γ levels in controls have been reported before in response to AWA and SEA.[16] It is possible that components of these antigenic mixtures bear pathogen-associated molecular patterns and react with pattern recognition receptors on immune cells, such as monocytes, B-lymphocytes, or natural killer (NK) cells. The IFN-γ response to AWA in uninfected subjects might be produced by NK cells, which can readily release IFN-γ in response to stimulation by pathogen-associated molecular patterns (PAMPs).[29] We do not know if these PAMPs are schistosome-specific or caused by endotoxin contamination.

The data suggest that 8 years after treatment of schistosomiasis, those who had Katayama syndrome in the past had stronger or less well-regulated lymphoproliferative and cytokine responses to schistosome antigens compared with those who were infected but remained asymptomatic. However, most differences did not meet conventional levels for statistical significance. In addition, all stimulation assays were done with *S. mansoni*-derived antigens. We can not fully rule out that *S. haemaobium*- or *S. intercalatum*-infected subjects would react less with *S. mansoni* antigens. Studies conducted on Senegalese patients living in an endemic area with single *S. haemaobium*, single *S. mansoni*, or mixed *S. haemaobium* and *S. mansoni* infections indicated that the antigens are cross-reactive when it comes to cytokine production. In other words, there was no consistent pattern showing that *S. haemaobium*-infected subjects respond better to *S. haemaobium* antigen than *S. mansoni* antigen or vice versa (unpublished data). Nevertheless, the percentage with proven *S. mansoni* infection was slightly higher in the group with Katayama syndrome than in the group without Katayama syndrome, and this may have influenced results.

We studied a limited number of cytokines that we believed to be important in the immune response in naive subjects. It has been argued that it makes biological sense for IL-13, IL-5, and IFN-γ to have a central role in the response to infection with *Schistosoma* spp.[16,30] Because of IL-13's function in signaling B cells to switch to IgE production and IL5's and IL-13's principal role in recruiting and activating eosinophils, these cytokines are important in the initial response to invading cercaria and later on, to schistosomula.[30] Eosinophilia is known to be associated with Katayama syndrome,[17,19] and the stronger IL-5 and IL-13 responses that we found may reflect stronger activation of eosinophils at the time of infection in the travelers with Katayama syndrome. The higher IFN-γ levels in response to SEA in travelers with Katayama syndrome may reflect a stronger immune response to the infection in this group.

This study shows that acute schistosomiasis induces a memory response that can be detected 8 years after treatment. Furthermore, we found that formerly infected travelers who had Katayama syndrome had an overall stronger action of the immune system to schistosome antigens compared with their asymptomatic counterparts. This is in line with the idea that Katayama syndrome is caused by a hyperreactive immune response to migrating schistosomula or eggs. Why some do and others do not mount such a hyperreactive immune response remains unknown. Differences in the genetic background or the antigen load during the acute infection offer plausible but unproven explanations.

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II. Novel vaccines

Chapter 6 Analysis of efficacy of CVD 103-HgR live oral cholera vaccine against all-cause travellers' diarrhoea in a randomised, double-blind, placebo-controlled study. *Vaccine. 2005 Oct 17;23(43):5120-6.*

Chapter 7 Intradermal fractional booster dose of inactivated poliomyelitis vaccine with a jet injector in healthy adults. *Vaccine. 2013 Aug 12;31(36):3688–94.*

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Analysis of efficacy of CVD 103-HgR live oral cholera vaccine against all-cause travellers' diarrhoea in a randomised, double-blind, placebo-controlled study

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC), which produces heat labile toxin (LT) and/or heat stable toxin (ST), is considered to be the most common known cause of travellers' diarrhoea (TD). Owing to the antigenic similarity between cholera toxin and LT, immunization with inactivated oral B-subunit/whole-cell cholera vaccine (BS–WC) offers short term (3 months) but significant (>67%) protection against TD caused by LT-related ETEC. Since it expresses the cholera toxin B (CTB) subunit, the live attenuated oral cholera vaccine strain CVD 103-HgR, may induce similar protection. A trial was performed to determine if CVD 103-HgR live oral cholera vaccine would provide a protective efficacy of at least 50% against TD. In addition, the protective efficacy of the vaccine against TD specifically due to LT–ETEC and LT/ST–ETEC was determined. Volunteers ($n = 134$) travelling to Indonesia, India, Thailand or West-Africa were randomised to receive either a placebo ($n = 65$) or the vaccine ($n = 69$). In the placebo group, 46% reported an episode of diarrhoea, compared to 52% in the vaccine group. No significant group differences were found with regard to incidence, duration or severity of all caused TD or ETEC-associated TD. However, ETEC-associated TD occurred earlier in the placebo group (median 5 days), compared to the vaccine group (median 15 days).

In conclusion, CVD 103-HgR live oral cholera vaccine failed to provide a 50% protection against TD. This study does not exclude that the vaccine may offer a short-lived protection against ETEC-associated TD. However, the power of the study was limited by the unexpected low incidence of LT–ETEC-associated diarrhoea (9% of all TD) compared to ST-associated TD (24% of all TD).

Keywords: Travel; Diarrhoea; Cholera vaccine

1. Introduction

Travellers from industrialized countries visiting (sub)tropical regions often develop diarrhoea. Large-scale studies among European and North American travellers to high-risk destinations, report an incidence rate of diarrhoea of 20–50% per 2 weeks' stay [1,2]. Though a self-limiting illness, travellers' diarrhoea (TD) can ruin holidays and cause substantial financial and emotional damage, creating a need for prophylactic and therapeutic agents. In both respects antibiotic drug therapy has proven effective. However, the use of antibiotics carries disadvantages when administered to a large number of people [3]. Therefore, consensus opts against the prophylactic use of antibiotics, and the need for a preventive agent that is both effective and safe, persists.

Although the prevalence of etiologic agents that cause TD differs from area to area, enterotoxigenic *Escherichia*

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coli (ETEC) is deemed to be the most common cause [4]. Based on a meta-analysis, the median isolation rate of ETEC associated TD is 42% in Latin America, 36% in Africa and 16% in Asia [3,5]. Despite the use of modern methods, in approximately 50% of cases, no pathogen is detected [3,4,6]. A significant proportion of this pathogen-undetected TD is probably caused by ETEC [7].

ETEC expresses a heat-labile toxin (LT), a heat-stable toxin (ST) or both (LT/ST). Reports on the proportion of ETEC strains producing a certain type of toxin vary. Approximately 25–30% of strains express LT, 35–45% ST and 30–35% LT/ST [8,9]. LT is very similar to cholera toxin in both structure and mode of action. It is composed of an enzymatically active (CTA) subunit surrounded by 5 identical binding (CTB) subunits. It binds to the same ganglioside receptors via its CTB moiety that are recognized by the cholera toxin, and its enzymatic activity is identical to that of cholera toxin. This explains why immunization with oral B-subunit/whole-cell cholera vaccine (BS–WC) can induce an increase in intestinal IgA directed against LT antigens [10]. Most likely these antibodies account for the effect found by previous studies, that showed that BS–WC offered significant (>67%) short-term (3 months) protection against diarrhoea caused by LT–ETEC and LT/ST–ETEC [11,12]. CVD 103-HgR, a live oral cholera vaccine, may induce similar protection. It contains a genetically modified strain of *Vibrio cholerae* O1, attenuated via deletion of about 95% of the *ctxA* locus encoding the toxic CTA subunit, and elicits seroconversion with high titres of vibriocidal antibody. Since the strain expresses normal quantities of immunogenic CTB, it is also known to induce a significant antitoxin immune response in intestinal fluid [13–16]. Owing to antigenic similarity between cholera toxin and LT, one may expect CVD 103-HgR to induce antibodies directed against LT, offering protection against diarrhoea caused by LT–ETEC and LT/ST–ETEC.

The use of CVD 103-HgR has some advantages over BS–WC. Firstly, it induces a strong immune response after only a single dose [17,18], as opposed to the multiple doses required for immunisation with BS–WC. Secondly, CVD 103-HgR possibly elicits higher vibriocidal titres than BS–WC [13]. However, the antitoxin response is probably similar to that induced by BS–WC.

We performed a randomised, double-blind, placebo controlled trial to determine if CVD 103-HgR live oral cholera vaccine would provide a protective efficacy of at least 50% against (severe) travellers' diarrhoea. In addition, the protective efficacy of the vaccine against travellers' diarrhoea specifically due to LT–ETEC and LT/ST–ETEC, was determined.

2. Materials and methods

2.1. Study design

The study was performed at the Leiden University Medical Center (LUMC). The primary outcome was the attack rate of TD in the placebo group compared to the vaccine group. The difference between the two groups, regarding attack rate of TD caused by LT–ETEC and LT/ST–ETEC was chosen as secondary outcome. The protocol (KV 9506) was approved by the ethical committee of the LUMC, The Netherlands.

2.2. Inclusion of subjects

Dutch volunteers were enrolled between May 1995 and February 1996. Travellers were recruited from the travel clinics of the LUMC ($n = 131$), the Municipal Health Centre at Leiden ($n = 5$) and the Harbour Hospital at Rotterdam ($n = 9$). All adults who made an appointment at the travel clinic between May 1995 and February 1996 and who were intending to travel to Indonesia, Thailand, the Indian subcontinent or West Africa (Gambia or Senegal) for a period of 1–4 weeks were invited to take part in the trial and were subsequently sent an informative letter concerning the study.

2.3. Exclusion criteria

The following subjects were excluded from the study. People suffering an acute or chronic inflammatory disease of the intestinal tract; prior recipients of WC–BS cholera vaccine or CVD 103-HgR vaccine; subjects receiving immunosuppressive drugs; persons known to be immunodeficient; anyone having received an experimental drug within the last 3 months; subjects participating in other clinical trials and women who were either pregnant or breast-feeding. Information on the concomitant use of medication, treatment or vaccination was obtained by way of a standardised questionnaire.

2.4. Randomisation

After having obtained written informed consent, subjects were stratified according to region, and were subsequently randomised (1:1) to two groups. For randomisation a computer-generated randomisation list, was used, which had been produced at the Berna Biotech AG (formerly Swiss Serum and Vaccine Institute), Bern, Switzerland. Sachets and suspensions of vaccine ($n = 100$) and placebo ($n = 100$), that were identical in appearance, were labelled by a coded number from 1 to 200. Within each stratum, for each permutation of 20, the weighing of randomisation was adjusted to 1:1 (vaccine to placebo). Participants were subsequently enrolled in the trial. At least 2 weeks prior to departure they consumed the appointed sachet. The key to the coded sachets was stored at the hospital pharmacy in a sealed envelope. The envelope was only to be opened by the investigator in case of an emergency that required knowledge of the identity of the trial medication in order to manage the participant's condition. At the end of the trial the coded envelope was returned to the Berna Biotech AG and checked to ensure that the seal had remained unbroken.

2.5. Vaccine and placebo

The vaccine consisted of a single dose of 5×10^8 colony forming units (CFU) of lyophilised CVD 103-HgR live oral cholera vaccine (CVD 103-HgR). CVD 103-HgR is an attenuated strain of *Vibrio cholerae* O1 derived from the wild-type classic Inaba strain 569B by deleting the genes that encode for the A subunit of cholera toxin and by inserting a marker gene encoding for resistance to Hg^{2+} into the *hlyA* locus of the bacterial chromosome. Genes encoding for the synthesis of the immunogenic, non-pathogenic, B-subunit remain intact. A placebo dose consisted of 5×10^8 heat killed *Escherichia coli* K-12. Both vaccine and placebo were administered in a glass of water together with a buffer containing 2.65 g $NaHCO_3$, 1.65 g ascorbic acid and 0.2 g lactose. A nurse supervised administration. Volunteers were urged not to eat or drink anything 1 h before and after vaccination.

2.6. Definition of travellers' diarrhoea

TD was defined as any episode of three or more unformed stools per 24 h, or two such bowel movements accompanied by vomiting, abdominal cramps or subjective fever, with an onset during travel until 3 days after returning home. Diarrhoeal episodes were registered from the time of getting on the plane. Diarrhoea was recorded as episodes, which were considered separate when the symptom-free interval was 5 days or more.

2.7. Recording incidence of diarrhoea and collecting stool specimens

All participants kept a diary of their defecation pattern during their stay abroad. On return they filled out a questionnaire, concerning defecation pattern, use of medication and information regarding travel, accommodation, and dietary hygiene. Each participant submitted a stool specimen. Subjects who had experienced an episode of diarrhoea during travel collected a sample during the first diarrhoeal episode, prior to having taken any medication. The remaining travellers collected and submitted a sample within 3 days after returning home. Written instructions were given on how to collect the stool specimen. The sample was preserved in a plastic vial on a specific transport medium, chosen because of its capacity to preserve ETEC for a minimum of 4 weeks (Para-Pak Enteric Plus system, Meridian diagnostics Inc., Cincinnati, OH, USA) [18–20]. After returning home, the vials were collected and sent to the laboratory for microbiology at the Academic Medical Center, Amsterdam where specimens were analysed for presence of enterotoxin producing *E. coli*.

2.8. Laboratory evaluation of stool samples

All samples, submitted by subjects who had experienced an episode of diarrhoea were examined for enterotoxigenic *E. coli*. In addition the first 28 samples, taken on return home, by people who had not suffered an episode of diarrhoea were subjected to the same examination. Stool samples were inoculated onto Cystine Lactose Electrolyte Deficient (CLED) agar plates. After 18 h of incubation at 37 °C, a sweep of the complete bacterial growth on the agar was collected using a sterile cotton swab, and stored in glycerol–pepton at −70 °C, as described previously [21]. This frozen material was inoculated on a CLED agar plate, from which a new sweep was taken. This material was diluted in PBS and subjected to PCR for detection of ETEC–LT, STIa and STIb genes, as described previously [21]. All PCR-positive samples were submitted to repeated PCR detection from the sweeps stored at −70 °C. The detection limit for ETEC is 10^2 CFU/g of feces [21].

2.9. Statistical analysis

The aim of this trial was to estimate the difference (δ) in chance of acquiring travellers' diarrhoea after having taken the placebo (p_p) compared to the vaccine (p_v). The attack rate of TD per group reflects these chances. The null hypothesis (H_0) implies that placebo and vaccine are equally effective in preventing TD ($\delta = p_p - p_v = 0$). The alternative hypothesis (H_1) states that $\delta \neq 0$. The number of subjects required for this trial was 100 per group (vaccine/placebo). This was calculated on the basis of a one-sided test with a power of the study of at least 0.9, a type I error of less than 0.025 and an expected incidence rate for travellers' diarrhoea of 35% with an expected protection rate of the vaccine of at least 50%. Proportions were compared using univariate analysis for numerical data and the χ^2-test for categorical data. Numerical data that were not normally distributed were analysed with Mann–Whitney *U*-test. The study was terminated after an ad hoc[1] interim analysis. During the interim analysis the key to the randomisation code remained blinded from the principal investigators.

3. Results

In total, 343 volunteers, meeting the inclusion criteria were approached, of which 198 either refused to take part or matched one or more of the prior mentioned exclusion criteria. At the moment of interim analysis, 145 volunteers had been stratified according to region and subsequently randomised to receive either placebo or vaccine. Since three individuals cancelled their journey, and eight did not fill out the questionnaire, 134 participants were evaluable. A total of 65 subjects received a placebo and 69 received CVD 103-HgR (Fig. 1). Except for the category 'duration of stay' and for

[1] Due to changes in the law regarding the use of genetically modified products, the study was temporarily put on halt, pending the outcome of an investigation of the vaccine and the study design. Though the trial was allowed to continue, the study was terminated based on the results of the interim analysis.

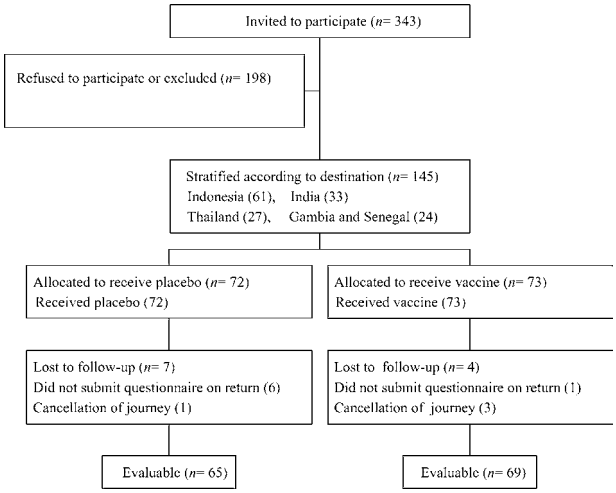

Fig. 1. Flow diagram of the progress through the phases of the randomised trial.

the subcategory 'accommodation with locals', demographic data and distribution of risk factors for diarrhoea did not differ significantly between the two groups (Table 1). Vaccine tolerability was excellent, with 10% of vaccines reporting mild abdominal discomfort compared to 17% in the placebo group.

3.1. Primary endpoint

Of the 134 participants, 66 (49%) reported at least one episode of diarrhoea. No significant difference was observed between the placebo and vaccination groups with respect to attack rate (Table 2). No significant differences existed

Table 1
Base-line characteristics of the study population consisting of 134 Dutch travellers

Parameter	Placebo ($n=65$)	Vaccine ($n=69$)	All ($n=134$)	p-value
Average age (years)[a]	38.7	40.3	39.5	NS
Sex (m/f)[a]	26/39	36/33	62/72	NS
Interval from vaccination to departure (days)[a]	18.0	17.1	17.6	NS
Average duration of stay (days)[a]	22.5	20.1	21.3	0.01
Travel destination (n)				
Indonesia[b]	30	28	58	NS
India[b]	12	17	29	NS
Thailand[b]	14	13	27	NS
Gambia + Senegal[b]	9	11	20	NS
Prior travel to (sub)tropics (n)[b]	45	48	93	NS
Antacid medication (n)	1	0		
Accommodation (n)				
Large hotel[b]	20	27	47	NS
Budget hotel[b]	18	13	31	NS
Guesthouse[b]	13	21	34	NS
Camping	0	2	2	
With locals[b]	14	6	20	0.04
Followed advise on diet and hygiene (n)[b]				NS
Always	27	34	61	
Sometimes	38	35	73	
Use of antibiotic prior to onset of TD	1	0	1	

Placebo: heat-killed *Escherichia coli*-K1; vaccine: a single dose of CVD 103-HgR live oral cholera vaccine; mean duration of stay abroad: 20 days, range (7–30) days; NS: not significant.

[a] Statistics: univariate analysis comparing placebo group to vaccine group.

[b] χ^2-test comparing placebo group to vaccine group; p-value significant at <0.05.

Table 2
Attack rate of travellers' diarrhoea (TD) among 134 Dutch travellers

Parameter	No. (%)				
	Placebo ($n=65$)	Vaccine ($n=69$)	Total ($n=134$)	p-value	ETEC associated diarrhoea ($n=17$)
Subjects with TD	30 (46)	36 (52)	66/134 (49)	NS	
Subjects with TD specified for					
Indonesia ($n=58$)	15	18	33/58 (50)	NS	11/33 (33)
India ($n=29$)	5	9	14/29 (48)	NS	4/14 (29)
Thailand ($n=27$)	6	3	9/27 (33)	NS	0/9 (0)
Gambia and Senegal ($n=20$)	4	6	10/20 (50)	NS	2/10 (20)

Placebo: heat-killed *Escherichia coli*-K12; vaccine: a single dose of CVD 103-HgR live oral cholera vaccine; mean duration of stay abroad: 20 days, range (7–30) days; statistics: χ^2-test comparing placebo group to vaccine group; p-value significant at <0.05; NS: not significant.

Table 3
Detection of enterotoxigenic *Escherichia coli* in faeces of 134 Dutch travellers to (sub)tropical destinations

Parameter	No. (% of analysed samples)						
	Travellers' diarrhoea			No travellers' diarrhoea			All ($n=134$)
	Placebo ($n=30$)	Vaccine ($n=36$)	Total ($n=66$)	Placebo ($n=35$)	Vaccine ($n=33$)	Total ($n=68$)	
Stool samples analysed	28	31	59	14	14	28	87
Sample negative for ETEC	21 (75)	21 (68)	42 (71)	13 (93)	11 (79)	24 (86)	66 (76)
Sample positive for ETEC	7 (25)	10 (32)*	17 (29)	1 (7)	3 (21)	4 (14)	21 (24)
ETEC LT only	1	0	1	0	0	0	1
ETEC LT and ST	2	3	5	1	3	4	9
ETEC ST only	4	7	11	0	0	0	11

Placebo: heat-killed *Escherichia coli*-K12; vaccine: a single dose of CVD 103-HgR live oral cholera vaccine; Mean duration of stay abroad: 20 days, range (7–30) days; samples: diarrheic stool specimens taken during episodes of diarrhoea, non-diarrheic specimens taken a maximum of 3 days after return home; statistics: χ^2-test comparing placebo group to vaccine group; p-value significant at <0.05.
* $p > 0.05$.

regarding number of episodes, time to first onset, duration or severity of diarrhoea (Table 4). In the placebo group 30 of 65 subjects (46%) developed diarrhoea, compared to 36 of 69 (52%) in the group of vaccines (Table 2). Comparison of the two groups, stratified according to travel destination, did not yield significant differences either (Table 2). The study was ended prematurely, because the primary endpoint, a vaccine efficacy of at least 50%, would not be reached by continuing the study until 200 subjects were included.

Table 4
Severity, number of episodes and duration of travellers' diarrhoea (TD) in 134 Dutch travellers to (sub)tropical destinations

Parameter	No.						
	ETEC associated diarrhoea					All diarrhoea	
	Placebo ($n=7$)	Vaccine ($n=10$)	Toxin			Placebo ($n=30$)	Vaccine ($n=36$)
			LT	ST	LT and ST		
Severity of episode of TD[a]							
2 stools/day	1	1	0	1	1	10	11
3–6 stools/day	6	3	1	5	3	15	16
>6 stools/day	0	6	0	5	1	5	9
Number of episodes of TD[a]							
1 episode	6	9				23	20
2 episodes	0	1				5	12
3 episodes	1	0				2	4
Mean duration (days) [range][a]	2.7 [1,11]	3.7 [1,10]				2.5 [1,14]	4.1 [1,24]
Median interval to onset of TD (days) [range][b]	5 [4,17]	15 [5,23]*				9 [4,25]	9 [3,25]

Placebo: heat-killed *Escherichia coli*-K12; vaccine: a single dose of CVD 103-HgR live oral cholera vaccine; mean duration of stay abroad: 20 days, range (7–30) days; a separate episode of TD is defined as an episode occurring after five consecutive days without diarrhoea.
[a] Statistics: χ^2-test comparing placebo group to vaccine group.
[b] Mann–Whitney U-test comparing placebo group to vaccine group.
* $p = 0.043$.

3.2. Secondary endpoint

Analysis could be performed on 59 stool samples obtained from the 66 participants who had reported an episode of diarrhoea. In the placebo group ETEC was isolated from 7 of 28 samples (25%), compared to 10 of 31 samples (32%) in the vaccine group (Table 3). The majority of sweeps (65%) were only positive in the PCR detecting the ST-genes. In the placebo group 4 sweeps contained only ST-, 2 LT/ST- and 1 LT-genes. For the vaccine group this was 7, 3 and 0, respectively (Table 3). This means that per 100 travellers, only 4.6 experienced an episode of LT–ETEC or LT/ST–ETEC associated diarrhoea. For all travellers, the (detected) incidence rate of ETEC associated TD was 11% in the placebo group and 15% in the group of vaccines. Travellers to Indonesia experienced the highest incidence of ETEC associated diarrhoea (33%), followed by travellers to India (29%) and West-Africa (20%). No ETEC diarrhoea was found in people visiting Thailand (Table 2). The median time from departure to onset of ETEC-associated diarrhoea was shorter in the placebo group (5 days, range 4–17 days) compared to the vaccine group (15 days, range 5–23 days) ($p = 0.043$) (Table 4). Six of 7 subjects from the placebo group developed diarrhoea within 12 days compared to 4 of 10 vaccinated travellers. However, there was no difference in time from departure to onset of diarrhoea when all TD were taken into account.

Of the samples obtained from travellers who had not suffered an episode of diarrhoea, the first 28 were analysed for ETEC. In the placebo group 1 of 14 contained ETEC, compared to 3 of 14 in the vaccine group (Table 3).

4. Discussion

Interim analysis of 134 travellers to different tropical destinations failed to demonstrate a 50% protective efficacy of CVD 103-HgR live oral cholera vaccine against all-cause travellers' diarrhoea. In addition, no significant differences between placebo and vaccine group were found with regard to time of first onset, duration, severity or recurrence of TD. A vaccine-induced protection against TD of at least 50% was chosen as primary endpoint because we assumed that a lower protection rate would not be relevant to clinical practice. The study was not continued until all 200 participants were included because a statistical significant difference would not have been reached with an attack rate of TD of 46% in the placebo group, even in the unlikely event that all additionally included vaccinated travellers were protected against diarrhoea.

Most travellers from the vaccine group developed ETEC-associated diarrhoea after two weeks. Therefore, this study does not exclude a short-lived protection of CVD 103-HgR against TD specifically caused by ETEC. The duration of protection was much shorter than observed for BS-WC (3 months) in a field trial in Bangladesh [11]. However, because of the high incidence of LT–ETEC and LT/ST–ETEC diarrhoea in this country, it is likely that BS–WC had boosted pre-existent immunity against ETEC resulting in longer protection. Further studies should confirm our observation and evaluate whether a second oral dose of CVD 103-HgR could boost the primary response and prolong protection.

Several potential limitations of this study require comment: (1) the detected incidence of LT-associated diarrhoea was much lower than expected. Only 4.6 subjects per 100 travellers experienced an episode of LT–ETEC or LT/ST–ETEC associated TD. The vaccine's protective efficacy is based on the putative production of cross reacting antibodies against LT. The low incidence of LT–ETEC associated diarrhoea may have limited the power of this study to demonstrate a protective effect of CVD 103-HgR on incidence, duration and severity of LT–ETEC or LT/ST–ETEC associated TD; (2) the preservation of the stool sample in a faecal transport medium may have adversely affected the recovery of ETEC. This would result in an underestimation of the true incidence of ETEC-associated diarrhoea. However, *E. coli* can be recovered from the faecal transport medium up to 49 days after inoculation in the laboratory [18–20]. The mean (±S.D.) interval between collection and microbiologic analysis in this study was 19 (±7.6) days (range 7–34 days). Furthermore, the attack rate of ETEC associated diarrhoea according to travel destination in the present study was in accordance with published literature [2,4,6]. Finally, PCR detection of LT- and ST-genes in sweeps of the complete bacterial growth is far more sensitive than the conventional DNA-probe hybridisation of *E. coli* like colonies [7,21]. Therefore, we do not think that the incidence of ETEC associated diarrhoea was underestimated; (3) seroconversion of the participants for anti-cholera toxin or anti-heat-labile enterotoxin was not documented in this study. No doubts exist concerning the placebo, as it has been proven not to elicit an antitoxin antibody response [22]. The biological activity of the vaccine was extensively tested in the laboratory prior to supervised administration. Dosage and method of delivery were similar to those known to induce an anti-cholera toxin antibody response in 72–83% of vaccinated healthy Swiss or American volunteers 21 days after vaccination [14]. The mean interval from vaccination to departure was 17.1 days allowing enough time to mount an immune response; and (4) other enteropathogens than ETEC have not been looked for. Several studies have found mixed infections with other pathogens along with ETEC in stools of travellers affected by TD [6,12,24]. CVD 103-HgR may not protect against TD caused by such mixed infections.

Remarkably 65% of all detected ETEC strains isolated from stool specimens of subjects with TD were sole producers of ST. Furthermore, none of the asymptomatic participants, whose specimens were analysed, carried ST–ETEC. This suggests that ST–ETEC is more pathogenic than LT–ETEC or LT/ST–ETEC.

In summary, a 50% protective efficacy against TD could not be demonstrated for CVD 103-HgR live oral cholera vaccine. This may be due to the low incidence of LT-producing

ETEC strains. The study does not exclude a short-lived protective effect against ETEC-associated TD. However, the small sample size, lack of antibody-response measurements and selective testing of faeces, limit the predictive power.

Future studies attempting to prevent TD through vaccination may focus on ETEC, as it remains the most common causative pathogen [5,6,7], but should target a broader range of strains, because ST–ETEC seems to have a higher incidence than suggested in earlier studies [8,9,23]. Recent studies have done just that by developing vaccines including colonization factor antigens expressed by ETEC [24]. Furthermore, it is recommended that future trials stating attack rate of TD as a primary outcome should include large numbers of travellers, or limit the investigation to countries for which detailed data concerning aetiology of TD is available.

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Intradermal fractional booster dose of inactivated poliomyelitis vaccine with a jet injector in healthy adults

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ABSTRACT

For global eradication of poliomyelitis, inactivated poliovirus vaccine (IPV) needs to become available in all countries. Using fractional-doses (reduced-doses) may impact affordability and optimize the utilization of the production capacity. Intradermal administration has the potential to lower the dose without reducing immunogenicity. A needle-free jet injector may be a reliable way to administer vaccines intradermally. The primary objective of this randomized controlled trial was to compare the immunogenicity and tolerability of fractional-dose intradermal IPV (Netherlands Vaccine Institute, NVI) booster vaccination administered with a jet injector (PharmaJet) to full-dose and fractional-dose intramuscular vaccination with a needle and syringe. Immunogenicity was assessed by comparing the differences in the post-vaccination $\log_2$ geometric mean concentrations of neutralizing antibodies (GMC) between the study groups. A total of 125 Dutch adult volunteers with a well-documented vaccination history were randomized to one of four groups: full-dose intramuscular needle (IM-NS-0.5), full-dose intramuscular jet injector (IM-JI-0.5), 1/5th dose intramuscular needle (IM-NS-0.1), 1/5th dose intradermal jet injector (ID-JI-0.1). Vaccination with the JI was less painful (87% no pain) than vaccination with a NS (60% no pain), but caused more transient erythema (JI 85%, NS 24%) and swelling (JI 50%, NS 5%). Intradermal vaccination caused less vaccination site soreness (ID 16%, IM 52%). At baseline all subjects had seroprotective antibody concentrations. After 28 days, GMC were slightly lower in the ID-JI-0.1 group than in the reference group (IM-NS-0.5). The differences were not statistically significant, but the stringent non-inferiority criterion (i.e. a difference of 1 serum dilution in the microneutralization assay) was not met. After one year, differences in GMC were no longer apparent. In contrast, intramuscular vaccination with a fractional dose administered with a needle (IM-NS-0.1) was statistically inferior to full-dose intramuscular vaccination. This shows that intradermal but not intramuscular delivery of fractional-dose IPV may be sufficient for routine polio vaccination.

1. Introduction

The new Global Polio Eradication Initiative has set a target for complete interruption of the transmission of poliovirus [1]. After eradication, cessation of oral poliovirus vaccine (OPV) is needed to prevent outbreaks due to circulating vaccine derived poliovirus [2,3]. Countries must then decide whether to stop all routine immunization against polio or to continue immunization with inactivated poliovirus vaccine (IPV). One of the prerequisites for cessation of the use of OPV is therefore to make IPV affordable and suitable for use in developing countries [4]. The worldwide production capacity for IPV is limited and the current weighted-average purchase price per dose of vaccine, when purchased by the United Nations Children's Fund, is $0.15 for trivalent OPV and approximately $3 for IPV [5]. Strategies to reduce this 20-fold cost increase include intradermal (ID) delivery of a fractional (reduced) antigen dose, intramuscular (IM) delivery of a fractional dose, or delivery of fewer doses. Administering vaccines intradermally is thought to enhance their immunogenicity because of the high density of antigen presenting cells in the dermis [6–9]. In a trial in the Philippines, a fractional dose of IPV administered intradermally with a needle at 6, 10 and 14 weeks and at 15–18 months, induced similar seroprotection rates but lower antibody titers than full-dose intramuscular IPV [10].

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Intradermal vaccination with a needle and syringe can be difficult, particularly in small children. A needle-free jet injector may be a reliable way to administer vaccines intradermally. It requires little training and reduces the risk of needle-stick injuries. In a trial in Oman, a fractional dose of IPV administered intradermally with a needle-free jet injector (Biojector® 2000) at 2, 4 and 6 months of age induced similar seroconversion rates but lower antibody titers than three full intramuscular doses [5]. In a similar trial in Cuba, in which infants were vaccinated at 6, 10 and 14 weeks after birth, which is a suboptimal immunization schedule for IPV [11,12], both the seroconversion rates and antibody titers were lower after fractional-dose intradermal vaccination than after full-dose intramuscular vaccination [13]. In both trials, parents preferred administration with a jet injector over injection with a needle [5,13]. No data are yet available on long-term protection and booster responses after vaccination with fractional-doses in infants.

These studies could not distinguish whether the intradermal site of administration or the lower antigen dosage were responsible for the lower immunogenicity of fractional-doses, because the study design did not include a third arm with fractional-dose IPV given intramuscularly. In anticipation of subsequent trials in infants as the primary target for polio eradication, this trial was designed to compare the immunogenicity and safety in adult volunteers with a well-documented vaccination history of a fractional booster dose of IPV administered intradermally with PharmaJet injection system, to both full- and fractional-dose IPV (Netherlands Vaccine Institute, NVI) injected intramuscularly with a needle and/or jet injector. The PharmaJet injection system is a handheld spring-powered injector and therefore suitable for use in developing countries.

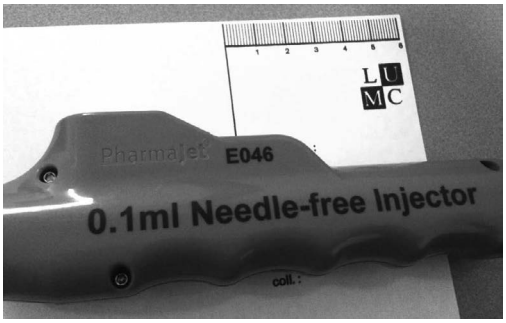
2. Methods

2.1. Ethics statement

All participants provided informed consent. The study was approved by the Dutch ethics committee, the Central Committee on Research Involving Human Subjects (protocol number NL29671.000.09; EU Clinical Trials Register EUDRACT 2009-015175-27; Netherlands Trial Register 2196).

2.2. Study design

This was a single-center, randomized, controlled, non-inferiority trial conducted at Leiden University Medical Center in The Netherlands, between August 2010 and February 2012. Subjects were vaccinated between August 2010 and January 2011. The primary objective was to evaluate the tolerability (vaccination site and systemic reactions) and to compare the immunogenicity 28 days after vaccination of a fractional booster dose of IPV administered intradermally with a needle-free jet injector (ID-JI-0.1), with standard full-dose intramuscular vaccination administered with a needle and syringe (IM-NS-0.5). Secondary objectives were (i) to compare the safety and immunogenicity of full-dose intramuscular IPV booster vaccination administered with a jet injector (IM-JI-0.5), with IM-NS-0.5, and (ii) to compare the immunogenicity of ID-JI-0.1, with fractional-dose intramuscular IPV administered with a needle and syringe (IM-NS-0.1). Healthy Dutch adult volunteers who had received exactly 6 combined DTP-IPV vaccinations according to the National Immunization Program (i.e. at age 3 months, 4 months, 5 months, 11 months, 4 years and 9 years) were eligible. Exclusion criteria were: any IPV booster dose after 10 years of age, any OPV dose.



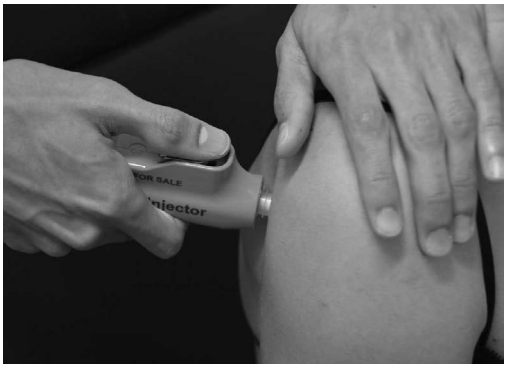
Photograph 1. PharmaJet Needle-free Jet Injection System for intradermal delivery. The ID injector used in this study was an investigational version of the FDA 510k-cleared v1.0 SC/IM device.

2.3. Vaccine and jet injector

Per participant we used one vial of IPV (NVI, lot 814AB, 0.5 mL per vial, expiration date: 05 Nov 2011) containing formaldehyde-inactivated poliovirus (strains Mahoney, MEF-1 and Saukett), type 1, 2 and 3: 40:8:32 D-antigen units respectively, and formaldehyde: 0.025 mg in phosphate buffer. The jet injector that was used was the PharmaJet Needle-free Jet Injection System. Separate jet injectors and single-use needle-free syringes were used for intramuscular and intradermal administration. The ID injector used in this study was an investigational version of the FDA 510k-cleared v1.0 SC/IM device. Modifications to permit ID delivery included a smaller main spring, a longer ejection pin to limit syringe fill volume to 100 μL, and the ability to continuously vary the main spring pressure through the use of spring preload system. With the exception of orifice diameter modifications, syringes were identical to SC/IM syringes (Photograph 1).

2.4. Randomization and procedures

The sponsor (NVI) prepared 125 sealed envelopes indicating allocation to one of the four treatment groups. The envelopes were numbered in random order using a random number generator (www.random.org). The study was not blinded. A single investigator included and vaccinated all participants (D.S.). The reference group, IM-NS-0.5, received one full-dose vaccination with IPV (40:8:32 DU in 0.5 mL) administered intramuscularly with a 25-gauge needle and 1.0 mL syringe. Study group IM-JI-0.5 received one full-dose (0.5 mL) vaccination administered intramuscularly with a jet injector. Study group IM-NS-0.1 received one fractional-dose vaccination with IPV (8:1.6:6.4 DU in 0.1 mL) administered intramuscularly with a 25-gauge needle and 1.0 mL syringe. Study group ID-JI-0.1 received one fractional-dose vaccination (0.1 mL) administered intradermally with a jet injector. Vaccinations were injected into the deltoid muscle of the right arm, except for intradermal vaccinations, which were injected in the skin overlying the posterior deltoid (Photograph 2). In all study-groups, we measured residual moisture, defined as vaccine remaining on, rather than in the skin, with a quantitative filter paper. Blood samples were taken at baseline (immediately before vaccination) and at day 7 (6–8), day 28 (25–31) and day 365 (330–400) after vaccination. For four days, participants filled out a diary on vaccination site and systemic reactions and recorded use of medication. Participants measured the size of vaccination site redness, swelling and induration using a caliper that was designed to measure the size of skin reactions. Adverse events occurring after four days were collected by



Photograph 2. Intradermal vaccination in skin overlying the posterior deltoid.

routinely inquiring after health-complaints at the 7- and 28-day blood collection.

2.5. Immunogenicity assay

The titer of neutralizing antibodies against poliovirus types 1, 2 and 3 was determined by microneutralization assay [14]. Sera were diluted in 24 two-fold dilution steps and in duplicate. Dilutions were incubated for three hours at 36 °C with $100CCID_{50}$ (cell culture infectious dose 50%) of poliovirus type 1, 2 or 3 (strains Mahoney, MEF-1 and Saukett) followed by an overnight incubation at 5 °C. Then, 2×10^5 Vero cells/mL were added to the serum/virus mixtures. After a seven-day incubation at 36 °C (5% CO_2) the results were read following fixation and staining with a crystal-violet solution with 5% formalin. The $\log_2$ titer was defined as the final serum dilution giving protection against $100CCID_{50}$ of challenge virus in which no CPE is present, resulting in a completely stained monolayer. Titers were converted to IU/mL by comparison with the titer of an in-house reference serum (IHS) of known potency. The potency of the IHS in IU/mL was determined by comparison with the titer of an International Standard Serum (NIBSC code: 82/585) as described previously [14]. To allow comparison between the groups, a $\log_2$ transformation was performed on the antibody concentrations in IU/mL and the mean was calculated which is referred to as the $\log_2$ geometric mean antibody concentration ($\log_2$ GMC). Titers of 1:8 are considered seroprotective and this has been shown to correspond to 0.080 IU/mL for type 1, 0.0180 IU/mL for type 2 and 0.075 IU/mL for type 3 poliovirus [15].

2.6. Statistical analysis

The primary immunogenicity endpoint was evaluated at day 28, by comparing the differences in the post-vaccination $\log_2$ GMC between group ID-JI-0.1 (minuend) and the reference group, IM-NS-0.5 (subtrahend). Non-inferiority was to be concluded if the lower limit of the 95% confidence interval (95% CI) for the difference did not exceed -1, which corresponds to a difference of 1 serum dilution in the microneutralization assay. Only if the margin was not crossed for any of the three poliovirus strains (PV1, PV2, PV3), the overall verdict was 'non-inferior'. Based on a standard deviation of the $\log_2$ GMC of 2.0, a one-sided alpha of 0.025 and a beta of 0.8, the sample size for each study arm was 30. The non-inferiority margin was based upon a combination of statistical reasoning and clinical judgment [16]. We assumed that all participants would already have a titer well above the level that corresponds to seroprotection since they had received 6 previous polio vaccine doses [17,18]. That is why the between-group difference in the $\log_2$ GMC at day 28 was chosen as the primary endpoint for immunogenicity. GMCs were analyzed in the per-protocol population with t-tests. Adverse events were described in the intention-to-treat population and analyzed with χ^2 tests. Statistical significance was defined as a p-value <0.05. Analyses were done with IBM® SPSS®, Statistics, Version 20.0.

2.7. Role of the funding source

IPV was produced and supplied by the NVI. Funding was provided by the ministry of Public Health, Welfare and Sport. The jet injectors and related materials were provided by PharmaJet®, which has a research and development agreement with NVI to support clinical trials *in kind*.

3. Results

A total of 125 adults were randomly assigned to one of four groups. One subject did not complete the visit at day 28 and was excluded from immunogenicity analyses, as were four subjects who followed a different childhood immunization program (Fig. 1). These five subjects were included in the safety analysis but not in the immunogenicity analysis. One year after vaccination, 79 subjects submitted an additional sample. The remaining 41 subjects were not included at this time-point; 20 had received pre-travel DTP booster vaccinations, 20 were lost to follow-up and 1 had received chemotherapy. Baseline characteristics are described in Table 1.

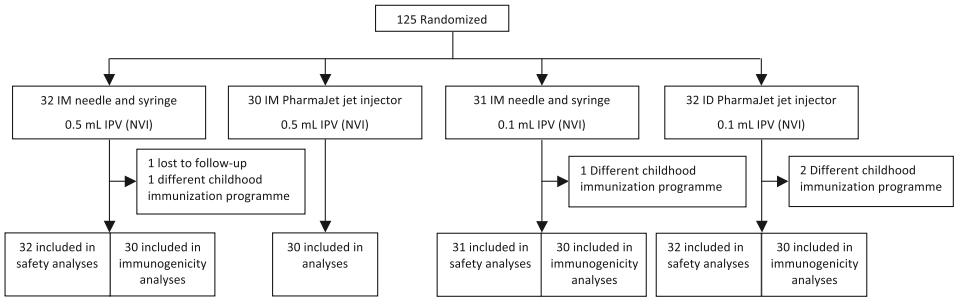


Fig. 1. Trial profile. IM, intramuscular; ID, intradermal; IPV, inactivated poliovirus vaccine; NVI, Netherlands Vaccine Institute.

Table 1
Demographic characteristics of volunteers assigned to full- (0.5 mL) or fractional-dose (0.1 mL) inactivated poliovirus booster vaccination, injected intramuscularly (IM) or intradermally (ID), with a needle and syringe (NS) or a jet injector (JI).

Characteristic	IM-NS-0.5 (n = 32)	IM-JI-0.5 (n = 30)	IM-NS-0.1 (n = 31)	ID-JI-0.1 (n = 32)
Female sex – n (%)	20 (63)	18 (60)	23 (74)	21 (66)
Mean age – years (SE)	21.1 (0.5)	21.8 (0.8)	21.6 (0.7)	21.5 (0.4)
Mean body mass index (SE)	22.2 (0.4)	22.0 (0.6)	22.4 (0.4)	22.3 (0.5)
Mean skin fold measurement – mm (SE)*	17.6 (1.4)	18.2 (1.6)	19.4 (1.3)	15.0 (1.0)
Current smoker – n (%)	4 (13)	7 (23)	4 (13)	5 (16)

The skin fold was measured at the injection site. Vaccinations were injected into the deltoid muscle of the right arm, except for intradermal vaccinations which were injected in the skin overlying the posterior deltoid. SE, standard error.

Table 2
Adverse events following administration of full- (0.5 mL) or fractional-dose (0.1 mL) inactivated poliovirus vaccine, injected intramuscularly (IM) or intradermally (ID), with a needle and syringe (NS) or a jet injector (JI).

	IM-NS-0.5 (n = 32)	IM-JI-0.5 (n = 30)	IM-NS-0.1 (n = 31)	ID-JI-0.1 (n = 32)
Vaccine delivery				
Pain – n (%)	13 (41)	6 (20)	12 (39)	2 (6)
Vagal reaction	0	0	1 (3)	0
Bleb diameter in mm – median (IQR)	NA	NA	NA	8 (8–8)
Spillage on skin in μL – median (IQR)	0 (0–17)	12 (2–45)	0 (0–2)	13 (8–40)
Systemic adverse events				
Fever – n (%)	0	0	1 (3)	0
Myalgia – n (%)	2 (6)	3 (10)	4 (13)	3 (9)
Fatigue – n (%)	8 (25)	6 (20)	10 (32)	10 (31)
Headache – n (%)	6 (19)	6 (20)	9 (29)	8 (25)
Vaccination site adverse events				
Erythema – n (%)	9 (28)	25 (83)[c]	6 (19)	28 (88)[c]
Maximum size in mm – median (IQR)	5 (5–15)	25 (15–35)	5 (5–6)	15 (10–15)
Duration in days – median (IQR)	2 (1–2)	3 (2–4)	1 (1–1.3)	4 (2.3–4)
Swelling – n (%)	0	12 (40)[c]	3 (10)	19 (59)[c]
Maximum size in mm – median (IQR) [range]	0	15 (11–33)	10 [5–65]	10 (10–15)
Duration in days – median (IQR) [range]	0	2.5 (2–3)	1 [1,2]	2 (2–4)
Induration – n (%)	3 (9)	11 (37)[d]	3 (10)	11 (34)[d]
Maximum size in mm – median (IQR) [range]	10 [5–25]	20 (10–20)	5 [5–65]	15 (10–20)
Duration in days – median (IQR) [range]	2 [2,3]	2 (2–3)	1 [1,2]	2 (1–3)
Soreness vaccination site – n (%)	16 (50)	17 (57)	15 (48)	5 (16)[c]
Arm stiffness – n (%)	13 (41)	9 (30)	11 (35)	5 (16)[d]

NA: not applicable. Medians, interquartile ranges (IQR) and ranges pertain to proportions that had the adverse event. p-Values for the comparison with the reference group: 0.09[a], 0.002[b], <0.005[c], 0.02[d] (χ^2 tests).

3.1. Vaccine delivery and adverse events

Intradermal delivery with the jet injector consistently produced blebs of 8 mm, which correspond to the diameter of the skin contact ring on the face of the needle-free syringe (Table 2). Vaccine residual moisture was minimal and more moisture was not associated with reduced immunogenicity. Of note, the measured residual moisture after vaccination with the jet injector was sometimes overestimated, as it also measured liquid adherent to the syringe face during filling, then transferred to the skin at the time of vaccine administration. Vaccination with a jet injector was less painful than vaccination with a needle (Table 2). Erythema, swelling and induration were more frequent after use of the jet injector. Soreness and arms stiffness were considerably less frequent after intradermal delivery with the jet injector than after intramuscular delivery with either a needle or jet injector (Table 2).

Table 3
Log_2 geometric mean antibody concentrations (GMC in IU/mL) at baseline and 7, 28 and 365 days after full- (0.5 mL) or fractional-dose (0.1 mL) intramuscular (IM) or intradermal (ID) inactivated poliovirus booster vaccination, administered with a needle and syringe (NS) or a jet injector (JI).

	IM-NS-0.5	IM-JI-0.5	IM-NS-0.1	ID-JI-0.1
At day 0 (baseline)	***n* = 30**	***n* = 30**	***n* = 30**	***n* = 30**
Poliovirus type 1	2.57 (2.04–3.11)	2.72 (2.12–3.31)	3.42 (2.74–4.11)[b]	2.98 (2.15–3.81)
Poliovirus type 2	3.12 (2.41–3.82)	3.28 (2.61–3.95)	3.30 (2.61–3.98)	3.58 (2.80–4.36)
Poliovirus type 3	0.87 (0.13–1.61)	0.59 (−0.29–1.47)	1.13 (0.35–1.91)	1.53 (0.63–2.42)
At day 7	***n* = 30**	***n* = 30**	***n* = 30**	***n* = 30**
Poliovirus type 1	5.74 (5.11–6.37)	5.13 (4.55–5.72)	5.25 (4.60–5.89)	5.29 (4.54–6.04)
Poliovirus type 2	6.82 (6.06–7.58)	5.93 (5.31–6.56)[b]	5.27 (4.47–6.08)[a]	6.08 (5.46–6.70)
Poliovirus type 3	5.88 (4.60–7.16)	4.62 (3.58–5.67)	3.86 (2.81–4.91)[a]	4.38 (3.74–5.02)[a]
At day 28	***n* = 30**	***n* = 30**	***n* = 30**	***n* = 30**
Poliovirus type 1	7.14 (6.45–7.83)	6.35 (5.83–6.86)[b]	6.06 (5.39–6.74)[a]	6.94 (6.02–7.87)
Poliovirus type 2	8.13 (7.27–9.00)	7.55 (6.89–8.21)	6.54 (5.70–7.38)[a]	7.71 (6.88–8.55)
Poliovirus type 3	7.26 (6.32–8.21)	6.44 (5.60–7.28)	5.61 (4.52–6.71)[a]	6.19 (5.43–6.95)[b]
At day 365	***n* = 22**	***n* = 21**	***n* = 17**	***n* = 19**
Poliovirus type 1	6.70 (5.87–7.62)	6.52 (5.70–7.34)	5.31 (4.48–6.14)[a]	6.71 (5.85–7.57)
Poliovirus type 2	5.87 (5.17–6.57)	5.57 (4.78–6.36)	4.44 (3.46–5.41)[a]	5.95 (5.14–6.76)
Poliovirus type 3	6.53 (5.66–7.40)	6.21 (5.26–7.15)	5.04 (4.10–5.98) [a]	5.92 (5.21–6.63)

p-Value for the difference in GMC in comparison with reference group (IM-NS-0.5): [0.01–0.05][a], [0.06–0.09][b].
Mean log_2 GMC with 95% confidence interval.

3.2. Immunogenicity

At baseline, all subjects had seroprotective antibody concentrations (Table 3). Baseline concentrations did not differ significantly between the groups. Seven days after vaccination, GMC increased for all poliovirus serotypes with a further increase at day 28 (Table 3). Reverse cumulative distribution curves of antibody titers, before and 28 days after vaccination are depicted in Fig. 2.

The primary immunogenicity endpoint was the between-group difference in the post-vaccination $\log_2$ GMC for each of the three poliovirus strains. At day 28, $\log_2$ GMC did not differ significantly between group ID-JI-0.1 and the reference group. The difference between ID-JI-0.1 (minuend) and IM-NS-0.5 (subtrahend) was −0.20 (95% CI −1.38 to 0.98) for PV1, −0.42 (95% CI −1.64 to 0.82) for PV2, and −1.07 (95% CI −2.31 to 0.17) for PV3 (Fig. 3). The lower limit of the 95% confidence intervals crossed −1, meaning that the pre-defined criterion for non-inferiority was not met. Formally the result can be classified as inconclusive regarding the question of non-inferiority [19]. Skin fold measurement, body mass index and spillage were not associated with the magnitude of the immune response (data not shown).

At day 28, $\log_2$ GMC were significantly lower in group IM-NS-0.1 (minuend) than in group IM-NS-0.5 (subtrahend): −1.08 (95% CI −2.07 to −0.09) for PV1, −1.59 (95% CI −2.82 to −0.37) for PV2, −1.65 (95% CI −3.13 to −0.17) for PV3 (Fig. 3). At day 28, $\log_2$ GMC did not differ significantly between group IM-JI-0.5 (minuend) and group IM-NS-0.5 (subtrahend): −0.79 (95% CI −1.67 to 0.08) for PV1, −0.58 (95% CI −1.69 to 0.53) for PV2 and −0.82 (95% CI −2.11 to 0.47) for PV3 (Fig. 3).

After one year, GMC remained high in all groups (Table 3). Antibody concentrations declined by less than one serum dilution for PV1 and PV3 and by approximately two serum dilutions for PV2. The rate at which antibody concentrations declined was similar in all four groups.

4. Discussion

Intradermal vaccination with a jet injector was less painful and caused less vaccination site soreness than vaccination with a needle. The jet injector caused more transient vaccination site erythema and swelling. This is in line with previous reports [20]. Fractional-dose intradermal vaccination was immunogenic, but titers were somewhat lower than after standard full-dose intramuscular vaccination. The differences were not statistically significant. After one year, the differences were no longer apparent. In contrast, intramuscular injection of fractional-dose IPV induced significantly lower titers than full-dose IPV.

The immunogenicity results are in line with previous studies in Oman and Cuba [5,13]. They are also in line with another recent trial in Cuba, in which infants who had not been vaccinated before received two ID fractional doses of IPV, delivered with a jet injector [21]. A single fractional dose produced seroconversion in almost half the infants and a priming response in almost all of those who did not undergo seroconversion. The authors argue, that for the post-eradication era, two doses of IPV given at the ages of 4 and 8 months could suffice. However, in another recent trial among Indian infants, supplemental fractional-dose ID IPV, delivered with an investigational PharmaJet injector was significantly less effective than full-dose IM vaccination [22]. Excessive undelivered vaccine as a result of marginal investigational device performance likely contributed to the low seroconversion and antibody titers in the ID group.

Our study shows that fractional-dose intramuscular IPV was significantly less immunogenic than full-dose IPV, even when used as a booster vaccination. Based on this result and the results of other studies, we conclude that dose reduction lowers immunogenicity but that fractional-dose intradermal vaccination is more immunogenic than fractional-dose intramuscular vaccination. The D-antigen content in IPV is not as superfluous for poliovirus type 3 as it is for type 1 and 2 [23,24]. This may be the reason why the response to type 3 poliovirus seemed weaker than to type 1 and 2 after intradermal vaccination.

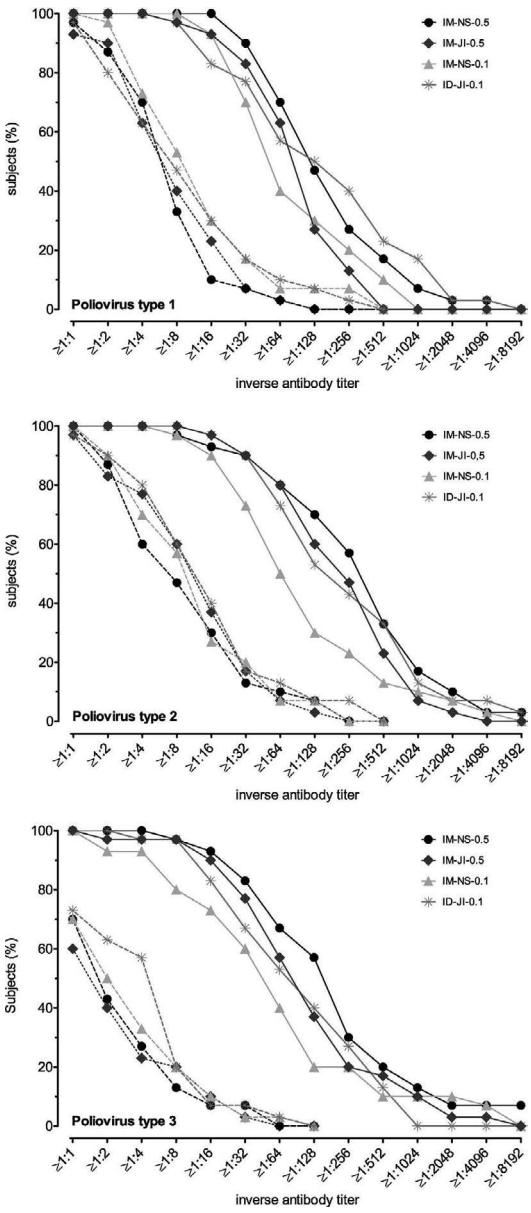


Fig. 2. Reverse cumulative distribution curves of antibody titers at baseline and at day 28. Dashed lines: baseline titers. Smooth lines: titers at day 28.

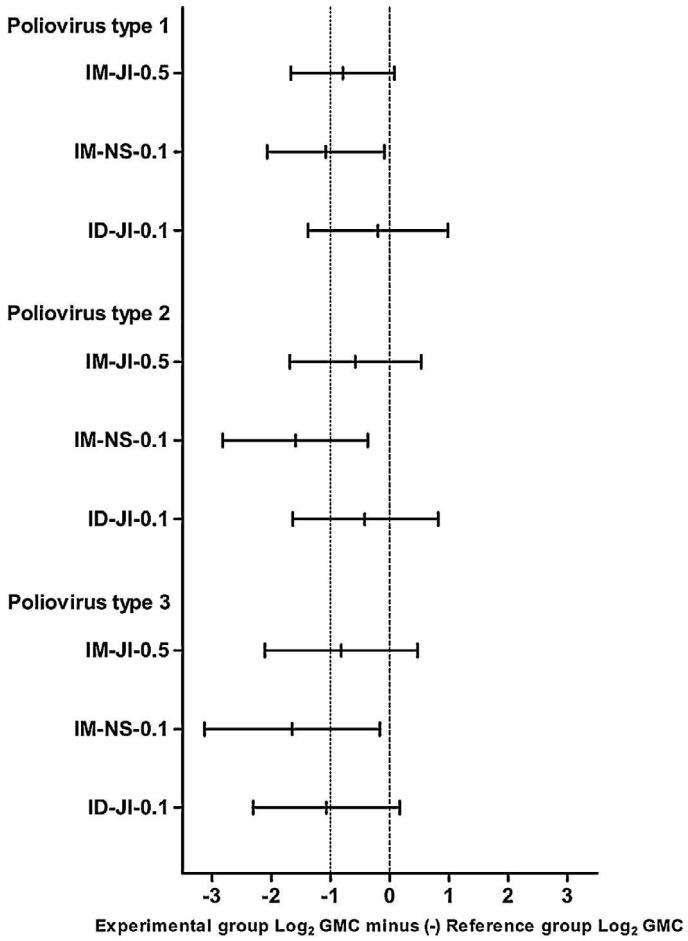


Fig. 3. Differences in the post-vaccination $\log_2$ geometric mean antibody concentration at day 28 in the study groups (minuend) in comparison with the reference group (IM-NS-0.5) (subtrahend). Mean differences with 95% confidence intervals. Zero indicates no difference. The non-inferiority margin was set at −1 (i.e. one titration step in the neutralization assay). Only if the margin was not crossed for any of the three poliovirus strains (PV1, PV2, PV3) the overall verdict was non-inferior.

The sample-size in preliminary studies is commonly based on a rule-of-thumb rather than a formal calculation. By using a non-inferiority design, we forced ourselves to pre-define the criterion by which fractional-dose IPV was to be judged vis-à-vis full-dose IPV. The pre-defined criterion for non-inferiority was not met. Ideally, one would want to base the primary outcome and non-inferiority margin on a clinically relevant endpoint such as the seroprotection rate. As expected, most participants in this study had baseline titers well above the level that corresponds to seroprotection. That is why the primary outcome and non-inferiority margin was based on the $\log_2$ GMC. We found that baseline antibody concentrations were higher and that the variance in antibody concentrations was larger than expected at the design stage of the study. This is exemplified by the fact that, even at baseline the confidence intervals for the between-group differences in antibody concentrations exceeded the pre-defined non-inferiority margin of one $\log_2$ GMC difference, i.e. one dilution step in the neutralization assay.

This study has a number of strengths. Firstly, the study population was homogenous and all participants had completed the same childhood vaccination schedule without any additional booster vaccinations. This increased the validity of the comparisons. Secondly, the study design made it possible to distinguish to what extent the route of administration and to what extent the dose was responsible for lower immunogenicity of fractional-doses. Furthermore, vaccination technique, residual moisture, bleb size and local vaccination site reactions were well documented. Lastly, results were reported in IU/mL, which facilitates comparison with other studies.

This study also has limitations. First, it was not blinded, which may have influenced results. Although Simon et al. describe a method with which blinding of such a trial is possible, this could not be done in our study, in which we used a different site for intradermal vaccination than for intramuscular vaccination [20]. Second, baseline antibody concentrations were higher than we had expected which influenced the statistical evaluation for

non-inferiority. Third, the mean baseline antibody concentration for PV1 was somewhat higher in the group that received fractional-dose intramuscular IPV. It seems unlikely that this influenced results in a significant manner, as the immune response to all three poliovirus strains was weaker in this group. Finally, all vaccines were delivered by a single user. Although this increases the validity of the comparisons by minimizing between-user differences in vaccine delivery, it limits the generalizability to real life practice.

5. Conclusion

Fractional-dose intradermal IPV booster vaccination using a PharmaJet injection system was well tolerated and immunogenic. Antibody titers in the fractional-dose intradermal group were slightly lower than after standard full-dose intramuscular vaccination. After one year, differences in antibody titers were no longer apparent. In contrast, one-fifth of a standard dose administered intramuscularly with a needle was statistically inferior to full-dose intramuscular vaccination.

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Contributors: DS, PV, AW, NR and LV designed the study. DS recruited the participants and conducted the study visits. DS, PV and AW were involved in data collection. PK performed the neutralization assay. DS did the data analysis. DS, PV, NR and LV drafted the manuscript. CB facilitated the study and reviewed and approved the manuscript. All authors gave final approval to the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.05.104.

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Ag85B–ESAT-6 adjuvanted with IC31® promotes strong and long-lived *Mycobacterium tuberculosis* specific T cell responses in volunteers with previous BCG vaccination or tuberculosis infection

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ABSTRACT

New TB vaccines are urgently needed because of the apparent lack of effect of the BCG vaccine on rates of adult contagious pulmonary tuberculosis and the risk of disseminated BCG disease in immunocompromised individuals. Since BCG appears to protect children, the primary target for vaccine development is a booster vaccine for adults but such vaccines ideally need to be able to efficiently prime mycobacterially naïve individuals as well as boost individuals previously vaccinated with BCG and those latently infected with TB. Protective immunity against *Mycobacterium tuberculosis* depends mainly on the generation of a Th1-type cellular immune response characterized by interferon-gamma (IFN-γ) production. In the present study, we monitored safety and IFN-γ responses in healthy BCG-vaccinated and prior or latently TB-infected individuals receiving a novel vaccine composed of the fusion protein Ag85B–ESAT-6 combined with the adjuvant IC31®, administered at 0 and 2 months. Vaccination caused few local or systemic adverse effects besides transient soreness at the injection site, but it elicited strong antigen-specific T cell responses against Ag85B–ESAT-6 and both the Ag85B and ESAT-6 components, that could be augmented by second vaccination. The strong responses persisted through 32 weeks of follow-up, indicating the induction of a persistent memory response in the vaccine recipients.

1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis*, an intracellular microorganism that is still considered to be among the world's most devastating pathogens. An estimated one third of the world's population is infected with TB, and 8–10 million cases of disease and 2–3 million deaths annually are caused by this pathogen [1]. Currently, the only widely available vaccine against TB is BCG, the live attenuated vaccine derived from *Mycobacterium bovis* by Calmette and Guérin early last century. BCG has shown to protect against some childhood forms of TB but the protective efficacy with regard to adult contagious pulmonary tuberculosis varies considerably, from 0 to 85% [2]. Adult pulmonary TB is the most infectious form of the disease and as a consequence, BCG has had little impact on the global TB epidemic. Improved second generation TB vaccines are therefore urgently needed [3].

Multiple new TB vaccines are being developed – which include recombinant BCG, attenuated *M. tuberculosis*, and DNA-based vaccines as well as recombinant proteins [4,5] and many of these have reached clinical trials. We recently published the first human phase I clinical trial data using a molecularly well-defined TB subunit vaccine, consisting of a hybrid protein of Early Secretory Antigenic Target (ESAT-6) and Antigen 85 (Ag85B) [6]. These antigens are strongly recognized by T cells from TB patients, are strongly conserved in clinical strains [7] and they have demonstrated protective efficacy in animal models [8–10]. However, because the protein component of the subunit vaccine is poorly immunogenic, the hybrid protein vaccine was adjuvanted with IC31®, a novel, two-component adjuvant system composed of the cationic polyaminoacid KLK and the oligodeoxynucleotide ODN1a [11–13]. This adjuvant was chosen on the basis of its ability to induce strong

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protective immunity in animal models of *M. tuberculosis* infection [11], by delivering a TLR9 ligand into the endosomal pathway [14] after comparison to the results of a substantial earlier adjuvant screening program [15]. The immunogenicity findings of the first trial showed that the adjuvanted Ag85B–ESAT-6 vaccine succeeded in inducing strong and long lasting Th1 cellular immune responses (>2 years after vaccination in a low endemic region) in volunteers without detectable prior recall responses to mycobacterial antigens [6]. This is encouraging, given earlier findings that protective immunity against mycobacteria depends on the generation of a Th1 cellular immune response characterized by the secretion of IFN-γ, and that the levels of IFN-γ appear to correlate with disease and recovery in TB patients [16,17]. Thus, even though IFN-γ by itself is clearly not the only factor required for immunity to *M. tuberculosis*, it is universally used as a marker of "vaccine take" [18].

For a TB vaccine that is to be employed in developing countries, showing safety and efficacy in mycobacterially naïve individuals is not enough, as globally most individuals have been sensitized by either prior BCG, by exposure to environmental mycobacteria or by latent or manifest TB infection. However, as the IC31®/H1 vaccine, containing both a new synthetic adjuvant and a novel recombinant protein had no prior data on safety of either component in humans, we adopted a cautious approach to testing. Therefore, only after the first clinical trial with the TB subunit vaccine in mycobacterially naïve individuals was shown to be safe and immunogenic, the vaccine was subsequently given to two groups of PPD (purified protein derivative) positive subjects. The first group included PPD positive BCG-vaccinated individuals with no known risk of *M. tuberculosis* infection and the second group consisted of PPD positive subjects with prior documented manifest or presumed latent TB infection. The primary objective of the present phase I trial was to evaluate the safety of the vaccine and the secondary objective was to evaluate the immunogenicity in PPD positive individuals.

2. Materials and methods

2.1. Ethics statement

All subjects gave informed consent for blood sampling, X-rays and tuberculin skin testing after verbal and written information was provided. The study protocol (EUDRACT No.: 2006-006366-42, ClinicalTrials.gov Identifier: NCT00929396, LUMC protocol no.: P07.132), the Investigator's Brochure and the Investigational Medicinal Product Dossier were approved by the accredited Ethical Review Board of LUMC and the relevant national authorities.

2.2. The investigational products

Ag85B–ESAT-6 is the recombinant fusion protein of Ag85B and ESAT-6, developed and manufactured by Statens Serum Institute (Copenhagen, Denmark). IC31® is a two-component adjuvant system developed by Intercell AG (Vienna, Austria), composed of the cationic polyaminoacid KLK and the oligodeoxynucleotide ODN1a in specific molar ratio of 25:1 KLK to ODN1a. KLK is composed of the amino acids lysine (K) and leucine (L). ODN1a is a single-stranded oligodeoxynucleotide based on alternating sequences of the nucleic acids inosine and cytidine. The final products were manufactured by Statens Serum Institute, in an accredited GMP facility and supplied to the study site as a sterile suspension for injection with a pH of 7.4. The injected volume was 0.5 ml. The vaccine was analyzed and released according to documented specifications before shipment to the clinical site. A GLP compliant repeated dose toxicity study in rabbits was conducted on Ag85B–ESAT-6 + IC31® (LAB Scantox, study no. 55926) in accordance with the CPMP Note for Guidance on preclinical pharmacological and toxicological testing of vaccines for human use (CPMP/SWP/465/95) providing guidance in the EU as of June 1998.

2.3. Study design and objectives

The study was an open label, single-centre, non-randomized phase I exploratory trial in PPD positive subjects. Two vaccinations of 50 μg Ag85B–ESAT-6 + IC31® (500 nmol KLK + 20 nmol ODN1a) were administered intramuscularly in the deltoid muscle, the first at time 0 of the study and the second at 2 months. The primary objective was to evaluate safety. The primary endpoints comprised sequential monitoring of a standard set of laboratory safety parameters (such as differential white blood cells, platelets, alanine aminotransferase, and creatinine), active solicitation for local and systemic adverse events following vaccine injections, and passive surveillance for solicited and unsolicited local and systemic adverse events by scheduled telephone interviews and self-reporting in a diary for up to 8 months after the first vaccination.

The secondary objective was to evaluate the immunogenicity of the vaccine. Cell-mediated responses were measured through detection of IFN-γ spot-forming cells by ELISPOT and of IFN-γ production in supernatants of peripheral blood mononuclear cells (PBMC) by ELISA, after stimulation with Ag85B–ESAT-6 protein, or with overlapping peptides of Ag85B and ESAT-6 (representing the individual components of Ag85B–ESAT-6). Reactivity was also monitored through detection of IFN-γ and TNF-α levels in plasma before and 7 days after the first and second vaccination. Finally, humoral responses were measured by ELISA for total IgG to the vaccine (Ag85B–ESAT-6).

2.4. Study subjects

The trial population consisted of 20 PPD positive volunteers divided into two groups: the 'BCG group' (BCG) and the 'TB infection group' (TBI). The BCG group consisted of 10 subjects (3 female, 7 male) who had been vaccinated with BCG >2 years before, whose tuberculin-skin-test (TST) was positive (range 6–15 mm or any documented value between 6 and 15 mm on medical file in the past), who did not have active, chronic or past TB disease as confirmed by chest X-ray at screening, a negative QuantiFERON®-TB Gold In Tube test and a negative 6-day lymphocyte stimulation test (LST: as described in [6]). The TB infection group consisted of 10 subjects (4 female, 6 male) who were either known to have been previously diagnosed with latent TB and whose TST was positive (≥10 mm, or documented ≥10 mm positive on medical file in the past), or of subjects with past documented (i.e., clinically manifest) TB infection but with no currently (>2 years) active disease as confirmed by chest X-ray at screening. According to the inclusion criteria, subjects with a past or latent TB infection should not have received treatment/chemoprophylaxis for TB within the preceding 2 years. They should also have evidence of prior *M. tuberculosis* infection as assessed by specific antigen recall responses. In the screening, many persons with documented past or latent TB infection tested QuantiFERON®-TB Gold negative. Therefore, besides 6 subjects with positive QuantiFERON®-TB Gold test, we selected 4 subjects with documented previous TB infection who did not have a positive QuantiFERON®-TB Gold test at screening, but who had positive antigen-specific responses to ESAT-6 or CFP10 as evidences by a response in the 6-day LST [6].

The general health of all participants was assessed by reviewing their recorded medical history, and performing a physical examination, chest radiography, and standard blood (including hepatitis B, hepatitis C and HIV testing) and urine tests. Exclusion criteria were having clinically relevant laboratory parameters outside of normal range, presence of a granulomatous disease (as evidenced by laboratory screening and chest radiography), being vaccinated with a

live vaccine within three months before the first vaccination, use of immune modulating drugs (e.g., glucocorticosteroids, immunosuppressive drugs or immunoglobulins) within the three months before the first vaccination, hypersensitivity to vaccine components, HBV, HCV or HIV sero-positive, pregnant women/planned pregnancy and/or breastfeeding within the trial period, or participation in another clinical trial. For the BCG group, 13 volunteers were screened and 3 were excluded: one because of an intention to become pregnant, one withdrew consent and one because of a positive TST > 15 mm. For the TB infected group, 16 volunteers were screened and 6 were excluded: one because of an intention to become pregnant, and 5 because of a negative QuantiFERON®-TB Gold in association with a negative LST. The median age was 49 (IQR: 24–54) years in the BCG group and 26 (IQR: 21–34) years, in the TB infected group, respectively. Volunteers were financially compensated as approved by the Institutional Review Board for the number and amount of blood and urine samples, inconvenience with respect to the intramuscular administration and for the time spent on trial visits and transportation to the study site.

The expected trial duration for each subject was 224 days (32 weeks or 8 months) following the first vaccination (day 0), which was preceded by a pre-vaccination screening period of approximately 10–14 days. The second vaccine dose was administered 2 months after the first. Each subject was expected to attend a total of 11 visits during the trial, two of which preceded the first vaccination. The final visit was planned eight months after the first and 6 months after the second (and final) vaccination.

2.5. Safety parameters

Volunteers remained under medical observation for 3 h after each intramuscular vaccination, to monitor possible immediate reactions. The volunteers were asked to record any symptoms and their body temperature in a diary. During the first week after each vaccination, symptoms and temperature (armpit) were recorded on a daily basis, thereafter on a weekly basis (the temperature being recorded in the evening). A medical examination of local reactions and temperature was performed on days 0, 1 and 7 and 42 after each vaccination. Extended physical examinations comprising ear, nose and throat, cardiovascular, pulmonary, neurological, gastrointestinal, urogenital and dermatological systems were performed at screening, just before the second vaccination and 98 and 224 days after the first vaccination. Adverse event recording took place from day 0 to day 224. On days 14 and 70 a standardized telephone interview was carried out.

Urine samples and blood sampling for hematologic and biochemistry clinical safety tests were taken before the vaccinations, 1, 7 and 42 days after the first and second vaccination, and 24 weeks after the last vaccination. All routine laboratory assays were performed by the certified central laboratories at LUMC according to standard procedures.

Adverse events were described in the Case Record Forms with onset date, duration, intensity of symptoms (grade I, II or III; i.e., mild, moderate, severe), outcome of the event and relationship (not related, possible, probable or certain related) of the event to the vaccine. Abnormal laboratory findings were scored for severity into severity grades 1–4 (based on "Toxicity grading scale for healthy adults and adolescent volunteers enrolled in preventive vaccine clinical trials" – FDA 2007 guidelines).

2.6. Quantiferon-TB Gold assay

Blood samples for testing with the QuantiFERON®-TB Gold in-tube assay were drawn before the first vaccination and at the last visit and performed according to the manufacturer's instructions with the result categorized as positive when the result was ≥0.35 IU/mL.

2.7. PBMC isolation and immunogenicity parameters

Baseline blood samples were collected two weeks before and on the same day as (i.e., immediately prior to) the first vaccination, and at 6 weeks of follow-up (i.e., 2 weeks before the second vaccination), and at weeks 14 and 32. Briefly, 40 ml of heparinized blood was centrifuged (5 min/322 g), the plasma removed for storage at −70 °C and cells resuspended and transferred to Leucosep filter tubes (Greiner-bio-one) containing 15 ml Ficoll (LUMC pharmacy #902861). After centrifugation, the fluid phase containing the PBMCs was removed and washed three times with 45 ml of sterile PBS (LUMC pharmacy). The PBMCs were aliquoted and stored in liquid nitrogen in medium (IMDM, Cambrax #BE112-722F) containing 20% fetal calf serum (FCS, PAA Laboratories #A15-043)/10% DMSO (Sigma #41650) until assaying. Assays were batch-processed with frozen cells to reduce variation. One ml aliquots of PBMC were thawed in a water bath at 37 °C, supplemented with 2 ml of 50% FCS/IMDM and centrifuged. PBMCs were pelleted, washed with 10 ml of serum-free culture medium (AIMV, Invitrogen #12055-91), resuspended in 1 ml medium, and counted. A minimum viability of 80% was considered acceptable for assay purposes. Assays using PBMC samples from each individual were performed in a single experiment or on the same day in case of different assays, to minimize temporal and inter-assay variation.

2.8. Quantifying IFN-γ spot forming units (SFU) after PBMC stimulation

To enable the determination of all longitudinal samples of single volunteers on a single day, limiting as far as possible inter-assay variability within subjects, it was necessary to start with frozen samples. In this protocol, cells were thawed and pre-stimulated for 16–18 h, followed by 24 h in the ELISpot plate. Of note, the pre-incubation step is done in a different plate, and samples are transferred to the ELISpot plate to reduce the background in the ELISpot and enhance the sensitivity of the assay when proteins or large peptides are used [19]. Briefly, 1×10^6 thawed cells/well were stimulated in 24 well plates with the same antigens and concentrations as described below for ELISA. All samples were assayed in triplicate. Incubation was done overnight in a fully humidified incubator at 37 °C, 5% CO_2. Subsequently, cells were resuspended and divided over 3 wells (250,000 cells/well, for antigen-stimulated cells, 100,000/well for PHA-stimulated cells) of a mixed cellulose ester-backed 96 well plate (MAHAS45, Millipore) which had been pre-coated with anti-IFN-γ-antibody (mAb 1-D1K, Mabtech, Sweden) and blocked with AIMV medium. Plates were incubated overnight in a fully humidified incubator at 37 °C, 5% CO_2. The next day biotinylated detector antibody (mAb 7-B6-1, Mabtech) was added and spots colored with alkaline phosphatase-conjugated streptavidin (Mabtech, Sweden) and Fast™NBT/BCIP (Sigma–Aldrich, The Netherlands), all according to the manufacturer's instructions. Substrate incubation was done at room temperature for 10 min and stopped by rinsing the plates with tap water. Plates were dried and spots were counted in the Bioreader 3000 pro (BioSys, Germany) using calibrated parameters.

2.9. Quantifying IFN-γ production (ELISA) after PBMC stimulation

After thawing, 150,000 cells/well were stimulated in triplicate in 96 well flat bottom plates (Greiner) with medium (AIMV, Invitrogen), 4 μg/ml PHA (Remel Europe), 5 μg/ml PPD (SSI, RT48 for *in vitro* use), 10 μg/ml Ag85B-ESAT-6 (SSI, GMP grade), an ESAT-6 peptide pool (9 peptides; 5 μg/ml of each), or an Ag85B peptide pool

(28 peptides; 5 μg/ml of each). The peptide pools consisted of 20-mer peptides, with an overlap of 10 amino acids. Every peptide was elongated at the N terminus with two lysine residues (K) to improve solubility. Cells were cultured for 6 days in a fully humidified incubator at 37 °C, 5% CO_2. Supernatants were harvested and stored at −20 °C until performing the IFN-γ ELISA. For the IFN-γ ELISA, Maxisorp™ plates (Nunc) were coated with mAb MD-2 (U-CyTech, The Netherlands) followed by blocking with 0.2% (w/v) milk powder (Fluka) in PBS (LUMC pharmacy). An IFN-γ standard (U-CyTech, The Netherlands) ranging from 5 to 5000 pg/ml was used, and to control for data consistency an external IFN-γ reference standard was included in all assays and demonstrated consistency over the assays (NIBSC, UK). Supernatants were diluted two-fold in 1% bovine serum albumin (BSA) in PBS. Following standard and sample incubation, biotinylated detector pAb (U-CyTech) was added. The ELISA was developed with streptavidin–horseradish peroxidase conjugate (CLB, The Netherlands) and tetramethylbenzidine substrate (BioSource, The Netherlands). Substrate incubation was done for 20 min at 37 °C. The reaction was stopped with H_2SO_4 and the OD was read at 450 nm.

2.10. Quantifying anti-Ag85B–ESAT-6 specific IgG antibodies in plasma by ELISA

Polysorb™ plates were coated with 4 μg/ml H1 or human serum albumin (Octalbine, Octapharma) as control for aspecific binding, followed by blocking with 1% (w/v) BSA (Sigma–Aldrich, UK)/1% (v/v) tween-20 (Sigma–Aldrich, UK) in PBS. A plasma dilution range was added to the wells, starting with a 1:12 dilution, followed by 2-fold dilution steps to 1:24,576 (12 dilutions in total). As a positive control, plasma from a treated TB patient was used; as negative control plasma from a healthy individual was used. After overnight incubation at 4 °C, HRP conjugated detector pAb (Dako, Denmark) was added. Development was done with TMB substrate (BioSource, Denmark) for 15 min at room temperature. The reaction was stopped with H_2SO_4, and the OD was read at 450 nm. Graphs with dilution factor versus OD450 yielded an S-curve. The antibody titre was defined as the dilution factor corresponding with the midpoint of the linear portion of this curve.

2.11. Quantifying IFN-γ and TNF-α levels in plasma by ELISA

Plasma was harvested after centrifugation of blood, and stored at −70 °C until performing the IFN-γ and TNF-α ELISA (Sanquin, Amsterdam). ELISAs were performed according to the instructions of the manufacturer.

2.12. Statistical analysis

The statistical analysis of the THYB-02 data was performed by JGConsult, an independent Contract Research Organisation in accordance with GCP and ICH-Guidelines.

Primary and secondary laboratory endpoints were evaluated within vaccination groups with respect to change from baseline to last visit using Wilcoxon signed rank test and between vaccination groups with respect to differences in change from baseline to end of trial using Wilcoxon rank sum test. Pairwise comparisons were conducted using Mann–Whitney. Adverse events were evaluated descriptively.

No formal sample size calculation was performed in this trial. A test significance level of 5% was used throughout the study. The statistical analysis was performed using SAS software (SAS®, Cary, NC 27513, USA, version 9.2 TS Level 1M0) on Platform Windows XP PRO Version 5.1.2600. Further subanalyses at SSI to produce the figures were conducted using Prism 4.0b MacOSX 10.5.8.

Table 1
Adverse events (AE), possibly vaccination-related, all classified as grade I, in BCG vaccinated and previously or latently TB infected subjects given two intramuscular injections with the Ag85B–ESAT-6 hybrid protein with IC31® adjuvant, relative to group size (n/N) or episode relative to persons affected.

Vaccination	Subjects			
	BCG vaccinated		TB infected	
	I	II	I	II
Any local adverse event (n/N)	5/10[a]	6/10	7/10	6/10
Stiffness	2	3	4	1
Soreness and/or pain at injection site	4	4	5	3
Erythema and/or swelling	1	1	1	5
Nodule at injection site	–	2	–	3
Any systemic adverse event (n/N)	2/10	1/10	0/10	0/10
Fever	1	1[b]	–	–
Tired and/or malaise	2	1[b]	–	–

[a] Number of subjects affected in each group/number of subjects in group.
[b] Reactions in same individual, temperature once elevated.

3. Results

3.1. Safety

All 20 subjects completed both vaccinations and the follow-up period. All subjects with at least one vaccination were included with all data in the safety analysis. No serious adverse events occurred in either of the two vaccination groups. In all, 52 local, potentially vaccine-related adverse events and 9 systemic, potentially vaccine-related adverse events were reported. Local vaccine-related adverse reactions included stiffness (defined as injection site movement impairment, $n = 6$ at first vaccination) and soreness and/or pain at injection site one day after vaccination ($n = 9$ at first vaccination). After the first vaccination such reactions were noted in about half of individuals in the BCG group and in the TB infection group (Table 1). After the second vaccination these reactions were more frequent. Most local adverse reactions were classified as mild (grades I–II) which means the event did not interfere with daily activities. In the BCG group however, one "injection site pain" reaction and one "injection site movement impairment" was classified as grade II, i.e., moderate, and one "injection site pain" reaction as severe (grade III) i.e., temporarily interfering with activities. All subjects with local reactions experienced full recovery within a maximum of 4 days. None of the subjects required analgesics. A small, cold nodule at the injection site was noted in 2 subjects in the BCG group and 3 in the TB infection group. This was without signs of attendant inflammation: local vesiculation, axillary lymphadenitis or fistula did not occur, and in all cases, the nodule had disappeared within one week. The systemic, potentially vaccine-related adverse events included fatigue ($n = 1$), malaise and feeling cold ($n = 2$) and were mild except for one individual where fatigue and malaise was classified as moderate.

Extensive follow-up of blood and urine parameters did not reveal any obvious trends within or differences between the two vaccination groups, or laboratory abnormalities with respect to change from baseline that could be related to the vaccinations.

3.2. Immunogenicity: IFN-γ ELISPOT and ELISA

Immune responses were evaluated by ELISA and ELISPOT. Responses were monitored to the Ag85B–ESAT-6 fusion protein, and also to the two individual components (ESAT-6 and Ag85B). The data in all cases are shown and analyzed without background subtraction.

For ELISA, there were no significant differences between any of the groups with regard to the negative (culture medium only: median value 8 pg/ml, Interquartile range 1 pg/ml) control or the

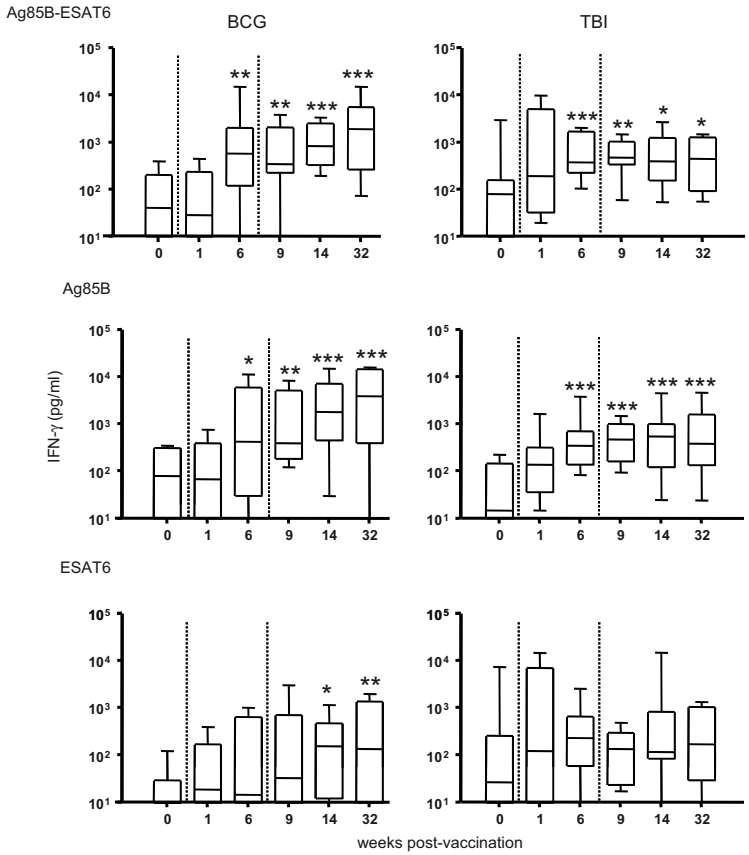

Fig. 1. IFN-γ production by PBMC as assayed by ELISA (expressed as pg/ml) after stimulation with Ag85B–ESAT-6 hybrid protein or peptide mixes of Ag85B or ESAT-6, in individuals given an intramuscular injection with the Ag85B–ESAT-6 hybrid protein antigen with IC31® adjuvant, at 0 and 2 months, with a subsequent 32 weeks follow-up. Subjects had either received a BCG vaccination in the past, or had previously been diagnosed with an *M. tuberculosis* infection (either latent of manifest) >2 years prior to the vaccinations (TBI). Vaccination time points are marked by dotted vertical lines. Samples for immune monitoring were taken just prior to (0) the first vaccination, six weeks after the first vaccination (6), and 1, 6, and 24 weeks after the booster vaccination. Results shown are medians and quartiles of assays performed in duplicate, with medians significantly different from those of the pre-vaccination bleeds marked (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

positive control (PHA: median value 15,000 pg/ml, interquartile range 226 pg/ml) (data not shown). Unlike in the previous study [6] – but as expected – these mycobacterially exposed individuals had detectable immune responses prior to vaccination (Fig. 1, time point 0). The magnitude of these responses were not significantly different between the groups for Ag85B, or the fusion molecule Ag85B–ESAT-6 (both had $p > 0.1$), but BCG vaccinees had a significantly lower response to ESAT-6 pre-vaccination than the TB infection group (Mann–Whitney, $p = 0.002$). By 6 weeks post-vaccination, however, there was no difference between the groups with regard to their ESAT-6 response ($p = 0.7$) and in both groups, it was higher after the booster vaccination than at baseline ($p = 0.05$).

Although baseline immune responses were higher as compared to the previous study in mycobacterially naïve individuals, for both Ag85B and Ag85B–ESAT-6 prior to vaccination, both groups showed significant increases in IFN-γ production to these antigens from 6 weeks post-vaccination ($p < 0.001$ for Ag85B–ESAT-6, $p = 0.007$ for Ag85B), and this elevated response was maintained without any apparent diminution up to 32 weeks after vaccination (Fig. 1).

The same overall pattern was observed when the response was assessed by ELISpot (Fig. 2): a detectable immune response to Ag85B and Ag85B–ESAT-6 was found prior to vaccination in both BCG-vaccinated and TB infected groups, which was efficiently boosted by the vaccination ($p < 0.0001$ for Ag85B–ESAT-6, $p < 0.001$ for Ag85B) in both groups. These p values were calculated using the 32-week endpoint, but significant increases were also seen at much earlier time points. As would be expected, the BCG vaccinees did not have a significant positive response to ESAT-6 prior to vaccination (Median response 0 SFU/million, interquartile range 22 SFU) while the TB infected individuals had a substantial response (median response 198 SFU/million, interquartile range 427 SFU, $p < 0.0001$ compared to BCG group). At 32 weeks, post vaccination, the response to ESAT-6 was significantly ($p < 0.0001$) higher than at baseline in the BCG group. The same is true in the TB group,

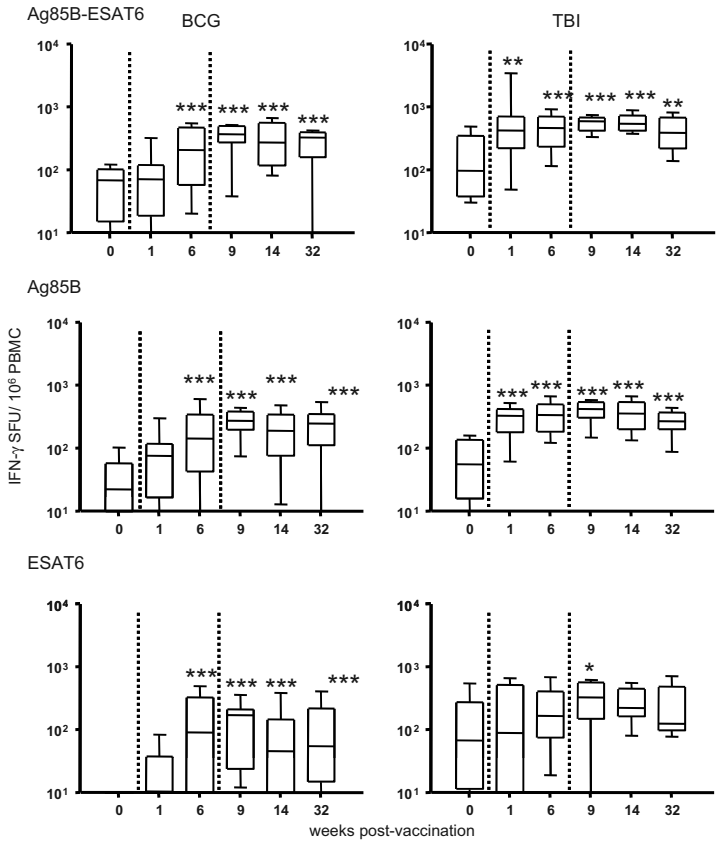

Fig. 2. IFN-γ production by PBMC as assayed by ELISPOT (expressed as spot-forming units per million peripheral blood mononuclear cells) after stimulation with Ag85B–ESAT-6 hybrid protein or peptide mixes of Ag85B or ESAT-6, in individuals given an intramuscular injection with the Ag85B–ESAT-6 hybrid protein antigen with IC31® adjuvant, at 0 and 2 months, with a subsequent 32 weeks follow-up. Subjects had either received a BCG vaccination in the past, or had previously been diagnosed with an *M. tuberculosis* infection (either latent of manifest) >2 years prior to the vaccinations (TBI). Vaccination time points are marked by dotted vertical lines. Samples for immune monitoring were taken and just prior to (0) the first vaccination, six weeks after the first vaccination (6), and 1, 6, and 24 weeks after the booster vaccination. Results shown are medians and quartiles of assays performed in triplicate, with medians significantly different from those of the pre-vaccination bleeds marked (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

though the difference was less pronounced due to higher baseline responses ($p < 0.03$).

We also tested the linker region between Ag85B and ESAT-6 to assess if any neo-epitopes were generated and were unable to detect any responses above background, whether humoral or cell mediated (data not shown). Thus, for both groups, in both assays, vaccination with Ag85B–ESAT in IC31® drove either the emergence of, or – in case of a preexisting response, an increase in – antigen-specific T cell responses that persisted up to the end of 32 weeks follow-up.

3.3. Kinetics of the response

While both groups increased their antigen-specific responses significantly after vaccination (Figs. 1 and 2), it also appeared that the kinetics of the response were somewhat different. This was particularly noticeable in the magnitude of the T cell responses against the hybrid protein at different times between the BCG-vaccinated (but TB naïve) group and the TB infection group (i.e., subjects either with past manifest TB or a clinical history indicative of latent TB infection). Prior to vaccination, the groups did not respond differently to the Ag85B–ESAT-6 fusion protein or to Ag85B. However, after vaccination, a relatively stronger T cell response to Ag85B–ESAT and Ag85B emerged in the TB infection group, compared to the BCG group, at the earliest time points, as assessed by ELISPOT ($p < 0.001$) (Fig. 3). This difference in antigen-specific ELISpot reactivity between BCG-vaccinated and the TB infection group remained significant following the second vaccination, until the numbers of IFN-γ positive cells in the ELISPOT converged by week 32. If the results from the previous clinical trial, THYB-01, where the same vaccine was given to individuals with no prior mycobacterial sensitization ($n = 11$) [6] are compared with the results from this study, it appears that the ELISpot response to Ag85B–ESAT and Ag85B in mycobacterially naïve indi-

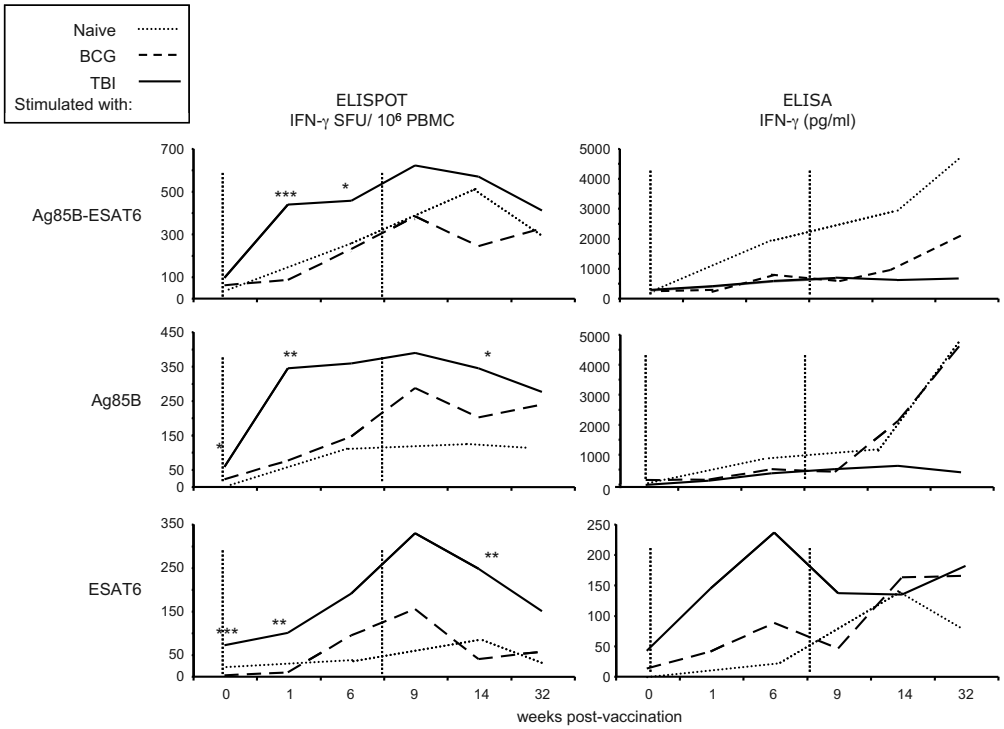

Fig. 3. Comparison of median IFN-γ responses over time in mycobacterially naïve (dotted line: data drawn from [6]) previously BCG-vaccinated individuals (dashed line) or in individuals previously been diagnosed with an *M. tuberculosis* infection >2 years prior to the vaccinations (solid line). IFN-γ production by PBMC was assayed after stimulation with Ag85B–ESAT-6 hybrid protein or peptide mixes of Ag85B or ESAT-6, in individuals given an intramuscular injection with the Ag85B–ESAT-6 hybrid protein antigen with IC31® adjuvant, at 0 and 2 months, with a subsequent 32 weeks follow-up. Subjects had either received a BCG vaccination in the past, or had previously been diagnosed with an *M. tuberculosis* infection (either latent of manifest) >2 years prior to the vaccinations. Vaccination time points are marked by dotted vertical lines. Samples for immune monitoring were taken just prior to (0) the first vaccination, six weeks after the first vaccination (6), and 1, 6, and 24 weeks after the booster vaccination. Results shown are medians of the assays performed in duplicate (ELISA: expressed as pg/ml) or triplicate (ELISpot, expressed as SFU per 10^6 PBMC), with medians significantly different between groups at the same time point marked (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

viduals most resembled that of the BCG group in the present study.

IFN-γ responses as assessed by ELISA, however, show a different trend, though the differences were not significant through the course of the study. The TB infection group responded more rapidly to Ag85B–ESAT and Ag85B in terms of the numbers of cells producing IFN-γ (Fig. 3), but not necessarily in the amount of IFN-γ produced. This difference was even more marked for the mycobacterially naïve recipients (Fig. 3 and [6]).

For ESAT-6, the pattern was slightly different, with the TBI group having a significantly higher baseline response ($p < 0.001$) prior to vaccination than the two groups with *M. tuberculosis* infection as assessed by ELISpot, but this difference diminished post-vaccination and was not significantly different thereafter. Again, despite higher levels of ESAT-6-induced IFN-γ in the ELISA, in the TBI group, the results were not significantly different between the three groups (Fig. 3).

These results indicate that after vaccination, all of the groups respond to vaccination by significantly increasing both the numbers of cells producing IFN-γ and the overall amount of IFN-γ produced, but that the kinetics of the response are different – not only between the groups but also between the different assays. This is interesting, given the trend (also seen in the prior study in mycobacterially naïve vaccinees [6]) for the magnitude of the ELISpot response to decline, even as the magnitude of the ELISA response increased, suggesting that the nature of the responding cells may be changing after vaccination, perhaps as a consequence of the development of long-term immune memory.

3.4. Humoral response to vaccination

With respect to a humoral response to the vaccinations no increase in anti-Ag85B–ESAT-6 IgG levels was observed after the first vaccination ($p > 0.15$) in the BCG group, but a small increase was seen in the TB group. However, after the booster vaccination, IgG antibodies reactive against Ag85B–ESAT-6 hybrid protein were significantly elevated above base-line by sampling at 14 weeks, in both groups. Thereafter, antibody levels decreased significantly again ($p < 0.05$) both in the BCG-vaccinated and TBI individuals (Fig. 4) and although in the TBI group, the over all response was greater and IgG levels remained above baseline at the final visit (32 weeks), the trend was clearly identical for both groups. There was no clear correlation at the individual level between the magnitude of IFN-γ and IgG responses.

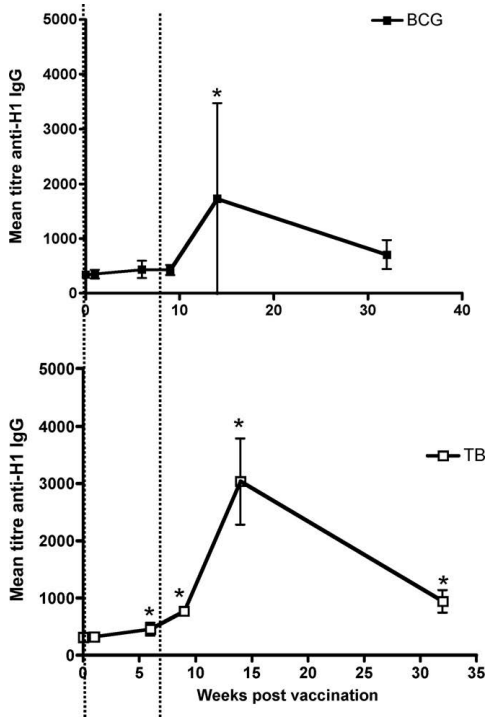

Fig. 4. IgG antibody production against the Ag85B–ESAT-6 hybrid protein in individuals given an intramuscular injection with the Ag85B–ESAT-6 hybrid protein antigen with IC31® adjuvant, at 0 and 2 months, with a subsequent 32 weeks follow-up. Samples for antibody measurement were taken just prior to (0) the first vaccination, six weeks after the first vaccination (6), and 1, 6, and 24 weeks after the booster vaccination. Vaccination time points are marked by dotted vertical lines. Results shown are geometric means and SD of the titre, from assays performed in triplicate, with medians significantly different from those of the pre-vaccination bleeds marked ($^*p < 0.05$).

3.5. Plasma levels of IFN-γ and TNF-α

Before the first vaccination and the week after each of the two vaccinations, blood was sampled for measurement of cytokine levels. In neither of the two groups was TNF-α above the limit of detection (i.e., <5 pg/mL). The same was largely true of IFN-γ, although in one instance in one individual in the BCG group, the level of IFN-γ detected was just above the limit of detection one week after first vaccination (data not shown). It can be concluded therefore, that plasma levels of TNF-α and IFN-γ are weakly – if at all – increased by vaccination. Consistent with this, plasma cytokine concentrations were not associated with occurrence or intensity of adverse effects.

3.6. Infrequent QuantiFERON conversion after vaccination with H1/IC31®

In the developed world, the QuantiFERON TB Gold test is now broadly used as a diagnostic assay for detection of TB infection. To assess the risk of conversion to a positive QuantiFERON TB Gold after intramuscular administration of the adjuvanted Ag85B–ESAT-6 vaccine to BCG-vaccinated individuals, the QuantiFERON TB Gold assay was done before vaccination and 24 weeks after the last vaccination. Test results of all subjects were negative before vaccination (as per the inclusion criterion in this group); one of the 10 had a positive response at the end of follow-up indicating that two injections of the adjuvanted Ag85B–ESAT-6 vaccine can induce a positive *in vitro* T cell response to ESAT-6 in a small percentage of BCG-vaccinated subjects. This is consistent with earlier results in mycobacterially naïve individuals [6]. In the TB-infected subjects, 6 out of 10 cases were QuantiFERON TB Gold positive before vaccination; of these, one subject tested negative after the last vaccination, whereas of 4 with a negative QuantiFERON TB Gold one subject reacted positively at the end of follow-up, indicative of boosting of the response by the vaccination. There was no obvious difference in the magnitude or kinetics of the immune response to any of the antigens between the QuantiFERON TB Gold converters and the rest of the group, though the numbers were too small to draw a definitive conclusion.

4. Discussion

This is the first study of a molecularly well-defined TB subunit vaccine adjuvanted with IC31®, a new adjuvant aimed at boosting the Th1-type immune reactivity, in BCG-vaccinated individuals and individuals with prior *M. tuberculosis* infection. The findings indicate that intramuscular injection of the SSI-produced Ag85B–ESAT-6 hybrid protein with adjuvant was safe and well tolerated. In TB-infected individuals, a single vaccine dose induced a strong cellular immune response to the hybrid protein; the strong reaction to the first vaccination is suggestive of a booster effect from prior *M. tuberculosis* exposure. In individuals who had received prior BCG vaccination, the ELISpot responses after the first vaccination lagged significantly behind those observed in the TB infected group, though they did eventually converge. In both groups, T cell responses were strongest against the Ag85B–ESAT-6 hybrid protein and Ag85B component, and following a second administration were sustained or even increased during the 32-week follow-up. The adjuvanted vaccine thus induced a strong and lasting Th1-type cellular immune response with a significant but perhaps less durable antibody response against the hybrid protein or its components.

The present study extends previous findings in mycobacterially naïve individuals that intramuscular injection of Ag85B–ESAT-6 hybrid protein with adjuvant IC31® is safe and well tolerated. In this study, as in the prior one, no serious adverse events occurred and systemic adverse events reported were all considered to be unrelated to the administration of the vaccine. In the two cases in which such a relation could not be excluded totally, the adverse events were mild or moderate and disappeared within one day. The only significant trial-related response that was observed in about half of the subjects consisted of a feeling of soreness and pain at the site of the injection, the day after administration of the vaccine. In most of those affected, the pain at injection site was generally mild, i.e., grade I, and disappeared within one to two days, did not hinder function of the extremity nor require analgesics, and was not accompanied by local lymphadenitis, febrile responses or systemic symptoms. To further evaluate the safety of vaccination, extensive hematological and biochemical assessments were conducted before starting, at the time of each of the two vaccinations, and after completing the long-term follow-up. The vaccine did not noticeably affect any hematological or biochemical measurement. Thus, the results of this clinical study demonstrate that there are no safety concerns associated with the administration of the subunit vaccine to PPD positive individuals – obviously within the limitations of any exploratory phase I study, which can exclude with certainty only very frequent adverse reactions. On

the other hand, the lack of any significant reactogenicity despite impressive immunogenicity of the adjuvanted vaccine can only be considered a breakthrough, given the current paucity of effective human adjuvants for the induction of cell-mediated immune responses [20]. Based on these results, a phase I trial to evaluate safety and immunogenicity in PPD negative volunteers, BCG vaccinees and volunteers with previous disease or latent TB infection is currently ongoing in Ethiopia: the first-ever vaccine phase I trial in Ethiopia.

The vaccination induced specific and strong Th1-type immune reactivity to the vaccine antigen that persisted and in many instances even increased up to 32 weeks following first vaccination. In *M. tuberculosis* infected individuals, assessment by ELISpot indicated that a single dose of antigen adjuvanted with IC31® induced a significant cellular immune response – in general, stronger than that observed previously in mycobacterially naïve subjects [6] or in BCG vaccinated subjects, and the response to H1 was further amplified by booster vaccination. In general, T cell responses were strongest against the Ag85B–ESAT-6 hybrid protein and secondarily against the Ag85B component and were sustained during a follow-up, suggesting induction of potent immunological memory. However, the differences in the kinetics of the response as assessed by ELISpot and ELISA observed here suggest that the nature of the immune response is slightly – but perhaps importantly – different between the groups, with those assumed to have a high level of prior exposure to mycobacterial antigens – the TBI group – responding to vaccination with a rapid increase in the number of cells making IFN-γ, but a much smaller increase in the amount of IFN-γ produced. In our previous study we found that individuals with no prior markers of mycobacterial sensitization responded by more slowly inducing antigen-specific cells (as assessed by numbers) but reaching a peak – in terms of IFN-γ concentration – higher than that seen in the TBI group [6]. The BCG group, who presumably fall in between the other two groups in terms of mycobacterial exposure also fell between them in terms of the difference in the number of IFN-γ producing cells compared to total amount of IFN-γ produced.

There are no defined surrogate biomarkers of protective immunity in TB. It appears clear that host defense against TB involves a number of factors, but that it crucially depends on establishment of a Th1-type immune response [21,22]. IFN-γ in particular appears to be essential, and the IFN-γ response is rapid and robust. Therefore, the number of IFN-γ-releasing *M. tuberculosis* antigen-specific T-cells and the amount of IFN-γ released – even though imperfect markers – remains widely used as a surrogate marker of potential vaccine efficacy in TB vaccines [18]. Although the mechanism controlling the composition of immune responses to *M. tuberculosis*-derived antigens is unclear, it is not unexpected that individuals with different immunological histories will respond differently to re-exposure to antigen. The data presented in Fig. 3 thus underline the importance of looking at vaccine induced immunity using different approaches and using well defined cohorts that can be differentiated with regard to their history of mycobacterial exposure.

Consistent with earlier findings in mycobacterially naïve individuals, the adjuvanted vaccine induced a strong and persistent Th1 cell immune response, with a relatively modest antibody response against the Ag85B–ESAT-6 protein, increasing after the second dose. The IgG response, though significant, showed a tendency to wane that was not observed for IFN-γ production. This suggests an initial response heavily biased towards the cell-mediated arm of the immune response. While this is unusual, it matches results obtained with this vaccine in mice, where relatively weak humoral responses were generated alongside high levels of antigen specific IFN-γ production [11], across a range of dosing regimens, possibly due to the apparent targeting of dendritic cells [12]. It is not clear if this response is specific for the *M. tuberculosis* antigens used, but if these characteristics of IC31® are confirmed for other types of vaccines, it may establish this adjuvant as one of the first adjuvants that may specifically induce a cellular immune response in humans. This is different from currently approved adjuvants like aluminum salts and MF59, both of which primarily promote a Th2 or humoral immune response [20,23–25], and also from the report from von Eschen and colleagues who recently reported that the mtb72F/AS02A adjuvanted protein vaccine gave a mixture of both Th1 T cell activity and a humoral response [26].

ESAT-6 is included in several widely used commercial diagnostic tests (the QuantiFERON and T.Spot.TB tests) and a positive response to ESAT-6 due to vaccination may interfere with follow up of potentially *M. tuberculosis* sensitized/infected patients. In the present study, vaccination was associated with conversion of the QuantiFERON assay in only one out of ten BCG-vaccinated volunteers. Even if subsequent trials gave higher levels of ESAT-6 responses, this is not anticipated to be a major problem: it should be realized that the employment of *in vitro* diagnostics for tuberculosis and vaccination against *M. tuberculosis* are quite distinct: the former is mostly used in western, low TB-incidence countries whereas the latter is aimed primarily towards TB endemic regions. Nonetheless, the interaction between diagnostic tests based on *in vitro* IFN-γ-production and the possible impact of the vaccine on the usefulness of the QuantiFERON assay is being studied in ongoing trials in Ethiopia.

Sustained responses seen here compare well with that induced by BCG vaccination in adolescents, where waning of the overall response is already be observed after 12 months [27]. Given the previous report of long lasting and persistent T cell responses in mycobacterially naïve individuals, and the present sustained T cell responses in PPD positive subjects, the Ag85B–ESAT-6 subunit vaccine may well be able to overcome what has previously been a stumbling block in vaccination against TB – the requirement for a long-lasting memory response [28,29]. Prolonged maintenance of immune memory elicited by an adjuvanted subunit vaccine is in good agreement with recent observations from a mouse study of Ag85B–ESAT-6 in an adjuvant consisting of cationic liposomes, and suggest that the adjuvants, perhaps through establishment of an antigen depot and subsequent slow release, or targeting of dendritic cells [12], may have unique abilities to maintain strong immune memory [30].

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The Data Safety Monitoring Board consisted of Prof. Dr. C.G.M. Kallenberg, MD, PhD, chair DSMB, Department of Internal Medicine, University Medical Center, Groningen, The Netherlands, (c.g.m.kallenberg@int.umcg.nl), David Lewis, professor, MD, FRCP, Centre for Infection, St George's University of London (sgjf300@sgul.ac.uk) and Dr. H.C Rűmke, PhD, Vaccine Center Rotterdam, Rotterdam, The Netherlands (h.rumke@erasmusmc.nl).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2010.12.135.

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A novel liposomal adjuvant system, CAF01, promotes long-lived *Mycobacterium tuberculosis*-specific T-cell responses in human

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ABSTRACT

Here, we report on a first-in-man trial where the tuberculosis (TB) vaccine Ag85B-ESAT-6 (H1) was adjuvanted with escalating doses of a novel liposome adjuvant CAF01. On their own, protein antigens cannot sufficiently induce immune responses in humans, and require the addition of an adjuvant system to ensure appropriate delivery and concomitant immune activation. To date no approved adjuvants are available for induction of cellular immunity, which seems essential for a number of vaccines, including vaccines against TB. We vaccinated four groups of human volunteers: a non-adjuvanted H1 group, followed by three groups with escalating doses of CAF01-adjuvanted H1 vaccine. All subjects were vaccinated at 0 and 8 weeks and followed up for 150 weeks. Vaccination did not cause local or systemic adverse effects besides transient soreness at the injection site. Two vaccinations elicited strong antigen-specific T-cell responses which persisted after 150 weeks follow-up, indicating the induction of a long-lasting memory response in the vaccine recipients. These results show that CAF01 is a safe and tolerable, Th1-inducing adjuvant for human TB vaccination trials and for vaccination studies in general where cellular immunity is required.

1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (MTB). A third of the world's population is infected with MTB, in 2013 there was a global estimated 8.6 million cases of TB and 1.3 million deaths caused by this pathogen [1]. Currently, the only available vaccine against TB is bacillus Calmette-Guérin (BCG), a live attenuated vaccine derived from *Mycobacterium bovis*. BCG protects against severe forms of childhood TB but its efficacy against pulmonary TB in adults is highly variable. Therefore, there is an urgent need for second generation TB vaccines [2,3].

Several novel vaccines are being explored, among which a prime-boost strategy using new TB vaccine candidates to boost BCG is considered a promising strategy [4]. In a recent phase IIb trial, an experimental vaccine MVA85A (modified vaccinia virus Ankara expressing antigen 85A) was given to infants who had previously been BCG-vaccinated, however the MVA85A vaccine failed to demonstrate efficacy against TB infection as well as TB disease emphasizing that there is a continued need for developing and testing novel vaccination strategies against TB [5]. We have published two human clinical trials investigating the Hybrid 1(H1) subunit vaccine; based on the hybrid protein of Early Secretory Antigenic Target (ESAT-6) and Antigen 85B (Ag85B) adjuvanted with IC31® (H1:IC31) [6,7]. These reports demonstrated that the H1:IC31 vaccine was safe and generated long-lasting antigen-specific Th1 T-cell responses against the hybrid protein [6,7].

Here we report on an independent H1 TB vaccine trial in which the adjuvant IC31® is replaced by the CAF01 adjuvant. CAF01 is a novel two-component liposomal adjuvant system composed of a cationic liposome vehicle (dimethyldioctadecyl-ammonium

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(DDA)) stabilized with a glycolipid immunomodulator (trehalose 6,6-dibehenate (TDB)) which is a synthetic variant of cord factor located in the mycobacterial cell wall. In addition to acting as an immunomodulator, TDB also ensures long-term stability of the DDA liposomes. Based on immunological data as well as physicochemical stability data the optimal weight ratio of DDA to TDB was found to be 5:1 [8]. In animal models, CAF01 promotes a broad and complex immune response characterized by multifunctional T-cells with a Th1 profile and possesses the same ability to induce long-lived immune responses as IC31® presumably through the establishment of a vaccine depot [8–13]. In preclinical studies, CAF01 also induced a Th17 response due to TDB signaling through the C-type lectin receptor Mincle [14]. CAF01 adjuvanted H1 vaccine was protective in animal models of TB [11–13,15], but safety and immunogenicity of a CAF01-adjuvanted vaccine has not yet been assessed in humans.

We report herein the first phase I clinical trial in human volunteers employing a CAF01-adjuvanted subunit TB vaccine (H1:CAF01), with safety as primary endpoint. The secondary objective of the trial was to evaluate the immunogenicity of H1:CAF01 in humans.

2. Materials and methods

An elaborated description of materials and methods can be found in the online supplement.

2.1. Ethics statement

All subjects volunteered to participate in the clinical trial and gave informed consent after verbal and written information was provided. The trial protocol (EUDRACT No.: 2008-006003-23, ClinicalTrials.gov Identifier: NCT00922363, LUMC protocol: P09.111), the Investigator's Brochure and the Investigational Medicinal Product Dossier were following good clinical practice (GCP) and the declaration of Helsinki and were approved by the accredited Ethical Review Board of LUMC and the relevant national authorities.

2.2. The investigational vaccine

CAF01 is a two-component liposomal adjuvant system developed by SSI [16,8,9,10]. One component, DDA, is a cationic quaternary ammonium salt and the other component, TDB, is a glycolipid. Both components are synthetically manufactured. When these two components are mixed and rehydrated in the specific weight ratio 5:1, DDA to TDB, the liposomal adjuvant is named CAF01. The H1 recombinant fusion protein of Ag85B and ESAT-6, is developed and manufactured by Statens Serum Institut (SSI, Copenhagen, Denmark). H1 sterile solution and CAF01 sterile suspension were manufactured by SSI, in an accredited GMP facility and supplied to the LUMC pharmacy in separate vials of relevant strengths. The vaccine was reconstituted by addition of the specified volume of adjuvant to the antigen concentrate, and injected into the deltoid muscle with a 25 mm 22–25 Gauge needle in a volume of 0.5 ml.

2.3. Trial design

The trial was an open label, single-center, non-randomized phase I exploratory trial in mycobacteria-naïve individuals defined by a negative TST (<10 mm, 2 units RT-23 PPD (SSI, Denmark)) and a negative Quantiferon®-TB Gold In-Tube test (QFT; Qiagen, Venlo, The Netherlands). All individuals were HIV negative. The trial comprised four vaccination groups. Subjects in group 1 received 50 μg H1 with no adjuvant, whereas groups 2–4 received the same amount of antigen with 125/25 μg, 313/63 μg and 625/125 μg CAF01, respectively. In all vaccination groups, the subjects were vaccinated on trial days 0 and 56. After the original trial was completed, a protocol amendment was approved (CCMO 12.1306/MA/26270, NL26270.000.09) and all trial participants were invited to attend a long-term visit 150 weeks after initial enrolment. Long-term visits were successfully conducted for 31 out of the original 34 volunteers that received 2 vaccinations within the appropriate time window. Timing of the long-term visit was on average 150.7 weeks (median 152.1 weeks; range 123–167 weeks) post primary vaccination and is referred to as '150 weeks' throughout the manuscript.

2.4. Trial subjects

The trial population consisted of 38 volunteers, healthy adult females or males between 18 and 55 years of age who had not been BCG vaccinated and who did not have active, chronic or past TB disease, and who had no MTB infection as confirmed by a negative QFT and a negative TST at screening. The general health of all participants was assessed by reviewing their recorded medical history, and performing a physical examination, and standard blood (including hepatitis B, hepatitis C and HIV testing) and urine tests.

The volunteers were financially compensated as approved by the Institutional Review Board for the number and amount of blood and urine samples, inconvenience with respect to the intramuscular administration and for the time spent on trial visits and transportation to the trial site.

2.5. Safety parameters

The subjects remained under medical observation for 3 h after each intramuscular vaccination, for possible immediate adverse reactions. During the first week after each vaccination, symptoms and evening armpit temperature were recorded on a daily basis, thereafter on a weekly basis. A medical examination of local adverse reactions and temperature was performed on days 0, 1, 7 and 42 after both vaccinations.

Adverse events were coded into system organ class (SOC) and preferred terms (PT) according to MedDRA version 10.0 and were classified into local (loco-regional) and systemic adverse events. The intensity of adverse events was graded as mild (grade 1/easily tolerated), moderate (grade 2/sufficient to interfere with daily activities) or severe (grade 3/preventing normal activity).

The relatedness of adverse events to the vaccination was graded as not related, possibly related, probably related or certainly related. Abnormal laboratory findings were scored for severity into severity grades 1–4 (based on "Toxicity grading scale for healthy adults and adolescent volunteers enrolled in preventive vaccine clinical trials" – FDA 2007 guidelines).

2.6. Quantiferon®-TB Gold In-Tube test

QFT testing was done according to the manufacturer's instructions and categorized as positive when the result was $\geq$0.35 IU/ml at baseline, and at 32 and 150 weeks after the primary vaccination.

2.7. PBMC isolation and immunogenicity parameters

Blood samples for cellular immunity and antibody determinations were collected at baseline and at 1 and 6 weeks after both vaccinations, and at weeks 32, 52 and 150 post the primary vaccination. Briefly, 40 ml heparinized blood was centrifuged on Leucosep tubes (Greiner-bio-one, Austria) containing 15 ml Ficoll (LUMC pharmacy #902861) (20 min/800 g), after centrifugation plasma was removed for storage at −70 °C and PBMCs were removed and washed three times with sterile PBS (LUMC pharmacy). PBMCs were aliquoted and stored in liquid nitrogen in RPMI (Invitrogen

#22409-015) containing 20% fetal calf serum (PAA Laboratories #A15-043, Netherlands)/10% DMSO (Sigma #41650). After defrosting a minimum PBMC viability of 80% was considered acceptable for assay purposes.

2.8. Flow cytometry

PBMCs were stimulated with pools from Ag85B or ESAT-6 peptides for 6 h or left unstimulated before staining for CD3, CD4, CD14, CD19, CD45RO, IFN-γ, IL-2, TNF-α, IL-22, IL-17A and CD154 (see online supplement) [18].

2.9. Quantifying IFN-γ producing cells

IFN-γ was determined using ELISpot from frozen samples to enable batch processing of longitudinally collected samples [19,20]. In this protocol, cells were thawed and pre-stimulated for 16–18 h, followed by 24 h incubation in the ELISpot plate [10] (see online supplement).

2.10. Multiplex analysis of cytokine release

PBMCs were stimulated 6 days with H1 fusion protein and a panel comprising cytokines (IFN-γ, IL-2, IL-4, IL-10, IL-13, IL-17A, IL-22, TNF-α), chemokines (IP-10, MIG, MCP-1, MIP-1b) and growth factors (VEGF and GM-CSF) were measured in undiluted cell culture supernatant samples using a Milliplex multiplex bead assay (see online supplement).

2.11. Statistical analysis

Clinical data were collected in CRFs, subject diaries and laboratory records. The statistical analysis of the data was performed by JG Consult, an independent Contract Research Organization in accordance with a statistical analysis plan and GCP and ICH-guidelines and documented in the clinical trial report. Here we report safety results and safety analysis based on the statistical trial report which was performed using SAS software (SAS®, Cary, NC 27513, USA, version 9.2 TS Level 1M0) on Platform Windows XP PRO Version 5.1.2600. Adverse events were evaluated descriptively.

Immunogenicity results shown here were analyzed at SSI and LUMC using Prism 6.04 for Windows (GraphPad Software, Inc., La Jolla, CA 92037, USA). Change from baseline to each observed visit within groups and comparisons between groups were compared using Kruskal–Wallis test with Dunn's correction. No formal sample size calculation was performed in this trial. An alpha <0.05 was considered significant throughout the trial.

3. Results

3.1. Trial subjects

Of 49 screened subjects 38 were included in the clinical trial. The safety population consisted of all included subjects. Mean ages were 20.7, 22.2, 30.5, and 24.6 years in vaccination groups 1, 2, 3 and 4, respectively, overall mean age of 24.9 years, ranging from 18–51 years. Seven subjects (7 females) were vaccinated with 50 μg H1 (no adjuvant), 10 subjects (2 male, 8 female) with 50 μg H1 + 125/25 μg CAF01 (low adjuvant group), 11 subjects (2 male, 9 female) with 50 μg H1 + 313/63 μg CAF01 (intermediate adjuvant group) and finally, 10 subjects (1 male, 9 female) with 50 μg H1 + 625/125 μg CAF01 (high adjuvant group). A total of 34 subjects were included in the per-protocol population and 7, 9, 10 and 8 from groups 1, 2, 3 and 4, respectively, were included in the immunogenicity analysis (Fig. 1). Long-term visits, 150 weeks after initial enrolment, were successfully conducted for 31 out of the original 34 per protocol trial subjects; 7, 9, 9 and 6 from groups 1–4, respectively.

3.2. Safety results

All 38 subjects with at least one vaccination were included in the safety analysis. No vaccine related serious or severe adverse reactions occurred during the trial. Loco-regional injection site reactions occurred more frequently in those given the CAF01-adjuvanted antigen, and mainly included stiffness (defined as injection site movement impairment) and pain at the injection site one day after the vaccinations (Table 1). Of note, these reactions were not more frequent after the second vaccination and there was no significant difference between the three adjuvant doses. In total, any local adverse reactions were distributed with 6 events in 2 (29%) subjects in the non-adjuvanted group 1, 26 events in 10 (100%) subjects in group 2, 24 events in 9 (82%) subjects in group 3 and 26 events in 9 (90%) subjects in group 4. None of the subjects required analgesics and all experienced full recovery within a maximum of 4 days. A small, cold nodule at the injection site was noted in 1 subject in the intermediate CAF01 dose group 3. No signs of attendant inflammation or local vesiculation, axillary lymphadenitis or fistula did occur, and the nodule had disappeared within one week. One subject in group 4 (in concomitant treatment with tramadol) did not receive the second vaccination due to rash and itch on knees, hips and elbows, as a relation to the trial vaccine could not be ruled out. Three individuals observed reactivity at the TST injection site after vaccination, two were in the mid- and high-dose adjuvant groups (reporting erythema and induration) and one was in the group receiving H1 without adjuvant (reporting erythema and swelling). A fourth individual observed erythema and induration at the site of the first vaccination after the 2nd vaccination (Table 1).

Systemic adverse reactions included headache, fatigue, malaise and fever in one subject given antigen only. Extensive follow-up of blood and urine parameters did not reveal any obvious trends within or differences between the three vaccination groups, or laboratory abnormalities with respect to change from baseline that could be related to the vaccinations. In the two subjects who developed a transient fever the day after vaccination, a small rise in C-reactive protein occurred that had subsided within a week.

3.3. Immunogenicity results

3.3.1. IFN-γ ELISpot

Stimulation with H1, Ag85B and ESAT-6 gave rise to an increased number of spot forming units (SFU) in all adjuvant groups (Fig. 2A and B). The highest proportion of responders to vaccination was seen in the low CAF01 group at week 32 and in the intermediate CAF01 group at week 32 and 52 (Fig. 2C). At this time point median responses were 301 SFU/per million PBMC (inter quartile range (IQR) 111–668 SFU) for H1; 308 SFU (IQR 108–558 SFU) for Ag85B and 39 SFU (IQR 9.5–136 SFU) for ESAT-6, $p < 0.05$ (Fig. 2B). No changes from baseline were seen in the non-adjuvant group at any time points. Overall, there was a clear trend in the adjuvant groups that responses increased after the first vaccination and that a second vaccination further increased the magnitude of responses (Fig. 2A).

3.3.2. Multiplex analysis of secreted biomarkers

To assess the breadth of the vaccine-induced immune memory, we performed an exploratory multiplex analysis of 14 cytokines and chemokines in supernatants of 24 h H1 stimulated PBMCs. We observed a broad induction of multiple cytokines and chemokines at both weeks 14 and 32 for the three groups vaccinated with

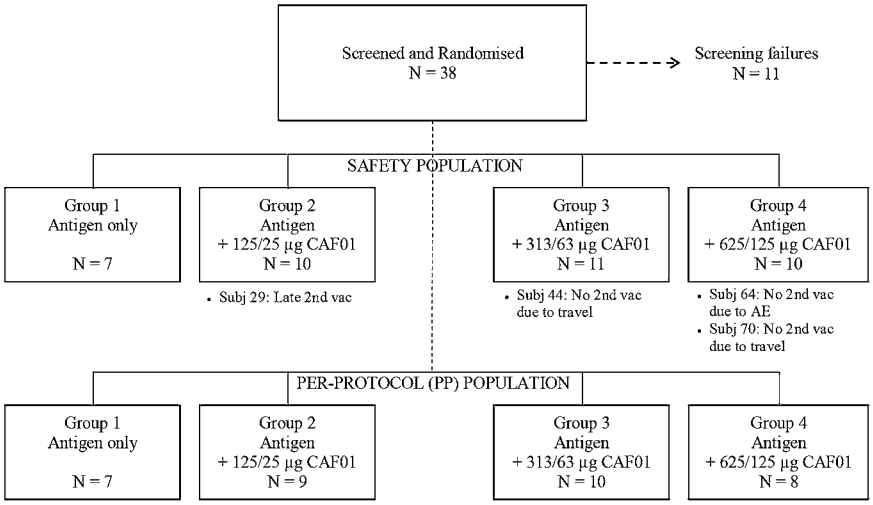

Fig. 1. Study overview and flow chart. Overview of volunteers screened and included into the clinical trial. All volunteers vaccinated were included in the safety population, whereas only those that received both vaccinations were included in the per-protocol population used for immunological analyses.

adjuvanted H1, responses in the intermediate CAF01 group are presented in Fig. 3 (all groups in supplementary Figure 1). The dominating markers were Th1 associated (IFN-γ, TNF-α, IP-10, MIG, MIP-1b and GM-CSF), but we also observed a substantial release of IL-13, but not IL-4. IL-2, IL-10 and IL-17 followed the same kinetic pattern, but levels were very low (<20 pg/ml) and failed to reach significance (Fig. 3 and data not shown). No clear pattern emerged for VEGF, IL-22 and MCP-1 (supplementary Figure 1).

Table 1

	Group 1	Group 2	Group 3	Group 4
	H1 only	H1 + 125/25 µg CAF01	H1 + 313/63 µg CAF01	H1 + 625/125 ug CAF01
Safety analysis set, *n*	7	10	11	10
Local adverse reactions; number of subjects (%), total number of reactions				
Reactions at site of injection				
Injection site stiffness	1 (14) 1	3 (30) 3	9 (82) 13	7 (70) 8
Injection site pain	0 (0) 0	10 (100) 15	2 (18) 2	5 (50) 6
Injection site swelling	0 (0) 0	2 (20) 2	2 (18) 2	4 (40) 5
Injection site erythema	0 (0) 0	2 (20) 2	1 (9) 1	3 (30) 3
Injection site induration	0 (0) 0	1 (10) 1	1 (9) 1	0 (0) 0
Injection site pruritus	0 (0) 0	1 (10) 1	0 (0) 0	1 (10) 2
Injection site bruising	0 (0) 0	1 (10) 1	0 (0) 0	0 (0) 0
Other local reactions				
Lymphadenopathy	0 (0) 0	1 (10) 1	1 (9) 1	0 (0) 0
Reactions at site of tuberculin skin testing				
Erythema	1 (14) 1[b]	0 (0) 0	1 (9) 2[b,a]	1 (10) 1[b]
Induration	0 (0) 0	0 (0) 0	1 (9) 2[b,a]	1 (10) 1[b]
Swelling	1 (14) 2[b]	0 (0) 0	0 (0) 0	0 (0) 0
Reactions at site of first vaccination				
Erythema	1 (14) 1	0 (0) 0	0 (0) 0	0 (0) 0
Induration	1 (14) 1	0 (0) 0	0 (0) 0	0 (0) 0
Any local adverse reaction	2 (29) 6	10 (100) 26	9 (82) 24	9 (90) 26
Systemic adverse reactions; *n* (%), total number of reactions				
Fatigue	2 (29) 3	1 (10) 1	0 (0) 0	3 (30) 3
Malaise	0 (0) 0	0 (0) 0	0 (0) 0	2 (20) 3
Pyrexia	1 (14) 1	0 (0) 0	0 (0) 0	0 (0) 0
Rhinitis	1 (14) 1	0 (0) 0	0 (0) 0	0 (0) 0
Musculoskeletal stiffness	0 (0) 0	0 (0) 0	1 (9) 1	0 (0) 0
Myalgia	1 (14) 1	0 (0) 0	0 (0) 0	0 (0) 0
Headache	1 (14) 1	1 (10) 1	1 (9) 1	2 (20) 3
Pruritus	0 (0) 0	0 (0) 0	1 (9) 1	1 (10) 1
Rash pruritic	0 (0) 0	0 (0) 0	0 (0) 0	1 (10) 1
Any systemic adverse reaction	3 (43) 7	2 (20) 2	3 (27) 4	4 (40) 11

Injection site stiffness reactions (MedDRA PT term Injection site movement impairment) were of mild intensity (easily tolerated) except for one of moderate intensity.

[a] One subject had erythema and induration at the 1st injection site after the 2nd vaccination.

[b] Three subjects had erythema, induration and or swelling at the Tuberculin injection site (of original PPD skin testing) after the 1st and/or 2nd vaccination.

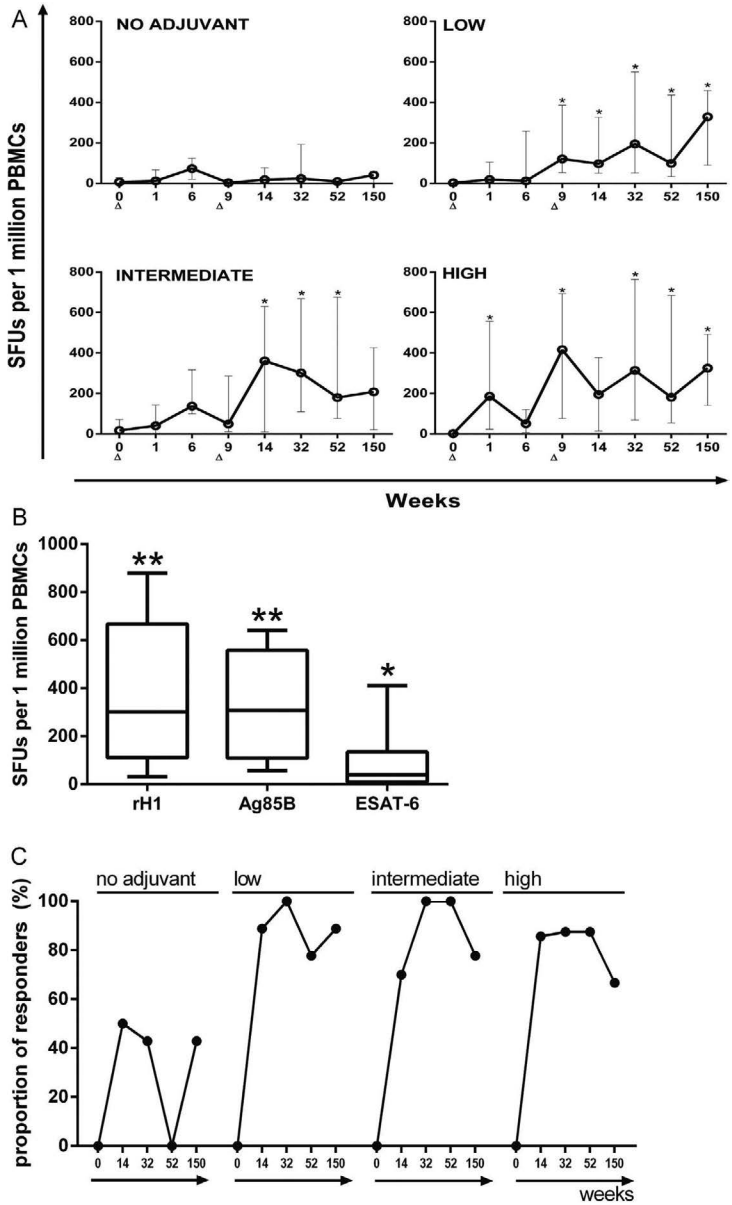

Fig. 2. IFN-γ spot forming units (SFUs) per 1 million PBMCs measured by ELISpot. (A) Healthy volunteers were given two doses of intramuscular injection of 50 μg of H1 antigen without CAF01 adjuvant ($n = 7$), low dose CAF01 adjuvant ($n = 9$), intermediate dose CAF01 adjuvant ($n = 10$) or high dose CAF01 adjuvant ($n = 8$). Vaccinations were done at week 0 and week 8 and PBMC samples were collected at weeks 0, 1, 6, 9, 14, 32, 52 and 150 and stimulated with H1 protein. Interconnected dots represent median, error bars interquartile range. Δ indicates vaccination, * indicates $p < 0.05$ compared to week 0 (Kruskal–Wallis, with Dunn's multiple comparisons test). (B) Healthy volunteers were given two doses of intramuscular injection of 50 μg of H1 antigen in intermediate dose CAF01 adjuvant ($n = 10$). Vaccinations were done at week 0 and week 8 and PBMC samples were collected at week 32. PBMCs were stimulated with H1 protein or Ag85B or ESAT-6 peptide pools and IFN-γ producing spots quantified using ELISpot. Responses are presented as median error bars interquartile range. * indicates $p < 0.05$, ** $p < 0.005$ compared to week 0 (Kruskal–Wallis, with Dunn's multiple comparisons test). (C) The proportion of responders to H1 stimulation was defined as the number of H1 induced IFN-γ-producing spots greater than the mean + 2.5 SD of unstimulated cells and was expressed as percentage of the group size.

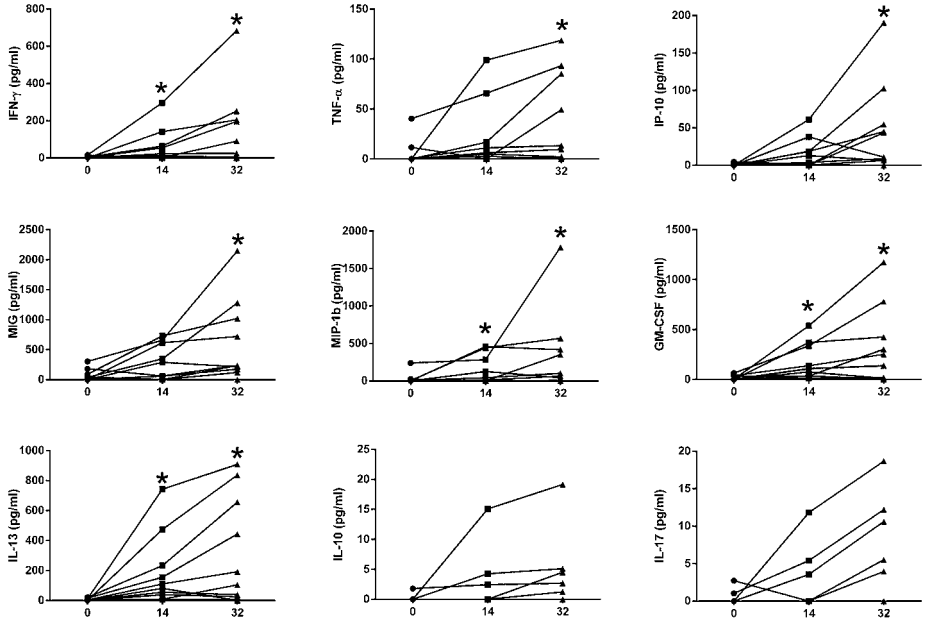

Fig. 3. Cytokine secretion in supernatants of 6 day H1 stimulated PBMCs. PBMCs were collected at baseline and at weeks 14 and 32 after two doses of intramuscular injection of 50 μg of H1 antigen. PBMCs were stimulated with H1 antigen for 6 days, supernatants were collected and cytokines and chemokines were measured using multiplex bead arrays. Only individuals from the intermediate dose CAF01 adjuvant are shown ($n = 10$). Responses are presented as individual measurements, * indicates $p < 0.05$ compared to week 0 (Kruskal–Wallis, with Dunn's multiple comparisons test).

3.3.3. Flow cytometry

To further assess the long-term immunogenicity of H1:CAF01, PBMC samples at week 150 were analyzed by Intracellular flow cytometry. Compared to the non-adjuvant group, intermediate and high dose CAF01 groups had increased frequencies of Ag85B-specific CD4 T-cells producing IFN-γ and/or IL-2 and/or TNF-α (Fig. 4A). Moreover, intermediate and high dose CAF01 groups induced significant TNF-α production, but only the intermediate CAF01 group reached significant levels of IL-2 (Fig. 4B) ESAT-6 specific CD4 T-cells were seen in the adjuvant groups, but responses were not significantly different from those in the non-adjuvant group. The most prevalent subset was IL-2/TNF-α double producing CD4 T-cells, and significantly increased frequencies of these cells were seen in the intermediate and high adjuvant groups compared to the non-adjuvant group (Fig. 4C). Responses were also detected in the triple positive subset and TNF-α single positive subset, but neither reached significance. No significant IL-17 responses to antigenic stimulation were detected (data not shown). No CD8 T-cell responses were observed following Ag85B or ESAT-6 stimulation (data not shown).

3.3.4. Antibody responses

No statistically significant changes from baseline were seen in any of the vaccination groups in IgG anti-Ag85B-ESAT-6 specific antibody titer (data not shown, methods in online supplement).

3.3.5. Quantiferon®-TB Gold conversion

QFT was performed at baseline at week 32, and 150 weeks after the last vaccination. All subjects were negative before vaccination (as per the inclusion criteria) and none in the non-adjuvanted group became QFT positive. However introducing CAF01 adjuvant in the vaccine caused 3 out of 8 (38%) individuals in the low CAF01 group to convert to a positive test, 6 out of 10 (60%) in the intermediate CAF01 group and 3 out of 8 (38%) in the high adjuvant group (Fig. 5). All but two of the QFT converters had reverted to negative at week 150. One QFT converter was lost to the extended follow up.

4. Discussion

This report describes the first clinical trial in humans investigating the TB vaccine H1:CAF01, combining a new liposomal adjuvant CAF01 with a well-defined TB subunit vaccine antigen H1. In this study, the vaccine was safe, well tolerated and generated long-lasting (3 years) T-cell responses, as monitored by IFN-γ ELISpot, intracellular cytokine staining and multiplex analysis of 14 secreted cytokines and chemokines.

Two vaccinations with H1:CAF01 did not lead to any serious adverse reactions. All adverse events that were assessed as related to the vaccination were mild or moderate and disappeared within days. The main H1:CAF01-related adverse event was stiffness and pain at the injection site, of mild to moderate severity, mostly the day after administration of the vaccine. A mild to moderate transient local reactogenicity of H1:CAF01 was anticipated based on the findings in nonclinical GLP toxicity studies and was also observed in previous vaccination studies in humans with the H1 antigen [6,7,21]. The vaccine did not consistently affect hematological or biochemical measurements. In conclusion, this clinical trial found no safety concerns associated with the administration of the CAF01-adjuvanted vaccine to healthy adults. As this was a phase I trial, the limitation to this conclusion is the limited number of

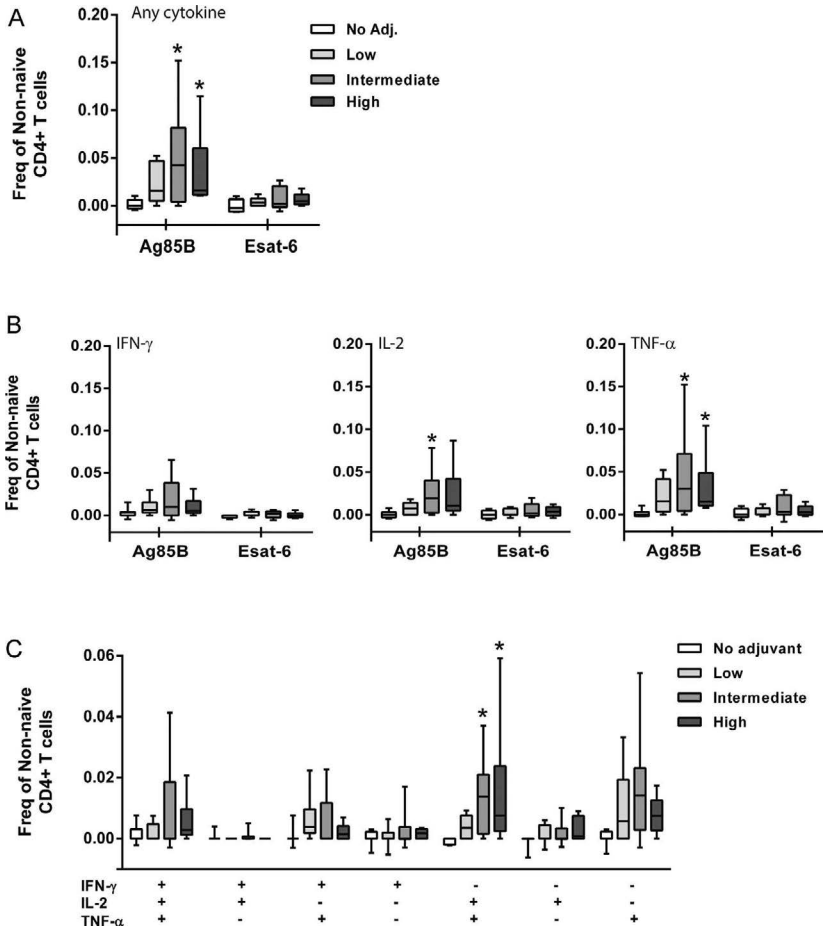

Fig. 4. Ag85B- and ESAT-6-specific CD4 responses in PBMCs collected at week 150 measured by flow cytometry. (A) Long-term visits at week 150 after vaccinations were successfully conducted for 31 out of the original 34 per protocol trial subjects; 7, 9, 9 and 6 from groups 1–4, respectively. PBMCs were isolated and frequencies of IFN-γ, IL-2 or TNF-α producing $CD4^+CD154^+$T-cells were measured after stimulation with Ag85B or ESAT-6 peptide pools. Responses are presented as box and whiskers, wherein the box represents median and interquartile range, whiskers range. For each individual, background values (DMSO) were subtracted. Kruskal–Wallis, with Dunn's multiple comparisons test, * indicates $p < 0.05$ compared to the no adjuvant group. (B) Frequency of IFN-γ (left), IL-2 (middle) or TNF-α (right) producing $CD4^+CD154^+$T-cells were measured after stimulation of week 150 PBMCs with Ag85B or ESAT-6 peptide pools. Responses are presented as box and whiskers, wherein the box represents median and interquartile range, whiskers range. For each individual, background values (DMSO) were subtracted. Kruskal–Wallis, with Dunn's multiple comparisons test, * indicates $p < 0.05$ compared to the no adjuvant group. (C) Patterns of single or combined production of IFN-γ, IL-2 or TNF-α by $CD154^+CD4^+$T-cells after stimulation of with Ag85B peptide pool are shown. The median frequency for each cytokine-producing cell subset is represented by the horizontal line, the interquartile range by the box, and the range by the whiskers. For each individual, background values (DMSO) were subtracted. Kruskal–Wallis, with Dunn's multiple comparisons test, * indicates $p < 0.05$ compared to the no adjuvant group.

subjects, and we can exclude with certainty only frequently occurring adverse reactions. On the other hand, the lack of any significant reactogenicity despite the immunogenicity of the CAF01-adjuvanted vaccine is important, given the paucity of effective human adjuvants for the induction of cell-mediated immune responses [22].

Successful vaccination against TB disease would be a major step to diminish TB disease burden and spread, however an important challenge remains to determine vaccine efficacy. Despite significant investments in the search for an accurate surrogate endpoint for protection against TB disease, no such biomarker has been identified. However, there is general consensus that an effective TB vaccine needs to be able to elicit at least a Th1 cell response which is essential for bacterial containment [23]. Importantly, due to the nature of the pathogen, a novel vaccine will need to induce long-lived protection, most likely through the induction of central memory T (T_{CM}) cells. Whereas IFN-γ production is the classical hallmark of Th1 cell responses and for many years has been used as the primary measurement in TB vaccine clinical testing, CD4 T-cells with a regenerative potential are typically IL-2

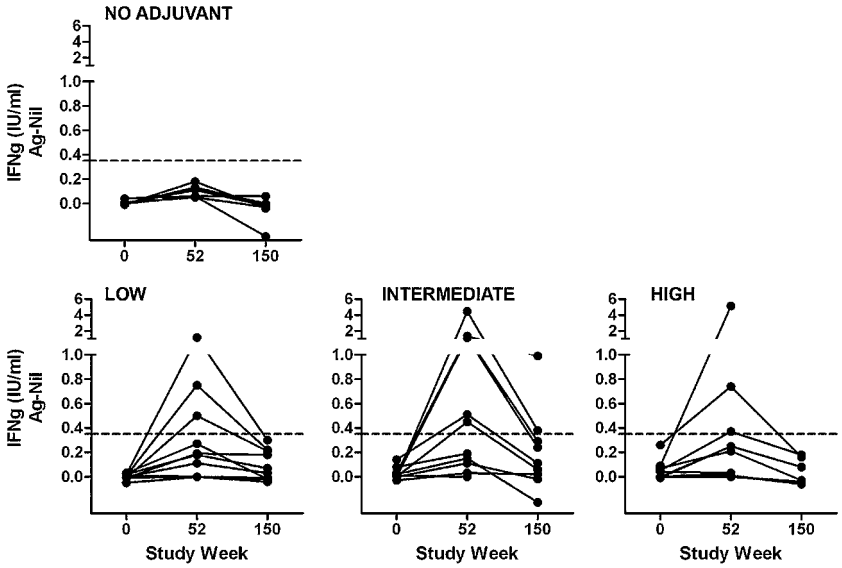

Fig. 5. Quantiferon TB Gold in tube (QFT) IFN-γ responses after vaccination with H1 alone and adjuvanted with low, intermediate and high CAF01 dose. Whole blood was collected in QFT blood collection tubes at baseline, week 52 and week 150 and incubated 16–24 h. Plasma was isolated by centrifugation and IFN-γ was determined using ELISA. Responses were classified as positive if the antigen-specific level (Ag-Nil) was ≥0.35 IU/ml.

positive and T_{CM} are usually functionally defined by the expression of IL-2 and CCR7/CD62L. Two vaccinations of H1:CAF01 induced a strong long-lasting cellular immune response to H1 and its two antigen components ESAT-6 and Ag85B. Responses were strongest to the Ag85B antigen, as observed previously also for H1:IC31 [6,7]. Measured by IFN-γ ELISpot, the vaccine led to increased responses at subsequent visits which were sustained also after 150 weeks, demonstrating a clear and long-term vaccine take in all three adjuvanted vaccine groups, but not in the non-adjuvanted group, as observed previously also for H1:IC31 [6,7]. This pattern was confirmed by the broad induction of mainly Th1 associated cytokines (IFN-γ, IL-2, TNF-α, GM-CSF) and chemokines (MIG, IP-10 and MIP-1β). Three years after vaccination, the intermediate and high H1:CAF01 dose groups showed significant numbers of antigen-specific CD4 T-cells secreting IL-2 and TNF-α, consistent with a central memory differentiation state, ready to become effector T-cells if required [24]. These results are in line with two recent and closely related TB vaccine trials investigating H1:IC31 in HIV-infected individuals, and H56:IC31 in healthy individuals with or without latent TB (Klaus Reiter, Gavin Churchyard, Thomas Scriba, personal communication), and recent results from a phase I/II trial of the subunit vaccine M72 adjuvanted in the liposome based $AS01_E$ [25]. These results underpin that estimates of vaccine immunogenicity based on IFN-γ detection alone will miss other relevant vaccine-induced immune responses. The prolonged maintenance of immune competence elicited by the CAF01-adjuvanted subunit vaccine is in good agreement with observations from mouse studies [11,12], and suggests that the adjuvant, likely through establishment of an antigen depot and subsequent slow release and targeting of dendritic cells [16], may have particular abilities to maintain immune memory [26]. In this regard, it is interesting to note that the development of immune profiles differ markedly between viral vectored vaccines and adjuvanted subunit vaccines with the latter having a slowly developing response dominated by IL-2/TNF-α double positive T-cells and with no tendency of a waned response over the three years observation time. Although MVA85A induces highly durable Th1 responses, peak responses were observed already 7 days post-vaccination [27] and with triple and double positive TNF-α/IFN-γ T-cells resembling a more effector-memory profile [28]. Whether this difference has any influence on the overall protective capability remains to be seen.

Significant amounts of IL-13 were also found in the intermediate and high dose CAF01 groups. IL-13 is traditionally associated with Th2-type immune responses and together with IL-4 involved in inflammatory disorders, however, a number of recent findings suggest a more complex lineation. Gallo and Katzman identified IL-13 producing CD4 T-cells in mice co-expressing IFN-γ and IL-17 generated both during autoimmune diseases but also upon immunization [29]. Although the induction of IL-13 in human vaccine trials is a relatively unexplored field, IL-13 responses has also been observed in volunteers receiving the Th1-promoting adjuvant MPL® [30] and synthetic HIV-1 peptides coupled to a palmytoil tail was found to induce both IFN-γ and IL-13 in a phase II trial [31]. These novel data show that IL-13 is an integrated component of a vaccine-induced Th1/Th17 response and an important role of IL-13 could be to down-regulate the vigorous inflammatory response induced by these novel generation adjuvants. We recently identified IL-13 secretion after vaccination with CAF01-based subunit vaccines in mice and the cellular origin and the regulatory role in balancing Th1/Th17 responses is currently under exploration (Dietrich, unpublished).

This trial demonstrated promising immunogenicity results, a good safety profile and no dose dependent adverse events. Immunogenicity data suggests that the intermediate and high dose of adjuvant induced superior T_{CM} profile, however this phase 1

safety trial was not designed for firm conclusion on dose selection. If these characteristics of CAF01 are confirmed for other disease targets, this adjuvant would be among the first candidates capable of inducing long-term memory cellular immune response in humans. This property is unique and not shared with currently approved adjuvants like aluminum salts and MF59, both of which primarily promote a Th2 or humoral immune response [22,32–34]. Based on results from animal models we expected CAF01 adjuvanted vaccines to also induce antibody responses to the vaccine antigen, however herein two vaccinations with H1:CAF01 did not induce significant IgG responses. Similarly, H1 in IC31® also failed to induce significant H1-specific IgG levels after two injections. We recently found specific IgG after a third administration of H56:IC31 (Hoff, Andersen, unpublished observation), suggesting that a third dose is required to induce IgG responses to this particular vaccine antigen in humans.

ESAT-6 is included in Interferon gamma release assay (IGRA) diagnostic test kits. In the present trial, similar to previous H1:IC31® trials, vaccination was associated with a transient conversion of the QFT in about half of the vaccinated subjects. Induction of ESAT-6 specific immune responses by vaccination with an ESAT-6-containing vaccine may very well interfere with current ESAT-6 based diagnostics. However, this may not pose a major diagnostic problem, as IGRAs are indicated in low endemic settings and TB vaccines will mainly be used in high endemic settings [35].

In conclusion, we report the first in man studies of the CAF01 adjuvant and demonstrate its safety in a phase I trial. Vaccination with CAF01 together with the H1 fusion protein resulted in long lasting T-cell immunity characterized by mainly IL-2 and TNF-α producing T-cells indicating that CAF01 is of relevance for future human vaccination studies.

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Conflict of interest statement: PA is co-inventor on a patent application claiming H1 as a vaccine and CAF01 as vaccine adjuvant. All rights have been assigned to Statens Serum Institut, a Danish not-for-profit governmental institute. BTC, EMA, IK, MR, SH and LVA are employed by Statens Serum Institut. The other authors involved in this study have no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.10.036.

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III. Vaccination in immunocompromised hosts

Chapter 10 Elderly subjects have a delayed antibody response and prolonged viraemia following yellow fever vaccination: a prospective controlled cohort study. *PLoS One. 2011;6(12):e27753.*

Chapter 11 Response to 2009 pandemic influenza A (H1N1) vaccine in HIV-infected patients and the influence of prior seasonal influenza vaccination. *PLoS One. 2011 Jan 31;6(1):e16496.*

Chapter 12 No cross-reactive serum antibodies to 2009 pandemic influenza A (H1N1) after seasonal influenza vaccination in the virus neutralization assay. *Unpublished supplemental data.*

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PLoS one

Elderly Subjects Have a Delayed Antibody Response and Prolonged Viraemia following Yellow Fever Vaccination: A Prospective Controlled Cohort Study

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Abstract

Background: Yellow fever vaccination (YF-17D) can cause serious adverse events (SAEs). The mechanism of these SAEs is poorly understood. Older age has been identified as a risk factor. We tested the hypothesis that the humoral immune response to yellow fever vaccine develops more slowly in elderly than in younger subjects.

Method: We vaccinated young volunteers (18–28 yrs, N = 30) and elderly travelers (60–81 yrs, N = 28) with YF-17D and measured their neutralizing antibody titers and plasma YF-17D RNA copy numbers before vaccination and 3, 5, 10, 14 and 28 days after vaccination.

Results: Ten days after vaccination seroprotection was attained by 77% (23/30) of the young participants and by 50% (14/28) of the elderly participants (p = 0.03). Accordingly, the Geometric Mean Titer of younger participants was higher than the GMT of the elderly participants. At day 10 the difference was +2.9 IU/ml (95% CI 1.8–4.7, $p = 0.00004$) and at day 14 +1.8 IU/ml (95% CI 1.1–2.9, $p = 0.02$, using a mixed linear model. Viraemia was more common in the elderly (86%, 24/28) than in the younger participants (60%, 14/30) (p = 0.03) with higher YF-17D RNA copy numbers in the elderly participants.

Conclusions: We found that elderly subjects had a delayed antibody response and higher viraemia levels after yellow fever primovaccination. We postulate that with older age, a weaker immune response to yellow fever vaccine allows the attenuated virus to cause higher viraemia levels which may increase the risk of developing SAEs. This may be one piece in the puzzle of the pathophysiology of YEL-AVD.

Trial Registration: Trialregitser.nl NTR1040

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Introduction

The live attenuated 17D yellow fever vaccine is regarded as one of the safest and most effective vaccines [1]. However, in immunocompromized individuals yellow fever vaccination can cause fatal adverse events [2,3]. A hampered immune response could allow the vaccine virus to replicate unrestrictedly, leading to vaccine-associated disease that resembles wild type yellow fever (yellow fever vaccine associated viscerotropic disease, YEL-AVD). YEL-AVD is fatal in 50% of cases [4]. In the last decade, a series of these serious and sometimes fatal adverse events following yellow fever vaccination has been reported [5–11]. The risk of YEL-AVD is increased for those with a history of thymectomy [12], male gender [13] and with increasing age. For vaccinees of 60–69 years this risk is estimated to be 1:100.000 doses and for vaccinees of ≥70 years it is 2.3–3.2:100.000, which is approximately a 4 and 11 fold higher risk than the risk for young adults [13,14]. The higher risk of YEL-AVD in elderly travelers has resulted in a more restrictive policy towards vaccinating travelers of 60 years and older, also advised by the World Health Organisation and Centers for Disease Control en Prevention [15–18]. In this group the risk of serious adverse events following vaccination is weighed against the risk of infection, using disease surveillance data of the WHO and reports of yellow fever outbreaks.

The biological mechanism for the association between adverse events and older age has not yet been elucidated [4]. Both innate and adaptive immune responses wane with increasing age [19]. This may allow the attenuated vaccine virus more time to replicate and cause adverse events in elderly subjects. In this study we focused on humoral immunity, as this is considered to confer protective immunity against yellow fever. We tested the hypothesis that the adaptive immune response to yellow fever vaccine develops more slowly in elderly than in young subjects.

Methods

The protocol for this trial and supporting checklist are available as supporting information; see Checklist S1 and Protocol S1.

Ethics statement

The protocol and consent forms were approved by the Dutch Central Committee of Human Research (CCMO) and by the Medical Ethical Committee of the Leiden University Medical Center (LUMC) in the Netherlands. The trial was registered under NTR1040 and ISRCTN42180653, (http://irsctn.org). Written informed consent was obtained from each participant prior to inclusion.

Objectives

This study was conducted to determine whether the adaptive immune response to yellow fever vaccine is slower to develop in persons of 60 years or older compared with persons aged 18 to 40 years. Primary outcomes were the humoral response to yellow fever vaccination, measured by Plaque Reduction Neutralization Test (PRNT), and Yellow Fever 17D (YF-17D) viraemia after vaccination, which was quantified by real time PCR (qRT-PCR). Secondary outcomes were adverse events.

Study design and Participants

In this prospective controlled cohort study, participants were recruited at the Travel Clinic of the Leiden University Medical Center (LUMC), and Municipal Health Centers of Leiden and The Hague, the Netherlands. Healthy volunteers aged between 18 and 40 years and eligible for inclusion into the control group were invited to participate. Participants in the control group were not necessarily planning to travel to a yellow fever endemic area. The study group consisted of healthy travelers aged 60 years or above, who had an indication for yellow fever vaccination based on their travel destination (National Coordination Center for Travelers' Health, LCR) [20]. Individuals who had previously received yellow fever vaccine or who had a compromised immunity due to underlying illness or immunosuppressive medication and those who were pregnant were excluded. The study was carried out between April 2008 and April 2009. Vaccinations were administered at the Travel Clinic of the LUMC by AR. The trial ended because the number of inclusions was met.

Yellow fever vaccine

The live, attenuated, 17D vaccine used in this study was manufactured on embryonated chicken eggs according to WHO regulations and stored according to manufacturer's guidelines. All administered vaccines originated from the same vaccine lot (Stamaril, Lot no B5355, Sanofi Pasteur, France). The vaccine was administered subcutaneously in the deltoid region of the right arm.

Data collection

At the time of inclusion, data on demographic characteristics of the participants were obtained. Blood samples for the determination of neutralizing antibodies (NA) and YF-17D viraemia were collected before (day 0), and 3, 5, 10, 14 and 28 days after vaccination. Participants were asked to document any injection site and systemic adverse events after vaccination in a three-week diary. Solicited symptoms were: erythema, pain and swelling at the site of injection, fever and myalgia. Non-solicited symptoms could also be reported.

Constant virus – varying serum dilution Plaque Reduction Neutralization Test (PRNT)

The tests were carried out in 6-well plates (Corning Inc., USA) using a slightly modified technique described originally by De Madrid and Porterfield [21]. Briefly, approximately 6×10^5 Vero cells/mL were seeded per well in 6-well plates and cultured to obtain a confluent monolayer. Coded sera were complement inactivated at 56°C for 1 hour. Pre-vaccination sera were tested in 1:16 dilution, to which 100 plaque forming units (PFU) of 17D-YF were added. Post vaccination sera were tested in two-fold dilutions starting from 1:4 to 1:1024. One hundred PFU of YF-17D virus were added to each serum dilution. All test sera were assayed in duplicate. After 1 hour incubation on ice, the mixtures of virus and serum were added to the Vero cell monolayers and incubated for 1 hour at 37°C. An overlay of 2×DMEM and 2% agarose was added. After 5 days of incubation at 37°C, the overlay was discarded and cell monolayers were stained with crystal violet. Plaques were counted by eye by a person who had no access to the sample code. Virus neutralization (VN) was calculated for each serum dilution (i) with the following formula: VN(i) = 100×1−(-number of PFU in diluted post vaccination serum/number of PFU in pre-vaccination serum (in a 1:16 dilution)). The serum dilution at which $\log_{10}$ neutralization index 0·7 (80% VN) occurred was taken as endpoint, as this corresponds to the World Health Organization (WHO) definition of protection [22]. A reference serum, obtained from the National Institute for Biological Standards and Control (http://www.nibsc.ac.uk/) was used for quantification of International Units per milliliter (IU/ml). In our hands a 0.7 log10 plaque reduction in 1:10 diluted serum corresponds to a titer of 0.5 IU/ml [95%CI 0.3–0.8 IU/ml] [23]. Similar values have been found by others [24]. Geometrical mean titers (GMT) were compared between the two groups.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Viral RNA was isolated from 200 µl plasma using a MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Molecular Diagnostics, Penzberg, Germany). cDNA was synthesized with 10 µl elute (200 µl total) in a professional ThermoCycler (Biometra, Germany), and quantitative reverse transcription-PCR (qRT-PCR) of YFV RNA was performed in a BioRad i-cycler IQ^{TM} real-time PCR detection system (BioRad, Veenendaal, The Netherlands). The following YFV specific primers and probe were used [25]:

YFV-1 (forward) AATCGAGTTGCTAGGCAATAAACAC
YFV-2 (reverse) TCCCTGAGCTTTACGACCAGA
YFV-P (probe) FAM-ATCGTTGAGCGATTAGCAG-BHQ

FAM (6-carboxyfluorescein) was used as 5′-reporter dye and BHQ (Black Hole Quencher) as 3′-quencher dye. In order to quantify YFV RNA, $\log_{10}$ dilutions of in vitro transcribed RNA standards were included as standard curves. RNA virus levels were calculated with standard curves from Cycle threshold (Ct) values to compare viraemia in both groups quantitatively, and were expressed as IU/ml.

Statistical methods

Power calculations were based on an expected 80% virus neutralization of 95% in the control group and 66% in the elderly group at day 14, based on previous observations at the Travel Clinic (unpublished data). With an α of 0·05 and β of 0·2, 26 participants per group were needed to confirm a significant difference under these assumed conditions. To take into account a possible attrition rate of 15%, 30 participants were included per

group. We analyzed the between group difference in GMT over the four time points (day 5, 10, 14, 28) using a mixed linear model. This model takes into account that each subject had repeated measurements of the antibody titer over time. More specifically, a unique identification number for each subject was entered as a random effect in the model and separate variables for all time points and for the groups (elderly versus young) were entered as fixed effects. Antibody titers below the detection threshold were assigned an arbitrary value of 0.05 IU/ml, which is twofold lower than the lowest detectable titer (i.e. 0.2 IU/ml). Where appropriate, Chi-square tests were used, and Wilcoxon's test for non-parametrical distributed numerical data. Statistical analysis was performed using a computer-assisted software package (SPSS version 16.0, SPSS Inc., Chicago, IL).

Results

Population

We enrolled 60 participants, none of whom withdrew prematurely. In 2 elderly participants, 17D-YF neutralizing antibodies were already present at day 0. In retrospect, these participants remembered that they were vaccinated against yellow fever many years ago. These two individuals were excluded from further analysis. In both groups 70% were female and 30% had visited flavivirus endemic countries in the past. The median age of the younger participants was 21 years (interquartile range 20–22.5) and of the elderly was 66 years (interquartile range 65–69). Although we invited persons of 18 to 40 years of age for the control group, the oldest participant in this group was 28 years old. Therefore the control group is defined as age 18–30 years. We recorded the incidence of previous travel to countries that are endemic for flaviviruses because past infections with other flaviviruses can cause cross-neutralization in the YF PRNT.

Neutralizing antibody response

At day 3 and 5 after vaccination, no neutralizing antibodies were found in any of the participants. Ten days after vaccination seroprotection was attained by 77% (23/30) of the young participants and by 50% (14/28) of the elderly participants ($p = 0.03$, Chi-square test) (figure 1). The average GMT taken over the four time points after vaccination was higher in the group of young participants compared with the group of elderly participants. The average difference in GMT was +1.7 IU/ml (95% CI 1.2–2.4, $p = 0.007$). At day 10 the difference was +2.9 IU/ml (95% CI 1.8–4.7, $p = 0.00004$) and at day 14 +1.8 IU/ml (95% CI 1.1–2.9, $p = 0.02$). At day 28 the difference was no longer statistically significant (+1.5 IU/ml, 95%CI 0.9–2.4, $p = 0.12$). Female participants in the elderly group had a higher antibody response 10 days after vaccination (female vs. male 0.04 IU/ml (95% CI 0.01–0.15) vs. 0.002 IU/ml (95%CI 0.0005–0.01), $p = 0.03$). Such a difference between men and women was not seen in the group of young participants.

Yellow fever vaccine virus RNA

YF-17D viraemia was measured by qRT-PCR at day 0, 3, 5, 10 and 14 (table 1). Viraemia was detected more often in elderly (24/28, 86%) than in young participants (18/30, 60%) ($p = 0.04$, Chi-square test). In addition, the elderly had higher viraemia levels detectable for longer periods and two had detectable viraemia at day 10, compared with none of the younger participants (table 1).

Adverse events

Participants reported the occurrence and duration of adverse events after yellow fever vaccination in a 3-week diary (table 2). In younger participants vaccination evoked erythema at the site of inoculation more frequently and for a longer period than in the elderly participants. In both groups, viraemia peaked at day 5. In the group of elderly participants the mean viraemia level at day 5 was higher in those who experienced a systemic adverse event (fever and/or myalgia) than in those who did not (viraemia level 31.3 versus 11.5 IU/ml, 95% CI for the difference 0.4–40.0 IU/ml), $p = 0.05$). In the group of young participants mean viraemia levels did not differ significantly between those who did experience

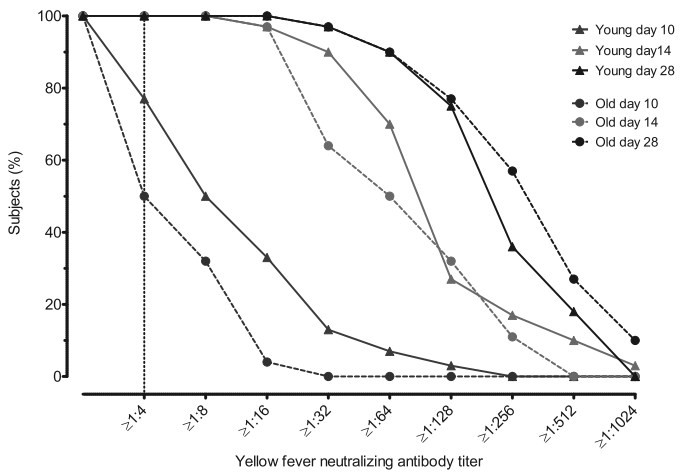

Figure 1. Neutralizing antibody response against YF-17D in young and elderly participants. Reverse cumulative distribution curves of yellow fever neutralizing antibody titers at 5, 10, 14 and 28 days after vaccination in 30 young and 28 elderly participants. Antibody titers were determined with Plaque Reduction Neutralization Tests and reflect the serum dilution at which 80% of virus was neutralized.
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Table 1. YF-17D viraemia measured by qRT-PCR in the elderly group compared to young participants.

YF-17D viraemia		Young N = 30	Elderly N = 28	p-value
Day 0	Number positive (%)	0 (0)	0 (0)	-
Day 3	Number positive (%)	6 (20)	11 (39)	0.1
	IU/ml (95% CI)	1.4 (0.9–1.9)	2.9 (2.1–4.4)	0.04
Day 5	Number positive (%)	16 (53)	23 (82)	0.02
	IU/ml (95% CI)	4.8 (0–10.7)	20.8 (10.2–31.5)	0.07
Day 10	Number positive (%)	0 (0)	2 (7)	0.2
	IU/ml (95% CI)	-	1.00 (0.8–1.2)	-
Day 14	Number positive	0 (0)	0 (0)	-
1 time point positive (%)		14 (78)	12 (50)	0.02
2 sequential time points positive (%)		4 (22)	12 (50)	

YF-17D RNA virus levels were calculated with standard curves from Cycle threshold (Ct) values and were expressed as IU/ml. Comparison of number of participants positive for viraemia was calculated by Fisher's Exact test. Comparison of quantitative viraemia (only of participants who had measurable viraemia) was calculated with Student's t-test. IU = International Units, 95% CI = 95% Confidence Interval.

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a systemic adverse event and those who did not (viraemia level 6.1 versus 3.9 IU/ml respectively).

Discussion

The main finding of this study was that after primary vaccination with 17D YF vaccine, elderly persons (≥60 years) were slower to develop an antibody response and had higher viraemia levels than younger persons. Only half of the elderly vaccinees had protective antibody levels 10 days after vaccination compared with over three quarters of younger vaccinees. In addition, GMT of neutralizing antibodies were significantly lower at 10 and 14 days after vaccination. The difference was less pronounced and no longer statistically significant 28 days after vaccination. Besides showing higher levels of viraemia in elderly subjects, our data also suggest that the duration of viraemia is prolonged in these subjects as two elderly participants and none of the younger participants had detectable viraemia at day 10.

These results provide insight into the etiology of the increased susceptibility to YEL-AVD after yellow fever vaccination in old age. Immunosenescence leading to an impaired ability to clear the vaccine virus has been put forth as a possible explanation for the increased risk of YEL-AVD in elderly people [26]. However, in a retrospective study of two large 17D vaccine trials involving 4,532 subjects, neutralizing antibody responses at 30 days after vaccination were equivalent in younger and elderly subjects. Due to the retrospective nature of that study, early responses (i.e. <30 days after vaccination) could not be compared and were assumed to be equal in both groups. Our results show that this assumption needs to be modified, as we show that elderly vaccinees are slower to develop an antibody response than younger vaccinees. This cannot entirely explain higher age as a risk factor for YEL-AVD, as viraemia levels peak at day 5, before the development of neutralizing antibodies. The innate immune response is probably also an important factor influencing viral replication after vaccination, as suggested by Silva and colleagues [27]. We think that the higher viraemia levels in elderly subjects may be due to a weaker innate immune response. Such a hampered innate immune response together with a slower humoral response could allow the YF-17D virus to replicate more efficiently and for a longer period of time increasing the chance of YEL-AVD. In this respect it is interesting to note that the incidence of adverse events at the injection site was lower in elderly than in younger subjects. If reactions at the injection site are the result of immune activation, observing less injection site adverse events in elderly subjects could reflect a weaker or slower innate immune response in elderly persons. Similar observations were

Table 2. Solicited adverse events after primary and booster YF-17D vaccination.

Adverse event (AE)			Young N = 30	Elderly N = 28	p-value
Injection site AE	Any	Yes (%)	9 (30)	4 (14)	0.15
		Days to onset (range)	0 (0-2)	0.5 (0-6)	0.6
	Erythema	Yes (%)	8 (27)	2 (7)	0.05
		Days duration (range)	2.5 (1-8)	2 (1-3)	0.4
	Swelling	Yes (%)	3 (10)	1 (4)	0.3
		Days duration (range)	2 (1-5)	2 (-)	1.0
	Pain	Yes (%)	3 (10)	2 (7)	0.7
		Days duration (range)	1 (1-3)	2 (2-2)	0.5
Systemic AE	Any	Yes (%)	12 (40)	8 (29)	0.4
		Days to onset (range)	0.5 (0-4)	5 (1-6)	0.002
	Myalgia	Yes (%)	12 (40)	6 (21)	0.4
		Days to onset (range)	1 (0-6)	5 (1-6)	0.12
	Fever	Yes (%)	3 (10)	4 (14)	0.6
		Days to onset (range)	0 (0-4)	5 (5-6)	0.03

Safety of vaccination expressed in various parameters. Numbers of days are medians. Fever was defined as self-measured temperature above 38 degrees Celsius. P-values based on Chi-square test and Wilcoxon's test. AE = Adverse event.

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made in an earlier study of yellow fever vaccination in elderly subjects [26].

Beside immunosenescence in elderly subjects, other factors contributing to YEL-AVD have been postulated. For example, it has previously been suggested that the vaccine virus reverts or mutates to a more virulent form during replication in a vaccinated individual, but extensive genetic analyses of the viral strains extracted from patients with YEL-AVD do not provide evidence to support this hypothesis [4]. The possibility of host genetic susceptibility for developing YEL-AVD seems more plausible. Pulendran and colleagues found a heterozygous CCR5Δ32 mutation in a patient who suffered from YEL-AVD [28]. Since the prevalence of heterozygosity of the CCR5Δ32 mutation in the general population is 15% [29] and the occurrence of YEL-AVD among yellow fever vaccinees is significantly less [13,14], additional host factors (e.g. immunosenescence) must also play a role in the development of YEL-AVD [30]. On the other hand, milder forms of YEL-AVD might occur more frequently, but might not be severe enough to be published, thus introducing publication bias. Supportive of the hypothesis of genetic susceptibility, other recently discovered genetic host factors, including complement protein C1qB and eukaryotic translation initiation factor 2 alpha kinase 4- (an orchestrator of the integrated stress response) predicted YF-17D $CD8^+$ T cell responses with up to 90% accuracy and a B-cell growth factor, TNFRS17, predicted the neutralizing antibody response with up to 100% accuracy [31].

Although occurrence of YEL-AVD is very rare, fear of this adverse event could reduce utilization of yellow fever vaccine. An "International Laboratory Network for Yellow Fever Vaccine-Associated Adverse Events" has been established in 2008, to complement the USA and the European Yellow Fever Vaccine Safety Working Groups [32]. Its goal is to determine the pathogenesis of severe adverse events following yellow fever vaccination through systematic and coordinated laboratory evaluation of reported cases. A greater understanding of the pathogenesis of YEL-AVD may lead to new approaches to prevent this serious complication. One strategy may be to inject less vaccine virus in a more immunostimulant manner (e.g. intradermally) [33]. Alternatively, inactivated YF-17D vaccine could be used to prime the immune response which can be boosted later with live attenuated YF-17D. This strategy has been successfully used in mice, hamsters and cynomolgous monkeys [34], and more recently Monath en co-workers have demonstrated an adequate antibody response against yellow fever following inactivated yellow fever vaccine [35].

The findings of our study can have the following practical implication: in travelers of 60 years and older, it would be prudent to vaccinate against yellow fever at least 14 days instead of 10 days before departure to guarantee that all vaccinees have obtained protective antibody levels.

Supporting Information

Protocol S1 Trial Protocol.
(DOCX)

Checklist S1 CONSORT Checklist.
(DOC)

Author Contributions

Conceived and designed the experiments: AHR SAJ JTD PJB LGV. Performed the experiments: AHR DS AWV XJ KD MG. Analyzed the data: AHR DS. Contributed reagents/materials/analysis tools: SAJ XJ PJB KD MG. Wrote the paper: AHR DS LGV. Revised manuscript: JTD.

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Response to 2009 Pandemic Influenza A (H1N1) Vaccine in HIV-Infected Patients and the Influence of Prior Seasonal Influenza Vaccination

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Abstract

Background: The immunogenicity of 2009 pandemic influenza A(H1N1) (pH1N1) vaccines and the effect of previous influenza vaccination is a matter of current interest and debate. We measured the immune response to pH1N1 vaccine in HIV-infected patients and in healthy controls. In addition we tested whether recent vaccination with seasonal trivalent inactivated vaccine (TIV) induced cross-reactive antibodies to pH1N1. (clinicaltrials.gov Identifier:NCT01066169)

Methods and Findings: In this single-center prospective cohort study MF59-adjuvanted pH1N1 vaccine (Focetria®, Novartis) was administered twice to 58 adult HIV-infected patients and 44 healthy controls in November 2009 (day 0 and day 21). Antibody responses were measured at baseline, day 21 and day 56 with hemagglutination-inhibition (HI) assay. The seroprotection rate (defined as HI titers $\geq$1:40) for HIV-infected patients was 88% after the first and 91% after the second vaccination. These rates were comparable to those in healthy controls. Post-vaccination GMT, a sensitive marker of the immune competence of a group, was lower in HIV-infected patients. We found a high seroprotection rate at baseline (31%). Seroprotective titers at baseline were much more common in those who had received 2009–2010 seasonal TIV three weeks prior to the first dose of pH1N1 vaccine. Using stored serum samples of 51 HIV-infected participants we measured the pH1N1 specific response to 2009–2010 seasonal TIV. The seroprotection rate to pH1N1 increased from 22% to 49% after vaccination with 2009–2010 seasonal TIV. Seasonal TIV induced higher levels of antibodies to pH1N1 in older than in younger subjects.

Conclusion: In HIV-infected patients on combination antiretroviral therapy, with a median CD4+ T-lymphocyte count above 500 cells/mm^3, one dose of MF59-adjuvanted pH1N1 vaccine induced a high seroprotection rate comparable to that in healthy controls. A second dose had a modest additional effect. Furthermore, seasonal TIV induced cross-reactive antibodies to pH1N1 and this effect was more pronounced in older subjects.

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Introduction

Most guidelines recommend annual influenza vaccination of all HIV-infected patients [1]. The rationale for this recommendation is that in the era of widespread use of combination antiretroviral therapy (cART) influenza is still associated with increased rates of morbidity in HIV-infected patients [2,3] and that vaccination prevents disease [4,5]. The immunogenicity of adjuvanted 2009 pandemic influenza A(H1N1) (pH1N1) vaccines in HIV-infected patients and the effect of recent and past trivalent inactivated influenza vaccines (TIV) is a matter of current interest. We measured the humoral immune response to a monovalent MF59-adjuvanted surface-antigen vaccine containing 7,5 µg hemagglutinin of strain A/California/7/2009 (H1N1) (X-181) (Focetria®, Novartis) in HIV-infected patients and in healthy controls. In addition we tested whether recent vaccination with seasonal TIV induced cross-reactive antibodies to pH1N1.

Methods

Ethics statement

This study was approved by the ethics committee of Leiden University Medical Center (protocol number 09.187). Subjects provided written informed consent for participation in the study and for the use of stored serum samples for the purpose of this study.

Study design and source population

This was a single-center prospective cohort study at Leiden University Medical Center in The Netherlands. The pH1N1 vaccine was administered twice to 58 adult HIV-infected patients (patients) and 44 healthy hospital employees (controls) in November and December 2009 (day 0 and day 21). Exclusion criteria were: use of systemic immunosuppressive medication,

ongoing febrile illness, pregnancy or laboratory confirmed pH1N1 influenza before the first vaccination. At inclusion, participants were asked whether they had experienced symptoms of influenza in the two preceding months. In addition, all participants filled out a standardized diary on symptoms of influenza during the 56 day follow-up period. Influenza-like illness was defined as sudden onset of fever of >38°C and cough or sore throat in the absence of other diagnoses [6]. Serum was collected at baseline, at day 21 (just before the second dose) and at day 56 (35 days after the second dose). In a subset of 51 participants (29 patients and 22 controls) serum was also collected at day 7. We retrieved stored serum samples of a subset of 51 HIV-infected patients who had been vaccinated with unadjuvanted 2009–2010 seasonal trivalent inactivated influenza vaccine (TIV) a month before receiving the first pH1N1 vaccination. In addition, we retrieved stored samples of 14 of these 51 HIV-infected patients who had also participated in an influenza vaccination trial in 2005 [7]. There were no such samples available of the healthy controls. The stored serum samples were used to measure whether 2009–2010 and 2005–2006 seasonal TIV induced cross-reactive antibodies to pH1N1 influenza.

Laboratory analysis and main outcome measures

Antibodies to the vaccine strain A/California/7/2009 (H1N1) and to the seasonal influenza vaccine strains A/NewCaledonia/20/1999 and A/Brisbane/59/2007 were measured using the hemagglutination-inhibition (HI) assay, according to standard methods [8]. Titers below the detection limit (i.e. <1:10) were assigned a value of 1:5. Geometric mean titers (GMTs) and seroprotection rates (defined as HI titers ≥1:40) were the main outcome measures. Seroconversion was defined by a post-vaccination HI titer of at least 1:40 combined with at least a four-fold increase in titer in accordance to European and international guidance [9,10].

Statistical methods

The between group difference in GMT taken over the three time points (day 0, 21, 56) was analyzed using a mixed linear model. This model takes into account that each subject had repeated measurements of the HI titer over time. We analyzed which variables predicted the level of post-vaccination GMT in the group of HIV-infected patients using a linear regression model with step-wise introduction of the continuous variables 'log of the HI titer at baseline', 'age in years', 'CD4+ T-lymphocyte count (cells/mm^3)', 'nadir CD4+ T-lymphocyte count (cells/mm^3)' and the categorical variables 'HIV-1 RNA' (<20 copy/ml, 20–400 copy/ml, >400 copy/ml) and 'gender'. Proportions were compared with Pearson χ^2 or Fisher's exact tests as appropriate. We explored which variables were associated with a baseline HI titer of ≥1:40 using a logistic regression model by step-wise introduction of the continuous variable 'age' and the categorical variables 'HIV-status' (i.e. infected or healthy control), 'gender', 'an influenza-like illness prior to inclusion', 'vaccination with 2009–2010 seasonal influenza vaccine', 'vaccination with 2008–2009 seasonal influenza vaccine' and 'vaccination with 2007–2008 seasonal influenza vaccine'.

In an exploratory analysis we looked at the effect of age on the level of cross-reactive antibodies to pH1N1 following 2009–2010 seasonal TIV using a linear regression model with step-wise introduction of the continuous variables 'age in years', 'CD4+ T-lymphocyte count (cells/mm^3)', 'nadir CD4+ T-lymphocyte count (cells/mm^3)' and the categorical variable 'HIV-1 RNA'. This analysis was restricted to HIV-infected patients who had received seasonal TIV before pH1N1 vaccine and who had no measurable HI titer to pH1N1 prior to receiving 2009–2010 seasonal TIV.

Results

Follow-up was complete for 98% (57/58) of HIV-infected patients and all healthy controls. The mean age of the patients was 52 (SD 11) years and of the controls 49 (SD 10) years. Of the patients, 91% (52/57) was on cART of whom 87% (45/52) had undetectable plasma HIV-1 RNA (<20 copies/mL) at baseline. The median CD4+ T-lymphocyte count was 507 (IQR 349-697) cells/mm^3 and only three patients had a count below 200 cells/mm^3. In the month preceding inclusion, 89% (51/57) of HIV-infected patients and 64% (28/44) of controls had been vaccinated with non-adjuvanted 2009-2010 seasonal TIV (Table 1).

Three patients (5%) and 3 controls (7%) reported an influenza-like illness in the two months preceding inclusion, of whom 2 patients and 1 control had a baseline HI titer ≥1:40. The baseline GMT was higher in patients (23, 95% CI 15–35) than in controls (12, 95% CI 8–16) (Figure 1a). At baseline, 44% (25/57) of patients and 23% (10/44) of controls had a HI titer ≥1:40. Titers above 80 were uncommon at baseline (Figure 1b).

Immunogenicity results are summarized in Figure 1 and Table 2. In a mixed linear model, the age-adjusted average GMT taken over the three time points after vaccination was a factor 1.6 higher in controls than in HIV-infected patients (95% CI 1.0-2.5, $p = 0.06$) (Figure 1a). In a linear regression model restricted to the HIV-infected patients, only higher baseline titers ($p = 0.02$) were associated with higher HI titers at day 21. This association was not seen at day 56.

The seroprotection rate, defined as a titer ≥1:40, was 88% (50/57) for HIV-infected patients three weeks after the first pH1N1 vaccination and 91% (52/57) after the second vaccination. For controls this was 93% (41/44) and 89% (39/44) respectively (Figure 1b). In a separate analysis, restricted to participants with a baseline titer below the detection limit, the seroprotection rate was 72% (18/25) for HIV-infected patients after the first and 88% (22/25) after the second vaccination. For the controls this was 89% (24/27) and 85% (23/27).

After the first vaccination only 53% (30/57) of HIV-infected patients achieved seroconversion compared with 73% (32/44) of controls. After the second vaccination this was 63% (36/57) and 70% (31/44) (Table 2). The GMT was lower in those who did not seroconvert than in those who did. The GMT in HIV-infected patients who did not seroconvert was 72 (95% CI 42–124) and was 161 (95% CI 122–212) in those who did seroconvert. For controls this was 61 (95% CI 25–147) and 347 (95% CI 233–516). As is to be expected, seroconversion rates were lower in those with high HI titers at baseline. In a separate analysis of 25 HIV-infected patients who had HI titers below the detection limit at baseline, 72% (18/25) achieved seroconversion after the first pH1N1 vaccination and 88% (22/25) after the second vaccination. For the 27 controls this was 89% (24/27) and 85% (23/27).

After the first vaccination, between day 0 and day 21, an influenza-like illness was reported by 5 HIV-infected patients (9%) and 6 controls (14%). Of these participants, 4/5 patients (80%) and 6/6 controls (100%) had a HI titer ≥1:40 at day 21. In addition, one patient and 1 control reported an influenza-like illness between day 21 and day 56 of follow-up. Both had HI titer ≥1:40 at day 56.

There were no serious adverse events following vaccination and HIV-1 RNA remained below the detection threshold in a random selection of 20 patients with undetectable viral loads at baseline.

All except 1 of the 35 subjects with a baseline pH1N1 titer ≥1:40 had received 2009–2010 seasonal TIV. Prior vaccination with 2009–2010 seasonal TIV (OR 14, 95% CI 2–113, p = 0.01) and higher age (OR 1.04, 95% CI 1.0–1.1 for an increase in age

Table 1. Participant Demographics.

	HIV-infected *n* = 57	Healthy Control *n* = 44
Male - *n* (%)	48 (84)	27 (61)
Age, years - *mean* (SD)	52 (11)	49 (10)
Age categories - *n* (%)		
18–44 years	14 (25)	12 (27)
45–59 years	26 (46)	28 (64)
>60 years	17 (30)	4 (9)
combination antiretroviral therapy (cART) - *n* (%)	52 (91)	-
baseline value CD4+ T-lymphocytes, cells/mm^3 - *median* (IQR)	507 (349–697)	-
CD4 category, at the time of vaccination *n* (%)		-
<350 cells/mm^3	14 (25)	-
>350 cells/mm^3	43 (75)	-
nadir CD4+ T-lymphocytes, cells/mm^3 - *median* (IQR)	143 (32–281)	-
baseline HIV-1 RNA - *n* (%)		-
<20 copy/ml	45 (79)	-
20-400 copy/ml	7 (12)	-
>400 copy/ml	5 (9)	-
past seasonal trivalent inactivated influenza vaccination - *n* (%)		
2009–2010	51 (89)	28 (64)
2008–2009	50 (88)	27 (61)
2007–2008	45 (79)	29 (66)

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by 1 year, p = 0.05) were associated with a baseline pH1N1 HI titer ≥1:40.

Using stored serum samples of 51 of the HIV-infected patients we measured the pH1N1 specific response to 2009–2010 seasonal TIV administered a median of 17 days (IQR 14–23 days) before the first pH1N1 vaccination. We found that the seroprotection rate to pH1N1 increased from 22% to 49% following vaccination with 2009–2010 seasonal TIV and that 31% seroconverted (Table 2). This effect was age dependent. In a regression analysis restricted to 40 HIV-infected patients who all had undetectable HI titers to pH1N1 prior to vaccination with 2009–2010 seasonal TIV, we found that 2009–2010 seasonal TIV induced higher HI titers in older than in younger subjects (HI titer increased by a factor 1.05 95% CI 1.01–1.08 for an increase in age by 1 year, p = 0.01). This effect was independent of the CD4+ T-lymphocyte count, nadir CD4+ T-lymphocyte count and HIV-1 RNA. Of note, we found no evidence indicating that the immune response to pH1N1 vaccine was augmented by prior vaccination with seasonal TIV.

In a subset of 14 HIV-infected patients we measured (cross-reactive) pH1N1 HI titers following three different influenza vaccinations (i.e. 2005–2006 seasonal TIV, 2009–2010 seasonal TIV and pH1N1 vaccine). In 2005 the seroprotection rate to pH1N1 for this cohort of 14 HIV-infected patients increased from 14% to 43% after vaccination with 2005–2006 seasonal TIV (Table 3). In 2009 the seroprotection rate to pH1N1 had dropped back to 7% but increased to 50% after vaccination with 2009–2010 seasonal TIV. The subjects who developed cross-reactive antibodies to pH1N1 after 2005–2006 seasonal TIV were not necessarily the same subjects who did so after 2009–2010 seasonal TIV (p = 0.5, Fisher's exact test for the association between seroconversion to pH1N1 following 2005–2006 seasonal TIV and 2009–2010 seasonal TIV).

Discussion

In HIV-infected patients on cART, with a median CD4+ T-lymphocyte count above 500 cells/mm^3, one dose of MF59-adjuvanted 2009 pandemic influenza A(H1N1) vaccine induced a

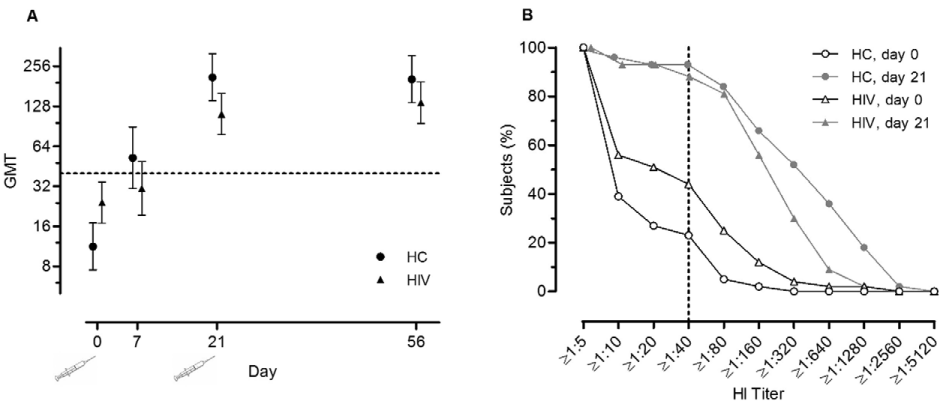

Figure 1. Immunogenicity of two doses of pH1N1 vaccine. Monovalent MF59-adjuvanted pandemic influenza vaccine (A/California/7/2009) administered to a group of 57 HIV-infected patients (HIV) and 44 healthy controls (HC). The vaccine was administered at day 0 (baseline) and at day 21. Age adjusted geometric mean titers with 95% confidence intervals at baseline, day 7, day 21 and day 56 (Panel A). Reverse cumulative distribution curves on hemagglutination inhibition assay at baseline and at day 21 (Panel B).
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Table 2. Humoral immune response to two doses of pH1N1 vaccine.

	HIV-infected $n=57$			Healthy Control $n=44$		
	prior 2009-2010 seasonal TIV			prior 2009-2010 seasonal TIV		
	Yes $n=51$	No $n=6$	All $n=57$	Yes $n=28$	No $n=16$	All $n=44$
pre-baseline, before 2009-2010 seasonal TIV (day –95)*						
HI titer ≥1:40 - *n* (%)	11 (22)	-	-	-	-	-
GMT – value (95% CI)	9 (7–12)	-	-	-	-	-
baseline, after 2009–2010 seasonal TIV but before 1st pH1N1 vaccine (day 0)						
HI titer ≥1:40 - *n* (%)	25 (49)	0	25 (44)	9 (32)	1 (6)	10 (23)
GMT – value (95% CI)	28 (18–42)	5 (-)	23 (15–35)	15 (9–25)	7 (5–9)	12 (8–16)
after 1st pH1N1 vaccine (day 21)						
HI titer ≥1:40 - *n* (%)	47 (92)	3 (50)	50 (88)	25 (89)	16 (100)	41 (93)
seroconversion – *n* (%)#	27 (53)	3 (50)	30 (53)	16 (57)	16 (100)	32 (73)
GMT – value (95% CI)	119 (87–163)	57 (16–193)	110 (81–150)	117 (69–198)	632 (422–947)	216 (139–334)
after 2nd pH1N1 vaccine (day 56)						
HI titer ≥1:40 - *n* (%)	47 (92)	5 (83)	52 (91)	23 (82)	16 (100)	39 (89)
seroconversion – *n* (%)#	31 (61)	5 (83)	36 (63)	15 (54)	16 (100)	31 (70)
GMT – value (95% CI)	138 (101–187)	107 (58–200)	134 (101–178)	117 (73–186)	572 (384–853)	208 (140–310)

Seroprotection- and seroconversion rates and geometric mean titers (GMT) to 2009 pandemic influenza A(H1N1) (pH1N1) virus for 57 HIV infected individuals and 44 healthy controls following vaccination with two doses of monovalent MF59-adjuvanted pandemic influenza vaccine (A/California/7/2009). Results are stratified by whether or not participants had been vaccinated with 2009–2010 seasonal trivalent inactivated influenza vaccine (TIV) before receiving the first pH1N1 vaccine.
*For 51 HIV-infected participants who had already been vaccinated with 2009–2010 seasonal TIV at baseline (day 0), we also determined HI titers to 2009 pandemic influenza A(H1N1) virus in stored serum samples that had been collected before they received 2009–2010 seasonal TIV.
#Baseline titers (day 0) were used as denominaters to determine seroconversion rates.
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high rate of seroprotection comparable to that in healthy controls. The second dose showed no effect on GMT 5 weeks after it had been administered, but it did have a modest additional effect on the seroprotection rate in HIV-infected patients. Post-vaccination GMT was lower in HIV-infected patients than in healthy controls. Furthermore we found that seasonal TIV induced seroprotection to pH1N1 in just under half of the participants and that this effect was more pronounced in older subjects.

There are three published studies and there is one set of preliminary data on the humoral response to a single dose of 2009 pandemic influenza A(H1N1) vaccine in comparable groups of HIV-infected patients (Table 4) [11–14]. This study is the first to report the effect of this particular vaccine in HIV-infected patients and the first to report the effect of a second dose in HIV-infected patients. It is also the only study on pH1N1 vaccine in HIV-infected patients that included a comparator control group. In two of the other studies with ASO3-adjuvanted vaccine, the seroprotection rate exceeds 90%. A third study reports a lower seroprotection rate. In a head to head comparison, squalene based adjuvanted influenza vaccine clearly outperforms unadjuvanted influenza vaccine in HIV-infected patients [11], as has also been found for healthy adults [15]. Due to relatively high baseline HI titers, the seroconversion rate in our study was lower than in other studies. A fourfold increase in titer is more difficult to achieve if the baseline titer is already high. This reasoning is in line with the fact that we found higher seroconversion rates for the participants who had undetectable pH1N1 HI titers at baseline. Our interpretation of the data is that most participants in our study were clinically protected following vaccination with MF59-adjuvanted pH1N1 vaccine.

In this study just under half of the participants had a HI titer ≥1:40 at baseline, i.e. at or above the threshold that defines seroprotection. Although the peak incidence of the influenza pandemic in the Netherlands coincided with the start of the vaccination campaign [16], less than 10% had a recent influenza-like illness before receiving the first pH1N1 vaccine. Therefore, it seems unlikely that infection with influenza accounted for the high seroprotection rate at baseline. There was a strong association between recent vaccination with 2009–2010 seasonal TIV and seroprotection at baseline. This association was confirmed by analyses of stored serum samples, which showed that 2009–2010 seasonal TIV induced cross-reactive antibodies to pH1N1 and that 2005–2006 seasonal TIV had a comparable effect. In other studies baseline seroprotection rates vary from 0 to approximately 30% [15,17–27]. Some studies do [17,20,26] and others do not [23,24] report an association between baseline HI titers to pH1N1 and prior vaccination with seasonal TIV. The fact that we found a stronger association between vaccination with seasonal TIV and induction of cross-reactive antibodies to pH1N1 than most other studies can be due to a number of reasons. Firstly, as opposed to most other studies, the majority of subjects in our study had received 2009–2010 seasonal TIV before inclusion. Secondly, the time between having received seasonal influenza vaccine and pH1N1 vaccine was much shorter in our study than in other studies. Lastly, we studied HIV-infected patients and it could be that this group produces larger quantities of cross-reactive

Table 3. (Cross-reactive) antibody titers following two different seasonal influenza vaccines in a cohort of 14 HIV-infected patients.

	HIV-infected ($n = 14$)*	
influenza strain used in HI assay	A/NewCaledonia/20/1999 (seasonal strain)	A/California/7/2009 (pandemic strain)
Before 2005–2006 seasonal TIV		
HI titer ≥1:40 - *n* (%)	7 (50)	2 (14)
GMT – value (95% CI)	39 (16–92)	10 (5–17)
after 2005–2006 seasonal TIV		
HI titer ≥1:40 - *n* (%)	11 (79)	6 (43)
seroconversion - *n* (%)	4 (29)	3 (21)
GMT – value (95% CI)	118 (52–272)	21 (10–45)
influenza strain used in HI assay	A/Brisbane/59/2007 (seasonal strain)	A/California/7/2009 (pandemic strain)
Before 2009–2010 seasonal TIV		
HI titer ≥1:40 - *n* (%)	12 (86)	1 (7)
GMT – value (95% CI)	55 (34–90)	6 (4–9)
after 2009–2010 seasonal TIV but before 1st pH1N1 vaccine		
HI titer ≥1:40 - *n* (%)	14 (100)	7 (50)
seroconversion - *n* (%)	2 (14)	6 (43)
GMT – value (95% CI)	103 (57–187)	23 (12–43)
after 1st pH1N1 vaccine		
HI titer ≥1:40 - *n* (%)	-	13 (93)
seroconversion - *n* (%)	-	8 (57)
GMT – value (95% CI)	-	114 (62–209)

Seroprotection- and seroconversion rates and geometric mean titers (GMT) to 2005–2006 seasonal influenza A(H1N1) virus, to 2009–2010 seasonal influenza A(H1N1) virus and to 2009 pandemic influenza A(H1N1) (pH1N1) virus for 14 HIV infected individuals following vaccination with seasonal trivalent inactivated influenza vaccine (TIV) in 2005 (A/New Caledonia/20/1999 (H1N1) like strain), with seasonal TIV in October 2009 (A/Brisbane/59/2007 (H1N1) like strain) and with a first dose of monovalent MF59-adjuvanted pH1N1 vaccine (A/California/7/2009) in November 2009.

*Population characteristics in 2009: 86% male, median age 48 years (IQR 47–66), 86% on cART, median CD4+ T-lymphocytes 532 cells/mm^3 (IQR 349–725), baseline HIV-1 RNA 71% <20 copy/ml, 14% 20–400 copy/ml, 14% >400 copy/ml. Population characteristics in 2005: 64% on cART, median CD4+ T-lymphocytes 473 cells/mm^3 (IQR 285–752), baseline HIV-1 RNA 57% <50 copy/ml, 14% 50–400 copy/ml, 29% >400 copy/ml.

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antibodies upon vaccination because of a less well regulated B-cell immune response [28,29].

Using virus neutralization assays, others have shown that cross-reactive antibodies that are induced by seasonal TIV are functional against pH1N1 [30]. This entails that these antibodies do confer protection against pH1N1. There is epidemiological evidence that supports this claim although there is also evidence to the contrary [31–34]. The surface hemagglutinin and neuraminidase proteins in recent seasonal trivalent inactivated influenza vaccines are antigenically very distant from those of pH1N1. Therefore seasonal TIV is generally not expected to confer a significant degree of cross-protection to pH1N1 [35]. Only older age by way of exposure to pre-1957 influenza strains has consistently been found to confer a relevant degree of cross-reactive antibodies to pH1N1 [17,36–39]. In this respect it is interesting that we found that seasonal TIV was more likely to induce cross-reactive antibodies to pH1N1 in older than in younger subjects, which contradicts the conclusion of Hancock et al. who found that seasonal TIV induces little to no cross-reactive antibody response to pH1N1 in any age group. We think that our findings show that seasonal influenza vaccines do not induce a relevant degree of cross protection to pH1N1 in (younger) immunologically naïve subjects but that seasonal influenza vaccines can boost relatively unrelated influenza specific memory B-cells. In older individuals who have been exposed to influenza strains or vaccines that are antigenically more related to pH1N1, such boosting induces measurable levels of antibodies to pH1N1, which may augment clinical protection against pH1N1.

This study has strengths and limitations. It was a prospective well controlled cohort study in a fairly homogenous group in which follow-up was complete for 99% of participants. This study is unique in that it shows the immune response to vaccination with pH1N1 and the effect of seasonal influenza vaccines in the same HIV-infected patients. Although symptoms of an influenza-like illness were systematically assessed, respiratory samples were not collected to confirm pH1N1 infection and therefore intercurrent infections can not be excluded. However, only 11 participants had an influenza-like illness between day 0 and day 21. Regarding the generalizability of our results: 91% of our HIV-infected patients were successfully being treated with combination antiretroviral therapy (cART) and very few HIV-infected participants had a CD4+ T-lymphocyte count below 200 cells/mm^3.

In conclusion, a single dose of MF59-adjuvanted 2009 pandemic influenza A(H1N1) vaccine in HIV-infected patients on cART with a median CD4+ T-lymphocyte count above 500 cells/mm^3 induced a high rate of seroprotection comparable to that in healthy controls. A second dose had a modest additional effect in HIV-infected patients but not in healthy controls. Post-vaccination GMT, a sensitive marker of the immune competence of a group, was lower in HIV-infected patients than in healthy controls, reflecting the underlying immunodeficiency. Furthermore we found that recent seasonal TIV induced a high rate of age-dependent cross-reactive seroprotection to pH1N1. We think that in general, seasonal TIV boosts pre-existent influenza specific memory B-cells. In older people who in the past have been

Table 4. Comparison of the immunogenicity of a single dose of 2009 pandemic influenza A(H1N1) vaccine in HIV-infected patients.

Study	Number of HIV-infected patients	Vaccine	Vaccine HA, content, µg	Age in years – mean (SD) or *median (IQR)*	CD4+ T-lymphocytes, cells/mm^3 - mean (SD) or *median (IQR)*	nadir CD4+ T-lymphocytes, cells/mm^3 - mean (SD) or *median (IQR)*	cART - (%)	HIV-RNA below detection limit – (%)*	Prior 2009-2010 seasonal TIV - (%)	HI titer ≥ 1:40 before pH1N1 vaccination - (%)	HI titer ≥ 1:40 after one pH1N1 vaccination - (%)	GMT before vaccination – value (95% CI) or *median (IQR)*	GMT after one accination – value (95% CI) or *median (IQR)*	serocon-version rate – (%)	seropro-tection rate – (%)#
Launay et al.[11]	154	ASO3-adjuvanted	3.75	*47 (39–54)*	*523 (387–752)*	NA	77	77	NA	7	95	8 (7–9)	202 (172–236)	92	95
	152	unadjuvanted	15	*47 (40–54)*	*548 (422–702)*	NA	78	73	NA	9	77	8 (7–9)	128 (104–158)	72	77
Orlando et al.[14]	253	ASO3-adjuvanted	3.75	47 (10)	570 (266)	NA	91	82	NA	26	92	*5 (5–40)*	*160 (80–320)*	83	92
Bickel et al.[12]	160	ASO3-adjuvanted	3.75	46 (10)	514 (246)	160(134)	90	70	11	14	75	9 (8–10)	94 (73–122)	69	75
Tebas et al.[13]	120	unadjuvanted	15	*46 (40–53)*	*502 (307–640)*	131 (37–253)	99	84	NA	25	69	NA	NA	56	71
Our study	57	M59-adjuvanted	7.5	52 (11)	*507 (349–697)*	*143 (32–281)*	91	79	89	44	88	23 (15–35)	110 (81–150)	53	88

*HIV-RNA detection limits vary between the different studies.
#Seroprotection rates pertain to all included subjects, irrespective of baseline HI antibody titers. NA: not available.
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exposed to influenza strains that are antigenically more alike to pH1N1, this effect induces measurable levels of cross-reactive antibodies to pH1N1. If such an effect is true and if it adds to clinical protection against pH1N1, it is an additional benefit of annual influenza vaccination.

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Author Contributions

Conceived and designed the experiments: DS LBSG LGV FPK. Performed the experiments: DS GFR. Analyzed the data: DS LBSG FPK. Contributed reagents/materials/analysis tools: GFR. Wrote the paper: DS GFR LBSG LGV FPK.

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No cross-reactive serum antibodies to 2009 pandemic influenza A (H1N1) after seasonal influenza vaccination in the virus neutralization assay

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Unpublished supplemental data

BACKGROUND

The surface hemagglutinin (HA) and neuraminidase proteins in recent seasonal trivalent inactivated influenza vaccines (TIV) are antigenically very distant from those of 2009 pandemic influenza A(H1N1) (pH1N1). Therefore seasonal TIV is generally not expected to confer a significant degree of cross-protection to pH1N1.[1] Only older age by way of exposure to pre-1957 influenza strains has consistently been found to confer a relevant degree of cross-reactive antibodies to pH1N1.[2-6] We were surprised to find that in our study, 2009–2010 seasonal TIV induced cross-reactive antibodies to pH1N1 in a sizeable proportion of subjects.[7] In the hemagglutination-inhibition (HI) assay, the seroprotection rate to pH1N1 increased from 22% to 49% following vaccination with TIV with 31% showing seroconversion. This effect was age dependent. Using virus neutralization assays, others have shown that cross-reactive antibodies that are induced by seasonal TIV are functional against pH1N1.[8] This suggests that these antibodies confer protection against pH1N1. There is epidemiological evidence that supports this claim, although there is also evidence to the contrary.[9-12] We determined whether the cross-reactive antibodies to pH1N1 that were detected in the HI assay, were also present in the virus neutralization (VN) assay.

METHODS

Study design and source population

From the original cohort, we selected 14 HIV-infected individuals who had been vaccinated with 2009–2010 seasonal TIV, a median of 19 days before being vaccinated with pH1N1 vaccine (interquartile range, IQR 15–24 days). Their pH1N1 antibody titer was below the detection limit before vaccination with seasonal TIV. Of these subjects 8 of 14 developed cross-reactive antibodies to pH1N1 after vaccination with TIV, according to the HI assay. Antibody responses before seasonal TIV (day -140), after seasonal TIV (day 0), after the first dose of pH1N1 vaccine (day 21) and after the second dose of pH1N1 vaccine (day 56) were measured with HI assays and VN assays.

Virus neutralization (VN) assay

50 µl volumes of heat-inactivated serum samples were diluted 1:10 and serially diluted two-fold and incubated with an equal volume of virus suspension containing 100 $TCID_{50}$ for two hours at 37 ◦C. The virus A/California/4/2009 (H1N1) was used. Subsequently the mixture was transferred to confluent MDCK cells grown in 96-well plates, incubated for two hours at 37 ◦C and then aspirated. The cells were washed once with infection medium and then cultured for 3–7 days at 37 ◦C. Then the culture supernatants were tested for HA activity as a measure for residual virus replication. The serum titers were expressed as the reciprocal of the dilution that still prevented virus replication. If there was no inhibition of virus replication, the titer was assigned a value of 1:5.

Hemagglutination-inhibition (HI) assay

Antibodies to the vaccine strain A/California/7/2009 (H1N1) were measured using the hemagglutination-inhibition (HI) assay, according to standard methods.[13] Titers below the detection limit (i.e., 1:10) were assigned a value of 1:5. Seroconversion was defined by a post-vaccination HI titer of at least 1:40 combined with at least a four-fold increase in titer in accordance to European and international guidance.

RESULTS

The median age of this group of 14 HIV-infected subjects was 57 (IQR 48–67) years. The median CD4+ T-lymphocyte count was 529 (IQR 324-706) cells/mm^3. The titers obtained in the VN assay correlated reasonably well with those obtained in the HI assay for most serum samples tested (Figure 1). Pearson's correlation coefficient was 0.64 (95% Confidence Interval, CI 0.46–0.77, p-value <0.0001). There were a number of samples with discrepant values. These were mainly seen if titers were at the lower end of the spectrum. Some of the samples with discrepant values had a negative HI titer and a weak VN titer. Others had a negative VN titer and a moderate HI titer. Most of the discrepant titers were seen in the pre-vaccination samples that had been

obtained during routine outpatient visits a median of 140 (IQR 65-205) days before vaccination with TIV and in the samples obtained after vaccination with TIV (day 0).

In the HI assay, none of the 14 subjects had antibodies to pH1N1 before vaccination with seasonal TIV. Eight subjects (57%) developed a cross-reactive anti-pH1N1 titer ≥1:40 after vaccination with TIV. No such response was seen in any of these subjects when measured with the VN assay. This was reflected in the geometric mean titers (GMT), as is depicted in Figure 2. In the HI assay, GMT for cross-reactive antibodies to pH1N1 increased from 5 (95% CI 5–5) to 30 (95% CI 15-61) after vaccination with seasonal TIV. In the VN assay there was no increase in cross-reactive antibody titers: pre-vaccination GMT 13 (95% CI 8-22), post-vaccination GMT 11 (95% CI 7-19).

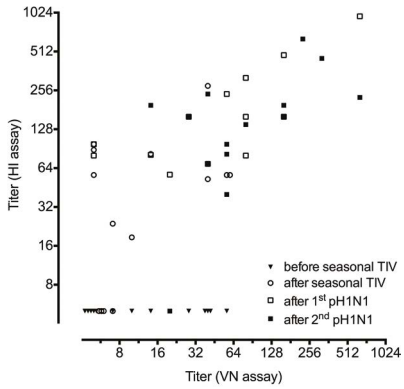

Figure 1. Comparison of serum antibody titers against influenza virus A/California/7/2009 (pH1N1) obtained in virus neutralization (VN) assay with those obtained in the hemagglutination-inhibition (HI) assay.

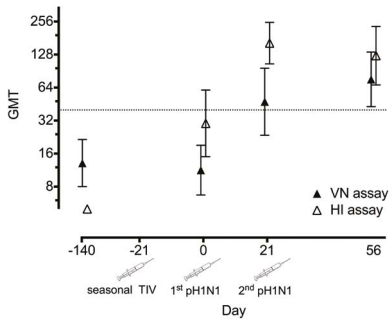

Figure 2. Comparison of geometric mean titers (GMT) for serum antibody titers against influenza virus A/California/7/2009 (pH1N1) obtained in virus neutralization (VN) assay with those obtained in the hemagglutination-inhibition (HI) assay, in response to seasonal TIV and to two doses of monovalent MF59-adjuvanted pandemic influenza vaccine (A/California/7/2009) (pH1N1), in a group of 14 HIV-infected patients.

DISCUSSION

Seasonal TIV did not induce cross-reactive antibodies to pH1N1 according to the VN assay. This was in contrast to the result obtained from the HI assay. The discrepancy between the results of the HI assay and VN assay, is unexpected, since HI titers for influenza virus antibody in human sera closely match VN titers.[14,15] However, VN and HI antibody repertoires do not fully overlap.[16] In our study, discrepant values were mainly seen if titers were at the lower end of the spectrum. Most of these samples had a negative HI titer and a weak VN titer. One can speculate on the cause of the discrepancy. Antibody assays such as the HI assay and VN assay have limited sensitivity to distinguish small difference in antibody titers, such as a two-fold dilution step difference. Since ours was a fairly small study, the play of chance may have magnified the inherent limitations of the assay and introduced a bias that led us to believe that TIV induced a significant degree of cross-protection to pH1N1 based on the HI assay results. Alternatively, aspecific binding of nonimmune or immune factors may prevent hemagglutination.[17]

The VN assay is a functional assay and is considered the gold standard. Therefore, we conclude that seasonal TIV did not confer a significant degree of cross-reactive protective antibodies to pH1N1. However, to complicate matters, there is compelling new evidence that supports our previous observation of a relevant increase in pH1N1 titer after vaccination with seasonal TIV. Li et al. have recently shown that memory B cells, reactive to pH1N1 are present in many people, before pH1N1 emerged.[18] They also show that pH1N1 influenza vaccination induces a recall response of certain memory B cells, that leads to broadly cross-reactive antibodies that bind to conserved regions of hemagglutinin.[18-20] One can speculate that in our study, seasonal TIV activated cross-reactive memory B cells in older individuals which led to the production of antibodies that cross-reacted with the HA protein of pH1N1. Maybe these antibodies were capable of inhibiting hemagglutination in the HI assay, but were not capable of neutralizing pH1N1 virus in the VN assay.

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Summary and discussion

SUMMARY AND DISCUSSION

The content of this thesis is based on research that was conducted at the travel and vaccination clinic at Leiden University Medical Centre. It covers a variety of topics relating to travel medicine and immunity. This final chapter starts by discussing methodological aspects of the various studies. Next it discusses the results of the studies on travel medicine and finally the chapters pertaining to immunity and vaccination.

METHODOLOGICAL ISSUES

Based on the method that was used, the chapters in this thesis can be categorised as either experimental studies (i.e. trials) (chapters 6, 7, 8 and 9) or non-experimental studies (i.e. observational studies) (chapters 1, 2, 3, 4, 5, 10 and 11). Experimental studies are defined as prospective follow-up studies in which the exposure to a determinant is manipulated and assigned at random, thus creating an index- and a reference group.[1] The purpose of random allocation is to create groups that differ only randomly at the time of allocation with regard to subsequent occurrence of the study outcome.[2] The goals of the study, rather than the subject's needs, determine the exposure assignment, so as to prevent 'confounding by indication'.[2] Furthermore, for ethical reasons, the treatment possibilities in an experimental study must be equally acceptable given current knowledge.[2] This is called the principal of equipoise.[3-9] Therefore in many trials, the reference group is treated with an active comparator as opposed to a placebo. For ethical reasons and for methodological reasons, it is essential that people who are assigned to a reference group are exposed to the best available active comparator. When feasible, clinical trials should attempt to employ blinding with respect to treatment assignment. This prevents certain biases that could affect assignment, compliance, treatment or assessment.[2] In an experimental study, the sample size depends on pre-defined assumptions and variables: (i) an assumption about the incidence of the primary endpoint, (ii) an assumption about the difference in the effect of treatment between the index- and the reference group., (iii) the magnitude of the type I error that is deemed acceptable (α) (i.e. asserting something that is absent) and (iv), the magnitude of the type II error that is deemed acceptable (β) (i.e. failing to assert what is present). Table 1 summarizes the methodology of the experimental studies that are described in chapters 6 and 7 of this thesis. Chapters 8 and 9 were open label, non-randomized phase I exploratory trials.

Non-experimental studies differ from experimental studies in that randomization is not used to assign treatments.[10] The researcher is an observer rather than an agent who assigns interventions.[2] There are four main types of non-experimental studies: cohort studies,

STUDY	STUDY PHASE	TRIAL TYPE	PRIMARY ENDPOINT	EXPERIMENTAL TREATMENT	STANDARD TREATMENT	EQUIPOISE	QUALITY OF STANDARD TREATMENT
VACCINE FOR TRAVELLERS' DIARRHOEA	3	SUPERIORITY TRIAL	INCIDENCE PROPORTION FOR ALL CAUSE TRAVELLERS' DIARRHOEA	CVD 103-HGR LIVE ORAL CHOLERA VACCINE	PLACEBO	☺	☺
POLIO VACCINE	3	NON-INFERIORITY TRIAL	DIFFERENCES IN THE LOG_2 GMC	1/5TH DOSE INTRADERMAL JET INJECTOR	FULL-DOSE INTRA-MUSCULAR NEEDLE	☺	☺

STUDY	RANDOMIZATION	CONCEALMENT OF ALLOCATION	PRE-DEFINED EXPECTED TREATMENT EFFECT	OBSERVED TREATMENT EFFECT	PRE-DEFINED SAMPLE SIZE	ATTAINED SAMPLE SIZE	PRE-DEFINED TYPE I ERROR	PRE-DEFINED TYPE II ERROR
VACCINE FOR TRAVELLERS' DIARRHOEA	COMPUTER GENERATED LIST	DOUBLE-BLIND	PROTECTION RATE ≥ 50%	PROTECTION RATE 0%	100 PER TREATMENT ARM	65 PER TREATMENT ARM	0.025 (ONE-SIDED)	0.1 (ONE-SIDED)
POLIO VACCINE	SEALED ENVELOPES NUMBERED IN RANDOM ORDER	OPEN-LABEL	LOWER END OF 95% CI FOR THE DIFFERENCE IN THE LOG_2 GMC LESS THAN -1	LOWER END OF 95% CI FOR THE DIFFERENCE IN THE LOG_2 GMC MORE THAN -1	30 PER TREATMENT ARM	30 PER TREATMENT ARM	0.025 (ONE-SIDED)	0.2 (ONE-SIDED)

GMC: GEOMETRIC MEAN CONCENTRATION. PV1: POLIOVIRUS TYPE 1, PV2: POLIOVIRUS TYPE 2, PV3: POLIOVIRUS TYPE 3.

TABLE 1

Summary of methodological aspects of the experimental studies that are described in chapters 6 and 7 of this thesis.

TABLE 2 (page 140)

Summary of methodological aspects of the non-experimental studies that are described in chapters 1, 2, 3, 4, 5, 10 and 11 of this thesis.

in which all subjects in a source population are classified according to their exposure status and followed over time to ascertain disease incidence; case control studies, in which investigators compare exposures between subjects with a particular disease outcome (cases) and people without that outcome (controls); cross-sectional studies, in which one ascertains exposure and disease status at a particular time; and ecological studies, in which the units of observation are groups of people.[2,11] The objective of an epidemiological study is to obtain a valid and precise estimate of the frequency of a disease or of the effect of an exposure on the occurrence of a disease in the source population of the study. Often, a further objective is to obtain an estimate that is generalizable to relevant target populations.[2] Errors in estimation may occur due to random- or systematic errors and are of influence on the internal validity of a study. Violations of internal validity can be classified into three categories: confounding, selection bias and information bias. Confounding produces relations that are factually right, but that cannot be interpreted causally because some underlying, unaccounted for factor is associated with both exposure and outcome.[11] Bias is a systematic deviation of a study's result from a true value. Typically, it is introduced during the design or implementation of a study and cannot be remedied later. Bias arises from flawed information or subject selection so that a wrong association is found.[11] Table 2 summarizes some methodological aspects of the non-experimental studies that are described in chapters 1, 2, 3, 4, 5, 10 and 11.

TRAVEL AND INFECTION

The first chapter of this thesis describes a study that was designed to improve the quality of medical electives.[12] It describes the health risks and the quality and comprehensiveness of pre- and post-travel care fore a group of Dutch medical students after an elective abroad. Most students engaged in procedures that constitute a risk of blood-borne viral infection, often in countries with high HIV prevalence rates. None of the participants took action following mucosal or percutaneous exposure to potentially infectious body fluids. This was also the case in a survey among British medical students.[13] Furthermore, the allocation of post exposure prophylaxis kits for HIV (PEP) was inadequate. Regarding other health risks: 20% stopped using mefloquine due to adverse effects, which left a sizeable proportion unprotected in countries that are hyperendemic for malaria. Post-travel screening for schistosomiasis, tuberculosis and methicillin-resistant *Staphylococcus aureus* (MRSA) was conducted for approximately half of all students who should have been screened. Based on the results of this study we adopted an integral set of measures to reduce the health risks associated with an elective abroad. Pre- and post-travel consultations as also the distribution of PEP has been centralized and standardized. Furthermore, student and supervisor in Leiden are required to fill out a checklist to assess whether the student is sufficiently experienced to participate in certain procedures, such as

STUDY	STUDY DESIGN	OBJECTIVE	RESPONSE RATE	STUDY SIZE	FOLLOW-UP RATE	STATISTICAL METHODS	POTENTIAL LIMITATIONS REGARDING STUDY DESIGN OR ANALYSIS	ASPECTS RELATING TO GENERALIZABILITY
HEALTH RISKS DURING MEDICAL ELECTIVES ABROAD	COHORT STUDY	QUANTIFY THE COMPREHENSIVENESS OF PRE- AND POST-TRAVEL CARE AND OF TRAVEL RELATED MORBIDITY.	74%	$n = 180$	-	INCIDENCE PROPORTIONS	INFORMATION BIAS (RECALL BIAS, RESPONDENT BIAS)	SINGLE CENTRE, STUDY
INCONVENIENCE DUE TO TRAVELLERS' DIARRHOEA	COHORT STUDY	QUANTIFY THE DEGREE OF INCONVENIENCE CAUSED BY TRAVELLERS' DIARRHOEA.	52%	$n = 406$	96%	INCIDENCE PROPORTIONS AND INCIDENCE RATES	INFORMATION BIAS (RESPONDENT BIAS)	SINGLE CENTRE STUDY
PRE-TRAVEL PREPARATION AND MORBIDITY IN PEOPLE WITH IBD	COHORT STUDY AND CASE-CROSSOVER STUDY	QUANTIFY THE COMPREHENSIVENESS OF PRE- AND POST-TRAVEL CARE AND OF TRAVEL RELATED MORBIDITY.	70%	$n = 277$	-	INCIDENCE PROPORTIONS AND MANTEL-HAENSZEL ODDS RATIO	INFORMATION BIAS (RECALL BIAS, RESPONDENT BIAS)	SELECTION BIAS (SELF-SELECTION BIAS)
POST-TRAVEL SCREENING FOR INTESTINAL PARASITES	COHORT STUDY	QUANTIFY THE INCIDENCE OF ASYMPTOMATIC PARASITIC INFECTION IN TRAVELLERS.	UNKNOWN	$n = 679$	82%	INCIDENCE PROPORTIONS	-	RECRUITMENT AT TWO TRAVEL CLINICS, HALF OF THE PARTICIPANTS WERE STUDENTS
IMMUNE RESPONSE TO SCHISTOSOME ANTIGENS	CASE-CONTROL STUDY	QUANTIFY THE TYPE AND STRENGTH OF THE MEMORY IMMUNE RESPONSE TO SCHISTOSOME ANTIGENS.	-	$n = 21$	-	MANN–WHITNEY TEST	-	-
IMMUNE RESPONSE TO YELLOW FEVER VACCINE	CONTROLLED COHORT STUDY	COMPARE THE IMMUNE RESPONSE TO YELLOW FEVER VACCINE BETWEEN YOUNG AND ELDERLY PEOPLE.	-	$n = 58$	100%	MIXED LINEAR MODEL, CHI-SQUARE TEST, WILCOXON'S RANK TEST	-	SINGLE CENTRE STUDY
IMMUNE RESPONSE TO INFLUENZA A VACCINE	CONTROLLED COHORT STUDY	COMPARE THE IMMUNE RESPONSE TO INFLUENZA VACCINE BETWEEN HEALTHY CONTROLS AND PEOPLE WITH HIV INFECTION.	-	$n = 112$	99%	MIXED LINEAR MODEL, CHI-SQUARED TEST	CONFOUNDING, INFORMATION BIAS	SINGLE CENTRE STUDY

IBD: INFLAMMATORY BOWEL DISEASE.

suturing or assisting in the operating theatre or delivery room. If new skills are to be acquired abroad, it should be specified beforehand whether the medical staff abroad has the time and facilities to supervise and teach new skills. Students also receive a brochure that describes how to act in case of exposure to potentially infectious body fluids. Upon return, all students fill out a standard short web-based checklist which assesses certain health risks, such as exposure to potentially infected body fluids and the risk of schistosomiasis and tuberculosis. The checklist results in a computer generated recommendation stating whether the student needs to contact the occupational health department or another care provider for a post-travel consult. Finally, the department of student affairs is creating a list of so called preferred partners. These are long standing partnerships with hospitals abroad, where medical staff are familiar with supervising foreign students and where student responsibilities and access to care are well-defined. A more comprehensive pre- and post travel survey will assess the effectiveness of the new policy. In addition this study will address other aspects, such as the incidence of culture shock, (traffic) accidents, violence and post-travel irritable bowel syndrome.

For most travellers to the tropics, diarrhoea is the most common health hazard. It can be a major nuisance but it is very seldom fatal. In **the second chapter** we assess the burden of illness due to travellers' diarrhoea in adults who travelled to the (sub)tropics for a median of 23 days.[14] We conclude that conventional definitions of travellers' diarrhoea encompass many mild cases (in our study at least a third of all cases) for which treatment is unlikely to provide a significant health benefit. We recommend that the degree of inconvenience should be incorporated as an endpoint in clinical studies on travellers' diarrhoea. This will enable scientists and policy makers to better distinguish 'significant' travellers' diarrhoea from mild travellers' diarrhoea, thus allowing for a more precise estimate of the size of the target population for vaccination or stand-by antibiotic prescription and of the benefit of such measures.

Chapter three describes a questionnaire study on travel experiences in which we investigated pre-travel preparation of Dutch patients with inflammatory bowel disease (IBD).[15] We also surveyed health problems encountered during travel and investigated whether travel increased the risk of an exacerbation of IBD. Faecal urgency and incontinence were the main IBD-related inconveniences. Onset of a new episode of diarrhoea was reported by 32%, which surprisingly is not higher than the incidence of travellers' diarrhoea in the general population.[16] Probably, people with chronic bowel disease are less inclined to regard gastro-intestinal complaints as new episodes of diarrhoea. We did not find that travel increased the risk of an exacerbation of IBD within a 2-month period after travel. However, the individual's self-reported number of exacerbations over the past 5 years may not be a valid marker for the expected incidence of an exacerbation after travel.

Lastly, pre-travel advice for IBD patients was often deficient. We recommend that physicians caring for patients with IBD raise awareness of the benefit of pre-travel counselling and that they refer patients to travel medicine clinics in a timely fashion. Sufficient time is required to check serology after hepatitis A vaccination in those who use systemic immunosuppressants. Even if seroprotection is not attained after one dose, a second dose is often effective, as has been shown in organ transplant recipients.[17,18]

Chapter four describes a study in which we aimed to determine the utility of routine post-travel screening of asymptomatic long-term travellers to the (sub)tropics for intestinal parasites using molecular diagnostics and for schistosomiasis using serology.[19] Only one infection with *Strongyloides stercoralis* was found in over 400 travelers and no infection with *Entamoeba histolytica* in over 500 travelers. The incidence of infection with Schistosoma spp. was higher. However, each case was associated with exposure to highly endemic lakes in Malawi and Tanzania. We conclude that routine screening of stool samples for parasitic infection is not indicated for asymptomatic people, who travel to the (sub)tropics for up to 3 months. Screening for Schistosoma spp. should be offered to travellers with fresh-water contact in endemic regions. Post-travel screening of specific groups of asymptomatic travellers, such as migrants, expatriates, or aid workers may yield higher infection rates.

Chapter six describes a randomized trial on the efficacy of a live attenuated oral cholera vaccine, CVD 103-HgR, to prevent all-cause travellers' diarrhea.[20] The vaccine failed to provide protection. The power of the study was limited by the unexpected low incidence of LT–ETEC-associated diarrhoea. Other studies that evaluated the protective efficacy of ETEC-specific vaccines also failed to demonstrate clinically important benefits.[21,22] Future studies attempting to prevent travellers' diarrhoea through vaccination should target a broader range of enteropathogenic *Escherichia coli* and other enteropathogens.[23-26] Newer vaccines have therefore included more colonization factor antigens that are expressed by *Escherichia coli*.[27-31] Furthermore, future trials should include large numbers of travellers, or limit the investigation to countries for which detailed data concerning aetiology of travellers' diarrhoea is available. Lastly we recommend that trials should incorporate the degree of inconvenience as a clinical endpoint.

IMMUNITY

Immunology from an evolutionary perspective

From the beginning of their existence, metazoan recruited a basic diversity of molecular categories able to interact with proteins, sugars or lipids, i.e. an innate immune system that was able to recognize pathogens. The interactions with pathogens were articulated to

signalling cascades that were sometimes shared with other functions, such as fertilization control, development, metamorphosis and regeneration pathways. These signals were coupled to a diversified set of effector mechanisms.[32-34] Later in evolution, jawed vertebrates developed a so-called adaptive immune system.[35] This system consists of a set of gene segments that are assembled during the ontogeny of lymphocytes. After selection, it provides each individual with an unparalleled diversity of recognition capacity.[32] According to Du Pasquier, the reason why most life-forms did not develop an adaptive immune system, may be related to the relative value of individuals for the survival of a species. In species with large progenies and in which individuals reproduce only once and relatively early, and in which older individuals are less important for the survival of the species, innate immunity may suffice. This avoids the complexity of an adaptive immune system.[32]

Immunity and vaccines against tuberculosis

In the realm of immunology, a leap of faith may be required to imagine it possible to apply vaccines to prevent infectious diseases, such as malaria and tuberculosis, to which no sterile immunity occurs in people who are infected with the wild type micro-organism. From an epidemiological viewpoint, it is not necessary to achieve complete or ''sterile'' eradication of bacteria to effectively reduce the incidence of active tuberculosis. The natural state of most humans is protective immunity, since only a minority (~5%) develop clinically active tuberculosis after infection.[36] Various host-derived factors increase the risk: malnutrition, aging, stress, type-2 diabetes, vitamin D deficiency and genetic factors that affect innate and adaptive immunity.[37-52] Furthermore, temporary or permanent skewing of the immune system due to co-infections influences cellular immunity and may increase the risk of developing active tuberculosis.[53-58] On the other hand, infection with *Mycobacterium bovis* may mitigate the risk of developing active tuberculosis. This has been demonstrated in the 1940s in Denmark, where the incidence of tuberculosis was compared between the island Zealand and South Jutland.[59,60] On Zealand, bovine tuberculosis had been eradicated by 1930, whereas in South Jutland it was still prevalent at the time. In Zealand the incidence of morbidity due to MTb was higher than in South Jutland.

Morbidity occurs after primary infection, or after reactivation of latent tuberculosis. In areas with a high incidence of MTb infection, re-infection is also an important cause of active tuberculosis. This has been shown by Dutch pathologists, who analyzed lungs of people who died of causes, other than tuberculosis, in the '30s, '50s and '60s.[61-63] Based on histology, they selected cases with a primary calcified complex in the apex of the lung, indicating past or latent MTb infection. They then looked how many of these cases also had morphologically different active complexes, which were categorized as reinfections

FIGURE 1

Tuberculosis in The Netherlands: incidence, mortality, number of patients that have been treated, total number of patients and estimated chance of contracting tuberculosis.
(Reproduced by permission of KNCV Tuberculosefonds. Source: CBS, CIb and NTR).

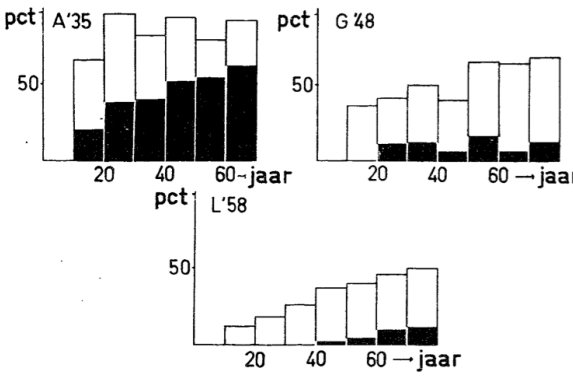

FIGURE 2

Prevalence of tuberculosis infection (total bar charts) and reinfections (filled sections of barcharts) by age and by year in which the studies were conducted.[63] (Reproduced by permission from Nederlands Tijdschrift voor Geneeskunde 1962. Copyright Bohn Stafleu Van Loghum).

with MTb. If these more active lesions were caused by reinfection and not by reactivation of latent MTb, such lesions should be less prevalent in the '60s than in the '30s and '50s, owing to the dramatic decrease in the incidence and prevalence of infection with MTb though time (FIGURE 1). This is exactly what they found (FIGURE 2). This observation has been corroborated by DNA fingerprinting of MTb isolates.[64,65]

Public health programs have had a large impact on the incidence of infection with MTb. This has been achieved by adequate treatment of people with active tuberculosis and by screening and treating the contacts that surrounds such a case. In a similar manner, preventing active tuberculosis with effective vaccines will impact the incidence of MTb infection, by reducing the reservoir of people who can transmit MTb. Vaccination strategies focus on preventing infection and/or active disease by inducing immunity to antigens that are expressed early in the course of infection, such as Early Secretory Antigenic Target (ESAT-6) and Antigen 85 (Ag85B). The subunit vaccine H1 consists of the fusion protein Ag85B-ESAT6.[66-68] To prevent active disease it also seems important to induce immunity to late stage antigens, that are expressed during bacterial dormancy.[52] The vaccine, H56 is based on this concept and combines Ag85B, ESAT-6 and Rv2660c. Rv2660 is expressed in late stage infection.[69]

Correlates of protection against MTb, vaccines and adjuvants

Despite increasing knowledge on the crucial role of individual cell types, genes and molecules in the protective host defence against MTb, we lack a true understanding of what exactly constitutes protection and protective immunity. This creates a roadblock for tuberculosis vaccine development and the identification of surrogate endpoints of protection, that can be used in clinical research.[70] Nevertheless, there is general consensus that a Th1 cell response is essential for bacterial containment during infection.[71] Inducing such a response with subunit vaccines, requires new types of adjuvants. Aluminum salts (i.e. alum), which were the only approved adjuvants until the end of the 20th century, promote the 'wrong' type of immune response, a Th2 antibody mediated response.[72] Initially, alum was added to vaccines, because it caused a precipitate (i.e. solid form), and because of the observation that precipitates improved vaccines' antigenic properties.[73] The mechanism governing the enhanced immunogenicity was thought to be the formation of a depot at the injection site, and subsequent slow release of antigen. This assumption has been disproven.[74] It seems that alum induces cytotoxicity and the release of host DNA, which acts as a damage associated molecular pattern (DAMP); an immunostimulatory signal.[75] Furthermore, alum allows host DNA to access the cytoplasm of dendritic cells (DCs), which activates pathways that promote MHC class II presentation and DC-T-cell interactions.[76] Activation of the inflammasome, directly by alum, or indirectly by local accumulation of uric acid, may also contribute to the adjuvant effect.[74]

In **chapter eight and nine** of this thesis, two clinical trials are described in which two new adjuvants were combined with a MTb subunit vaccine.[77,78] The first adjuvant, IC31® was developed by Intercell AG (Vienna, Austria) and consist of the artificial antimicrobial peptide KLK and the oligodeoxynucleotide ODN1a. KLK acts as a vehicle, enhancing uptake into antigen presenting cells (APC). ODN1a stimulates Toll-like receptor 9 (TLR9) signalling and activates APC. This causes a mixed Th1 and Th2 type response.[79-82] The second novel adjuvant, CAF01, was developed by Statens Serum Institute (Copenhagen, Denmark). It consists of liposomes formed by N,N'-dimethyl-N,N'-dioctadecylammonium (DDA) and of the synthetic immunomodulator α,α'-trehalose 6,6'-dibeheneate (TDB), which is inserted into the lipid bilayers.[83-85] DDA liposomes target cell membranes of APC, which subsequently leads to enhanced uptake and presentation of antigen and a weak Th1 cell response.[86] TDB is a synthetic analogue of the mycobacterial cell wall component trehalose 6,6'-dimycolate (TDM) often referred to as cord factor. TDB stabilizes DDA liposomes and enhances the Th1 and Th17 cell response.[84,85,87-89] This is mediated by recognition of TDB by the C-type lectin Mincle, which induces IL-1 production which in turn induces MyD88-dependent Th1/Th17 cell responses.[90-92]

H1-IC31® induced a long-lasting Th1 cell response in naïve subjects, characterized by IFN-γ producing lymphocytes.[93] The immune response was faster and generally stronger in subjects who had been vaccinated with BCG in the past and in subjects with past or latent MTb infection.[77] H1-CAF01 also induced a robust and long lasting Th1 cell response.[78] Despite these encouraging results, these surrogate immunological endpoints are not true correlates of protection. This is exemplified by a recent large phase 2b trial in which a vaccine consisting of a recombinant strain of modified Vaccinia Ankara virus that expresses Ag85A, induced excellent immune responses, but failed to protect South African infants against active tuberculosis.[94] Commenting on this result, Dr. Dye and Dr. Fine write: "The stakes are high. The venture is costly and risky, but has a huge potential payoff. We need to go on playing the high-stakes game."[95]

Poliovirus eradication, fractional doses and adjuvants

The Global Polio Eradication Initiative is another high-stakes venture.[96] Through thoughtful work, dedication and concerted effort, polio cases have decreased by over 99% since 1988, from an estimated 350 000 cases then, to 416 reported cases in 2013.[97] Furthermore, the last case of infection with poliovirus type 2 occurred in 1999 and of poliovirus type 3 in 2012.[97] In 2015, only 3 countries (Afghanistan, Nigeria and Pakistan) remain polio-endemic, down from more than 125 in 1988. War and displacement of people are currently the main obstacles to achieving complete interruption of the transmission of poliovirus. After eradication, cessation of oral poliovirus vaccine (OPV) is needed to prevent outbreaks due to circulating vaccine derived poliovirus.[98-100] IPV is a factor 20 more expensive than OPV.

Therefore, one of the prerequisites for cessation of the use of OPV is to make IPV affordable and suitable for use in developing countries.[101] Using fractional (reduced) doses may impact affordability and optimize the utilization of the production capacity for IPV. Intradermal administration has the potential to lower the dose without reducing immunogenicity. A needle-free jet injector may be a reliable way to administer vaccines intradermally. In **chapter seven** of this thesis, a study is described that found that fractional-dose intradermal IPV booster vaccination using a jet injection system was well tolerated and immunogenic.[102] Antibody titres in the fractional-dose intradermal group were slightly lower than after standard full-dose intramuscular vaccination. A way to further increase immunogenicity of fractional-dose IPV, may be to add an adjuvant. In mice, an IPV-CAF01 formulation has been tested.[103] IPV-CAF01, containing 2 D-Units (DU) of poliovirus type 1, 2 and 3 was compared to unadjuvanted IPV with either 2 or 20 DU of poliovirus type 1, 2 and 3. Intramuscular (IM) delivery of fractional-dose adjuvanted vaccine induced stronger antibody responses than IM fractional-dose unadjuvanted vaccine. The response to the fractional-dose adjuvanted vaccine was as strong as the response to the full-dose unadjuvanted vaccine. Furthermore, the adjuvant also induced an increased cellular response, as measured by multiplex cytokine analysis. In another experiment, IPV-CAF01 was injected simultaneously at an intradermal and an intramuscular site. Interestingly, this elicited an intestinal immune responses against poliovirus, measured as faecal IgA. This is important, because intestinal immunity shortens the time during which an infected person sheds poliovirus.[104-106] In the IPV vaccination trial described in this thesis, mucosal immunity was a secondary endpoint, which remains to be analyzed.

Digging up memory

In our study on IPV vaccination there was a fast and strong antibody response; i.e. a ≥ 40 fold increase in antibody titre within 7 days after a booster vaccination. This is typical of a memory immune response, which is characterized by a logarithmic increase in antibody titre within days after re-exposure to an antigen, combined with avidity maturation. Such a memory response depends on long lived-memory B cells.[107,108]

In a primary humoral immune response to a novel antigen, antigen-specific helper T cells that have been activated by antigen-bearing dendritic cells trigger some antigen-specific B cells to migrate towards follicular dendritic cells (FDCs), initiating the germinal centre reaction. In GCs, B cells receive additional signals from follicular T cells (Tfh) and undergo massive clonal proliferation, switch from IgM towards IgG, IgA or IgE, undergo affinity maturation and differentiate into plasma cells secreting large amounts of antigen-specific antibodies.[109] At the end of the GC reaction, a few plasma cells exit nodes/spleen and migrate to survival niches, where they survive through signals provided by supporting stromal cells.[110]

The duration of antibody responses is proportional to the number of long-lived plasma cells generated by immunization. In absence of subsequent antigen exposure, antibody persistence may be reliably predicted by the antibody titres that are reached 6–12 months after immunization, i.e. after the end of the short-term plasma cell response. This is illustrated by the accuracy of mathematical models predicting the kinetics of anti-HBsAg and anti-hepatitis A antibodies.[109,111,112] Long-lived plasma cells preferentially reside in niches in the bone marrow,[113] in the spleen[114] and in the tonsils.[115-117]

In parallel to plasma cells, memory B cells are generated in response to T-dependent antigens, during the GC reaction. When memory B cells exit the GC, they migrate to extrafollicular areas of the spleen and lymph nodes.[118] This migration occurs through the blood, in which post-immunization memory B cells are transiently present on their way towards lymphoid organs. The spleen harbours most memory B cells, followed by tonsils, bone marrow and peripheral blood.[119] Their phenotype does not differ in the different compartments. Memory B cells do not produce antibodies and do not protect, unless re-exposure to antigen or cross-reacting antigens drives their differentiation into antibody producing plasma cells. Since the affinity of surface Ig from memory B cells is increased, their requirements for reactivation are lower than for naïve B cells. Memory B cells may thus be recalled by lower amounts of antigen and without CD4+ T cell help. Therefore this reactivation is characterized by a rapid increase of the antibody titer.[109]

As we observed in a study on influenza vaccination in **chapter eleven** of this thesis, rechallenge with influenza subunit vaccines often fails to induce a typical booster humoral response.[120] This is peculiar, since influenza vaccines do induce a memory response with memory B cells and long-lived plasmablasts that can produce IgG antibodies with high levels of somatic hypermutation.[121,122] However, in individuals who have been primed by past vaccination or influenza infection, the recall response may be negatively influenced by residual cross-reactive anti-influenza antibodies. Upon vaccination, antigen-antibody complexes may reduce the load of antigen available for B cell binding. Alternatively, antibodies may have a negative feedback on B cells. Consequently, individuals with residual antibodies to a given antigen may only show a limited increase of their antibody response; such that vaccine responses are better described by the proportion of individuals above a given threshold than by those showing a 2- or 4-fold increase of antibody titers.[109] In chapter eleven geometric mean titers and seroprotection rates (defined as HI titers $\geq$1:40) were the main outcome measures.

Don't just do something, stand there.

Much in medicine remains uncertain. When faced with an incomplete pathophysiological model of a mechanism of disease and with an incomplete understanding of the effects

of treatment modalities, clinicians must rely on controlled studies to determine what is best. When such studies are lacking or when the results cannot be generalized to an individual patient, individual and collective experience must be combined with an understanding of pathophysiology to decide what is good. However, even impeccable logic doesn't always suffice and may have grave consequences.[123] To decide what is wisest, a doctor requires conscious knowledge of his inclination for cognitive error and of the fundament of intuition and reason. "*I call that man awake who, with conscious knowledge and understanding, can perceive the deep unreasoning powers in his soul, his whole innermost strength, desire and weakness, and knows how to reckon with himself.*"[124,1]

[1] *Wach nenne ich den, der mit dem Verstand und Bewusstsein sich selbst, seine innersten unvernünftigen Kräfte, Triebe und Schwächen kennt und mit ihnen zu rechnen weiß.*

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Nederlandse Samenvatting

NEDELANDSE SAMENVATTING

Het onderzoek dat in dit proefschrift wordt beschreven werd grotendeels verricht op de vaccinatiepolikliniek in het Leids Universitair Medisch Centrum (LUMC). In die kliniek verlenen gespecialiseerde verpleegkundigen advies aan mensen die van plan zijn op reis te gaan. Dankzij de nauwe samenwerking met de afdeling Infectieziekten, heeft men expertise over specifieke groepen reizigers, zoals mensen met een verminderde afweer en mensen met een chronische ziekte. De unieke locatie van een vaccinatiecentrum binnen een academisch ziekenhuis faciliteert het verrichten van onderzoek. Zo is er een ervaren groep artsen en verpleegkundigen, een constante stroom reizigers en de kennis en infrastructuur die nodig zijn voor microbiologisch, virologisch en parasitologisch onderzoek. Voorbeelden van onderzoek dat voortkomt uit de mogelijkheden die de vaccinatiepolikliniek biedt zijn: studies over vaccinatie tegen malaria,[1,2] gelekoorts,[3-6] reizigers diarree,[7,8] poliomyelitis[9] en hepatitis B,[10] vaccinatie van patiënten met een verminderde afweer,[11-14] onderzoek naar de incidentie van dragerschap van multiresistente micro-organismen in de darm na een reis[15] en onderzoek naar het nut van het routinematig screenen van mensen op darmparasieten na een reis naar de tropen.[16]

REIZEN EN INFECTIE

Volgens 'the World Tourism Barometer', reisden in 2014 meer dan een miljard mensen naar het buitenland.[17] Meer dan 500 miljoen mensen reisden naar een opkomende economie.[1] Naar verwachting zal dit aantal in 2015 stijgen met 3 tot 4%, waarbij de sterkste groei plaats zal vinden in Midden- en Zuid-Amerika, in Azië en in Oceanië. Jaarlijks reizen er naar schatting twee miljoen Nederlanders naar zogenaamde 'low and middle income countries', één miljoen naar Turkije (800.000) en Egypte (200.000), 400.000 naar landen in Azië, 400.000 naar Zuid-Amerika en 90.000 naar 'Sub-Saharan Afrika'.[18,19] Deze aantallen zijn aan verandering onderhevig, onder andere door politieke instabiliteit in bepaalde regio's. Reizen gaat gepaard met een aantal risico's voor de gezondheid. Reisgerelateerde ziekten zijn die ziekten, waarvan de kans op het oplopen ervan toeneemt door een veranderde blootstelling aan risicofactoren als gevolg van een tijdelijke wijziging van de verblijfsplaats. Data over de *incidentie* van reisgerelateerde morbiditeit is samengevat door Steffen et al. en door Cobelens (FIGUUR 1 EN 2 ▶ INTRODUCTIE, PAGINA 14 EN 15)[20,21,22] Het GeoSentinel Surveillance Network publiceert data over regionale verschillen in *proportionele morbiditeit*; het aantal reizigers met een bepaalde ziekte gedeeld door het totale aantal zieke reizigers.[23,24,25] Diarree is de voornaamste oorzaak

[1] Classificatie gebaseerd op het International Monetary Fund (IMF), zie Statistical Annex van de IMF World Economic Outlook van April 2012, pagina 177, at www.imf.org/external/pubs/ft/weo/2012/01.

van reisgerelateerde morbiditeit.[21,26] Ongevallen zijn de voornaamste oorzaak van mortaliteit.[26-28]

Hoofdstuk één van dit proefschrift betreft een studie naar de gezondheidsrisico's die studenten lopen tijdens een medische stage in het buitenland en naar de kwaliteit en volledigheid van het medische reisadvies en de medische zorg voor en na de reis.[29] Zoals mag worden verwacht verrichtte het merendeel van de studenten procedures die gepaard gaan met een verhoogd risico op besmetting met, via het bloed overdraagbare virale ziekten. Vaak was dat in landen met een hoge prevalentie voor HIV infectie. Het is zorgelijk dat geen van de studenten de juiste stappen ondernam na een spat- of prikaccident. Dit kwam overeen met de bevinding van een studie onder Britse medische studenten.[30] Verder bleek de toewijzing van 'post exposure prophylaxis' kits voor HIV (PEP) inadequaat te zijn. Wat de overige gezondheidsrisico's betreft: 20% stopte voortijdig met het gebruik van mefloquine vanwege bijwerkingen. Hierdoor waren zij niet beschermd tegen malaria, vaak in hoog-endemische landen. Na de reis werd, van de studenten die onderzocht hadden moeten worden, slechts de helft onderzocht op asymptomatische infectie met schistosomiasis, tuberculose en methicilline-resistente *Staphylococcus aureus* (MRSA). Op basis van de resultaten van dit onderzoek is een aantal maatregelen genomen om de gezondheidsrisico's voor studenten, die op stage gaan, te verminderen. De medische advisering voor en na de reis is gecentraliseerd, evenals de uitgifte van PEP. Verder dienen de studenten en hun Leidse supervisoren vooraf aan de stage een formulier in te vullen om te bepalen of de student bekwaam wordt geacht om bepaalde procedures uit te voeren, zoals hechten en assisteren bij operaties en bevallingen. Indien een nieuwe procedure tijdens de stage aangeleerd wordt, dient duidelijk te zijn of de begeleiders in het buitenland voldoende tijd en mogelijkheden hebben om de student te begeleiden. De studenten ontvangen ook een folder waarin staat hoe ze dienen te handelen in geval van een spat- of prikaccident. Na terugkomst vullen de studenten op het internet een korte vragenlijst in, om te bepalen of zij contact op moeten nemen voor een 'post-travel' consult. Tot slot maakt de dienst onderwijs- en studentenzaken een lijst van klinieken in het buitenland waar studenten bij voorkeur heen kunnen gaan voor een stage. Een vervolgonderzoek wordt uitgevoerd. Bij dit onderzoek wordt ook aandacht geschonken aan andere aspecten, zoals het psychosociale welbevinden, de incidentie van ongevallen en de incidentie van het prikkelbare darm syndroom na een episode van reizigersdiarree.

Diarree is de voornaamste oorzaak van reisgerelateerde morbiditeit.[21,26] Het kan erg vervelend zijn, maar is zelden dodelijk. In het **tweede hoofdstuk** wordt een studie beschreven, die tot doel had om te bepalen hoe groot het ongemak is dat reizigers ondervinden wanneer zij diarree oplopen tijdens een reis met een mediane duur van 23 dagen.[8] Onze conclusie is dat de gangbare definitie van reizigersdiarree ruim is. Ongeveer

een derde van de gevallen van reizigersdiarree was mild van aard en veroorzaakte weinig ongemak. Het is onwaarschijnlijk dat behandeling van dit soort gevallen zal resulteren in gezondheidswinst. Wij bevelen aan om de mate van ongemak als eindpunt te gebruiken in klinische studies naar behandeling van reizigersdiarree. Zodoende zullen onderzoekers de groep met 'significante' reizigersdiarree beter kunnen onderscheiden van de groep met 'niet-significante' reizigersdiarree. Dit zal beleidsmakers in staat stellen om met een grotere nauwkeurigheid de omvang van de groep reizigers te bepalen, die baat zou kunnen hebben bij maatregelen die tot doel hebben om diarree op reis te voorkomen of te behandelen. Tevens zal het effect van deze maatregelen nauwkeuriger kunnen worden geschat, indien de maatregelen worden beperkt tot de groep met 'significante' reizigersdiarree.

Hoofdstuk drie richt zich op mensen met de ziekte van Crohn of colitis ulcerosa.[31] In een retrospectieve studie werd met vragenlijsten onderzocht hoe deze groep reizigers zich voorbereidt op een reis en welke gezondheidsklachten zij ervaren tijdens een reis. Tevens werd onderzocht of het reizen het risico doet verhogen op een opvlamming van de darmziekte. De beschikbaarheid van sanitaire voorzieningen was het voornaamste punt van zorg en ongemak, aangezien mensen met deze darmaandoeningen last kunnen hebben van frequente en sterke defaecatiedrang en van incontinentie. Een derde van de deelnemers rapporteerde dat zij tijdens de reis een episode met reizigersdiarree hadden doorgemaakt. Verrassend genoeg was dit qua incidentie niet groter dan de incidentie in de algemene bevolking.[32] Het reizen leidde niet tot een hoger risico op een opvlamming van de darmziekte gedurende de eerste twee maanden na de reis. Echter, deze meting kent beperkingen, aangezien het aantal zelfgerapporteerde exacerbaties in een periode van 5 jaar vooraf aan de reis misschien geen betrouwbare schatting oplevert van de te verwachten incidentie van een exacerbatie na een reis. Tot slot werd geconstateerd dat de voorbereiding op een reis en het medische advies vooraf aan een reis gebreken vertoonden. Wij raden aan dat zorgverleners, mensen met een inflammatoire darmziekte bewust maken van het belang van een bezoek aan een reizigerspolikliniek voordat zij op reis gaan naar de (sub)tropen en dat zij hen op tijd verwijzen. Er is namelijk meer tijd nodig om, in geval van het gebruik van medicatie die de afweer onderdrukt, te controleren of iemand na vaccinatie beschermd is en om zonodig een tweede dosis toe te dienen.[33,34]

Hoofdstuk vier betreft een studie naar het nut van het routinematig screenen van mensen op darmparasieten na een reis naar de tropen, met moleculaire technieken en serologie.[16] Slecht één van 400 reizigers raakte geïnfecteerd met *Strongyloides stercoralis*. Geen van 500 reizigers had een infectie met *Entamoeba histolytica* opgelopen. De incidentie van infectie met *Schistosoma* spp. was hoger (6%), waarbij elk geval was geassocieerd met blootstelling aan hoog endemische meren in Malawi en Tanzania. Concluderend is het na een reis

van 3 maanden of korter naar de (sub)tropen niet nodig om asymptomatische reizigers routinematig te onderzoeken op darmparasieten. Uitzondering hierbij is dat screening op schistosomiasis wel is gewenst bij reizigers die zoetwatercontact hebben gehad in hoog-endemische gebieden.

IMMUNITEIT

In **hoofdstuk vijf** wordt de humane immuunrespons tegen *Schistosoma* spp. onderzocht.[35] Hierbij werd gebruik gemaakt van een uniek cohort van twintig Nederlandse reizigers, die in het verleden tijdens een groepsreis in Mali, schistosomiasis hadden opgelopen.[36] Sommige geïnfecteerde reizigers maakten destijds een acuut ziektebeeld door dat bekend staat als het Katayama syndroom en anderen niet. Acht jaar later werden bloedmonsters verzameld en werd in vitro de cellulaire en humorale immuunrespons tegen schistosoma worm- en ei-antigenen gemeten. De immuunrespons werd vergeleken tussen de groep die het Katayama syndroom had doorgemaakt en de groep die een asymptomatische infectie had doorgemaakt. Mensen die het Katayama syndroom hadden doorgemaakt bleken acht jaar later een sterkere immuunrespons te hebben tegen de schistosoma antigenen dan de mensen die een asymptomatische infectie hadden doorgemaakt. De verschillen waren echter niet significant volgens de gangbare statistische grenswaarde.

De meest gangbare oorzaak van diarree op reis is de enterotoxine producerende *Escherichia coli* (ETEC). Omdat het ziekmakende, hitte-labiele toxine dat door ETEC wordt gemaakt qua structuur lijkt op het choleratoxine, bieden choleravaccins een zekere mate van kruisbescherming tegen infecties met ETEC. **Hoofdstuk zes** betreft een onderzoek naar de werkzaamheid van een levend verzwakt oraal cholera vaccin, CVD 103-HgR, om diarree tijdens een reis te voorkomen.[7] In deze studie hadden de 69 reizigers die het vaccin hadden gebruikt even vaak diarree als de 65 reizigers die placebo hadden gebruikt. De interpretatie van het resultaat werd beperkt door de onverwacht lage incidentie van LT–ETEC geassocieerde diarree in de groep die deelnam aan het onderzoek. Andere onderzoeken naar vaccins tegen ETEC zijn eveneens ineffectief gebleken.[37,38] Een effectief vaccin tegen reizigersdiarree dient waarschijnlijk te beschermen tegen meerdere enteropathogene *Escherichia coli* en ook tegen andere enteropathogenen.[39-42] In nieuwere vaccins, die worden ontwikkeld, worden derhalve ook antigenen van kolonisatiefactoren toegevoegd. Dit zijn antigenen die tot expressie worden gebracht door veel verschillende typen *Escherichia coli*.[43-46]

Onderdelen van het aangeboren en het verworven mucosale immuunsysteem beschermen het lichaam tegen pathogenen die via de mucosa proberen binnen te dringen. Sommige pathogenen, zoals enteropathogene *Escherichia coli*, veroorzaken ziekte door de mucosa binnen te dringen of te beschadigen. Andere pathogenen, zoals poliovirus doorkruisen

de mucosa om uiteindelijk op een andere plek in het lichaam ziekte te veroorzaken. Overdracht van poliovirus vindt plaats via de faeco-orale route. Een infectie met het wild type poliovirus veroorzaakt mucosale en systemische immuniteit.[47] Het levend verzwakte orale poliovirus vaccin (OPV) bootst een infectie met wild-type virus na en veroorzaakt ook een vergelijkbare immuunrespons.[48] Daarentegen ontstaat er na intramusculaire vaccinatie met het geïnactiveerde poliovirus vaccin (IPV) uitsluitend een systemische immuunrespons, zonder mucosale respons.[48,49] IPV is desalniettemin een zeer effectief vaccin, omdat het een betrouwbare en sterke systemische immuunrespons opwekt en zodoende voorkomt dat het poliovirus zijn weg weet te vinden naar het centrale zenuwstelsel. Dit verschil tussen OPV en IPV vertaalt zich in het feit dat mensen, die gevaccineerd zijn met OPV, minder makkelijk een herinfectie met wild-type poliovirus oplopen en in geval van infectie gedurende een kortere periode het virus uitscheiden, dan mensen die gevaccineerd zijn met IPV.[50,51] Het gebruik van OPV kent echter ook nadelen. Zo treedt er bij vaccinatie met het trivalente vaccin interferentie op tussen de drie polio subtypen waardoor de respons tegen subtype 1 en 3 zwakker is dan tegen subtype 2.[52] Een ander nadeel is dat het levende verzwakte virus dat in OPV zit, soms terug kan muteren naar het wild type virus.[53-55] Daarom dient het gebruik van OPV uiteindelijk gestaakt te worden, om bestendige wereldwijde erradicatie van polio te bewerkstelligen. Het is aannemelijk dat deze transitie gepaard zal gaan met het vervangen van OPV door IPV. IPV is echter 20 keer zo duur als OPV en de productiecapaciteit is niet groot genoeg om de hele wereld van vaccin te voorzien. Daarom is het van belang om IPV goedkoper en geschikter te maken voor gebruik in ontwikkelingslanden.[56] Dit kan wellicht worden gedaan door een fractie van de gebruikelijke dosis toe te dienen. Intradermale toediening kan in potentie het gebruik van een lagere dosis mogelijk maken, zonder dat dit ten koste gaat van de mate van bescherming. Het gebruik van een naald-vrije jet injector, die door gebruik van hoge druk een dunne straal vloeistof in de huid of spier kan toedienen, kan een betrouwbare methode zijn om op grotere schaal intradermaal te vaccineren. **Hoofdstuk zeven** betreft een studie waarin 125 proefpersonen een booster vaccinatie met IPV kregen toegediend. De proefpersonen werden gerandomiseerd naar één van vier groepen: intramusculaire vaccinatie met een naald en spuit met een volledige dosis (IM-NS-0.5), intramusculaire vaccinatie met een jet injector met een volledige dosis (IM-JI-0.5), intramusculaire vaccinatie met een naald en spuit met een vijfde van een volledige dosis (IM-NS-0.1), intradermale vaccinatie met een jet injector met een vijfde van een volledige dosis (ID-JI-0.1).[9] Na 28 dagen was de gemiddelde antistoftiter in de ID-JI-0.1 groep iets lager dan in de referentiegroep (IM-NS-0.5). De verschillen waren niet statistisch significant, maar voldeden niet aan de strikte norm voor non-inferioriteit die vooraf was gesteld (i.e. een maximaal verschil van minder dan 1 serum verdunningsstap in de microneutralisatie assay). Een jaar later waren de kleine verschillen in antistoftiter verdwenen. In tegenstelling tot dit resultaat, was de gemiddelde antistoftiter in de IM-

NS-0.1 groep statistisch inferieur aan die in de referentiegroep (IM-NS-0.5). In een andere studie is recent het adjuvans CAF01 toegevoegd aan IPV en getest op muizen.[57] IPV-CAF01, met 2 D-Units (DU) poliovirus type 1, 2 en 3 werd vergeleken met IPV zonder adjuvans met ofwel 2 of 20 DU poliovirus type 1, 2 en 3. De respons op intramusculaire toediening van een fractionele-dosis van IPV-CAF01 was even sterk als de respons op een volledige dosis IPV en was sterker dan de respons op een fractionele dosis van IPV. Bovendien induceerde IPV-CAF01 niet alleen een humorale immuunrespons, maar ook een cellulaire respons.

Het opwekken van een cellulaire Th1 immuunrespons is essentieel om weerstand op te wekken tegen *Mycobacterium tuberculosis* (MTb).[58] Om een dergelijke respons op te weken met 'subunit' vaccins zijn nieuwe hulpstoffen (adjuvans) nodig. De **hoofdstukken acht en negen** betreffen klinische trials waarin twee verschillende nieuwe adjuvans werden gecombineerd met een MTb subunit vaccin, H1.[59,60] Het subunit vaccin H1 bestaat uit het fusie eiwit Ag85B-ESAT6.[61-63] Dit zijn twee antigenen die in een vroeg stadium van de infectie tot uiting worden gebracht. Het eerste adjuvans dat werd onderzocht is IC31®. Dit werd ontwikkeld door Intercell AG (Wenen, Oostenrijk) en bestaat uit het artificiële antimicrobiële peptide KLK en de oligodeoxynucleotide ODN1a. KLK fungeert als een vehikel om de opname in antigen presenterende cellen (APC) te bevorderen. ODN1a stimuleert Toll-like receptor 9 (TLR9) en activeert APC. Dit leidt tot een gemengde Th1 en Th2 type respons.[64-67] Het tweede nieuwe adjuvans dat werd onderzocht is CAF01. Dit werd ontwikkeld door het Statens Serum Instituut (Kopenhagen, Denemarken). Het bestaat uit liposomen, die gevormd worden door N,N'-dimethyl-N,N'-dioctadecylammonium (DDA) en uit de synthetische immunomodulator α,α'-trehalose 6,6'-dibeheneate (TDB), die ingevoegd wordt tussen het lipide dubbelmembraan.[68-70] DDA liposomen richten zich op cel membranen van APC en dit leidt tot toegenomen opname en presentatie van antigenen en een zwakke Th1 cel respons.[71] TDB is een synthetisch analoog van trehalose 6,6'-dimycolate (TDM), een mycobacterieel celmembraan onderdeel, dat vaak 'cord factor' wordt genoemd. TDB stabiliseert DDA liposomen en bevordert een Th1 en Th17 cel respons.[69,70,72-74] Dit wordt gemedieerd door herkenning van TDB door het C-type lectin Mincle, hetgeen IL-1 aanmaak stimuleert en een MyD88-afhankelijke Th1/Th17 cel respons induceert.[75-77]

In de klinische trials die worden beschreven in dit proefschrift, veroorzaakte H1-IC31® een bestendige Th1 cel respons bij MTb naïeve proefpersonen. Deze respons werd gekarakteriseerd door IFN-γ producerende lymfocyten.[59] Bij de proefpersonen die in het verleden waren gevaccineerd met BCG en bij de proefpersonen die in het verleden een MTb infectie hadden doorgemaakt trad de immuunrespons sneller op en was meestal ook sterker. H1-CAF01 veroorzaakte eveneens een krachtige en bestendige Th1 cel respons

bij MTb naïeve proefpersonen.[60] Ondanks deze bemoedigende resultaten, dient een kanttekening te worden geplaatst. De surrogaat immunologische eindpunten die in deze onderzoeken werden gebruikt kennen geen volledige correlatie met bescherming tegen MTb. Zo werd in een recente grote fase 2b onderzoek in Zuid Afrika, waarin een vaccin werd onderzocht bestaande uit een recombinant veranderd Vaccinia Ankara virus dat Ag85A tot expressie brengt, gezien dat het vaccin een goede immuunrespons opwekte maar geen bescherming bood tegen actieve tuberculose.[78] Om actieve ziekte te voorkomen is het waarschijnlijk ook belangrijk om immuniteit op te wekken tegen latere stadium antigenen, die tot expressie worden gebracht gedurende het slapende stadium van de bacterie.[79] Het vaccin, H56 is gebaseerd op dit concept en combineert Ag85B, ESAT-6 en Rv2660c. Rv2660 wordt tot uiting gebracht gedurende het late stadium van infectie.[80,81]

Mycobacterium tuberculosis kan een latente infectie veroorzaken en in sommige gevallen pas decennia later reactiveren. De reactivatie treedt vaker op als de afweer verzwakt raakt, zoals kan gebeuren bij veroudering. De leeftijdsafhankelijke veranderingen in het immuunsysteem worden samengevat met het begrip 'immunosenescence'. Dit proces kan op verschillende wijzen worden bestudeerd; bijvoorbeeld door de immuunrespons na een gestandaardiseerde infectie te vergelijken tussen jongeren en ouderen. Vaccinatie met het levend verzwakte gelekoortsvaccin biedt de mogelijkheid om een 'gestandaardiseerde infectie' te veroorzaken. Het virus uit het vaccin vermenigvuldigt zich in de naïeve gastheer. In zeldzame gevallen kan het ernstige ziekte veroorzaken; ofwel gelekoorts geassocieerde neurotrope ziekte, hetgeen zich manifesteert als ofwel een encefalitis, ofwel viscerotrope ziekte hetgeen de verschijnselen van een wild type infectie nabootst.[82,83] Neurotrope ziekte treedt met name op bij zuigelingen. Het risico op viscerotrope ziekte daarentegen neem toe met de leeftijd. In vergelijking met jong-volwassenen is het risico op deze zeldzame bijwerking 4 keer groter bij mensen van 60-69 jaar oud en 13 keer groter bij mensen van 70 jaar of ouder.[84] Op grond van deze gegevens is er terughoudendheid met het gebruik van het gelekoortsvaccin bij reizigers ouder dan 60 jaar. **Hoofdstuk tien** betreft een gecontroleerde studie naar de verschillen in de immuunrespons tussen een groep van 30 jongeren en een groep van 28 ouderen, na toediening van het gelekoortsvaccin.[6] Bij de oudere personen kwam de humorale respons later op gang. Bovendien ontstond bij de groep ouderen vaker een viremie. Aangezien de verschillen in de incidentie en de mate van viremie optraden op dag vijf na vaccinatie, nog voordat het adaptieve immuunsysteem voldoende tijd heeft gehad om te reageren, is het aannemelijk dat er een leeftijdsafhankelijke afname is van de innate immuunrespons.

Er geldt een contra-indicatie voor het gebruik van levend-verzwakte vaccins bij mensen met een immuunstoornis, zoals bij mensen met een HIV infectie en een laag aantal CD4 positieve T-cellen. Deze mensen hebben een grotere kans op vaccin-gerelateerde

morbiditeit, omdat hun immuunsysteem minder goed werkt, waardoor de virale replicatie niet goed wordt geremd. Dit betreft een beperking in het vormen van een adequate immuunrespons tegen met name neo-antigenen. Hiermee wordt bedoeld, antigenen die het immuunsysteem niet eerder is tegengekomen. In 2009 was er een pandemie met een influenza virus dat qua antigenen sterk verschilde met voorgaande jaren en waartegen de meeste mensen weinig antistoffen hadden; het zogenaamde 2009 pandemische influenza A(H1N1) virus (pH1N1). Het virus verspreidde zich snel over de wereld en werd beschouwd als een belangrijk gevaar voor de volksgezondheid. Dit leidde tot een snelle ontwikkeling en gebruik van vaccins tegen pH1N1. Hoewel de immuunrespons tegen influenza vaccins vrij uitvoerig is onderzocht onder mensen met een HIV-infectie, waren de omstandigheden in 2009 om een aantal redenen anders: i) het hemagglutinine antigen werd beschouwd als een neo-antigen; ii) het vaccin werd twee keer toegediend in plaats van een keer; en iii) de meeste mensen in Nederland waren een maand eerder al gevaccineerd met het standaard trivalente geïnactiveerde influenza vaccin (TIV). In 2009 verrichtten wij een observationele gecontroleerde studie waarin de humorale immuunrespons tegen pH1N1 vaccin werd onderzocht bij mensen met een infectie met HIV en bij gezonde controles. Tevens werd onderzocht of TIV kruisbeschermende antistoffen tegen pH1N1 opwekte en of een tweede vaccinatie een typische booster respons veroorzaakt. **Hoofdstuk elf** beschrijft het resultaat van dit onderzoek. Bij de meeste mensen met een HIV infectie (91%) ontstond seroprotectie. De tweede vaccinatie had een klein additioneel effect op de titer en de respons had niet het typische patroon van een boosterrespons met de typerende snelle en hoge titerstijging kort na een tweede vaccinatie. Aanvankelijk leek het erop dat TIV al leidde tot kruisreactieve antilichamen en een mate van seroprotectie tegen pH1N1. Echter na aanvullend onderzoek, waarin een virusneutralisatie werd gebruikt om influenza specifieke antistoffen te meten bleek dit niet het geval, zoals wordt beschreven in **hoofdstuk twaalf**.

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CURRICULUM VITAE

Darius Soonawala werd op 23 januari 1977 geboren in Leiden. In 1995 behaalde hij het eindexamen aan het Stedelijk Gymnasium in Leiden. In 1996 werd de propedeuse economie behaald aan de Universiteit van Amsterdam (cum laude) en in 1997 startte hij met de studie Geneeskunde aan de Rijksuniversiteit Leiden. In 2001 werd het doctoraal examen behaald en in 2003 het artsexamen (beiden cum laude). De eerste stappen in het onderzoek werden gezet op de afdeling fysiologie in Leiden bij dr. L.J.S.M. Teppema en in Edinburgh op de afdeling psychiatrie en radiologie bij prof. dr. K.P. Ebmeier. De opleiding tot internist werd gestart in 2004 in het Leyenburg ziekenhuis (opleider dr. R.H. Kauffmann), vervolgd in het Rode Kruis Ziekenhuis (opleider dr. R.M. Valentijn) en afgerond in 2012 in Leiden (opleider prof. dr. J.T. van Dissel). In 2012 volgde de registratie als nefroloog (opleiders prof. dr. A.J. Rabelink en prof. dr. J.W. de Fijter). Het onderzoek dat wordt beschreven in dit proefschrift werd verricht tussen 2008 en 2011, onder de supervisie van prof. dr. L.G. Visser en prof. dr. J.T. van Dissel. Gedurende het eerste jaar van de onderzoeksperiode werd tevens een project verricht over 'non-inferiority' studies, met dr. O.M. Dekkers en prof. dr. J.P. Vandenbroucke. Sinds 2013 is hij werkzaam als staflid op de afdeling Nefrologie in het Leids Universitair Medisch Centrum. Darius Soonawala is getrouwd met Leonie de Haan en samen hebben zij twee zonen, Abel en Benjamin.