Landon James Watt

From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Tuesday, 25 February 2014 4:00 a.m.
To: Helen Petousis-Harris
Subject: Doc and Video Conf

Dear Helen,

I hope this finds you well. And thank you so much for your excellent support. It has been extremely helpful for us.

We would be truly grateful if you could send us your presentation no later than 12pm (noon) 25th Feb NZ time so that we can send it to a printing center to be distributed during the meeting.

Further, we wish do a testing for the video conference machine as early as 2pm (NZ time) tomorrow.

We look forward to talking to you again soon.

Warm regards,

Koji Nabae

Landon James Watt

From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Thursday, 27 February 2014 3:31 a.m.
To: Robert Pless; SAHINOVIC, Isabelle; Robert Pless; mew2@cdc.gov; Helen Petousis-Harris; ZUBER, Patrick Louis F.; jbeytout@chu-clermontferrand.fr; LAMBACH, Philipp
Cc: 阿部 圭史(abe-keishi); 難波江 功二(nabae-koji)
Subject: HPV mtgs in Japan
Attachments: Dr Helen Petousis-Harris.pdf; Prof Jean Beytout.pdf; Summary of 25 Feb Symposium.pdf

Dear colleagues,

The two meetings on HPV vaccine in Japan today went very well.

I wish to express our sincere appreciation to all of you, particularly Prof Beytout who came all the way to Japan (in economy class!) and made an excellent and very convincing presentation (attached file) and Helen, despite the technical difficulty, generated a straightforward and punchy argument (attached file).

The media has already picked up the events. The tone is very neutral and some reports that these arguments were dismissed for lack of scientific evidence.

http://www.tv-tokyo.co.jp/mv/mplus/news/post_61006
http://www3.nhk.or.jp/news/html/20140226/k10015558551000.html

And I thank colleagues in WHO GACVS (Philipp, Patrick, Isabelle, Rob and Melinda) for your enormous support and encouragement. We really hope that our committee would come to a conclusion in a month or two and we can move forward with our immunization program.

I will send you a summary of the meeting today once ready. In the meantime, I attach an internal report of the symposium that took place yesterday (not for wider circulation, please keep it only among GACVS members).

Millions of thanks again and I look forward to talking to you soon.

Warmest regards,

Koji

資料4

Public hearing session on HPV safety.
Tokyo, Japan 26 February 2014

Helen Petousis-Harris. PhD
Director of Immunisation Research and Vaccinology
Immunisation Advisory Centre
The University of Auckland
Convenor, NZ Expert Advisory Group on Vaccine Safety

THE UNIVERSITY OF AUCKLAND
FACULTY OF MEDICAL AND HEALTH SCIENCES

Overview

- Dr Lee claims to have tested vials of Gardasil vaccine for the presence of vaccine derived HPV L1 gene DNA sequences and to have detected it.
- Dr Lee claims to have tested post mortem samples from a single person for the presence of HPV DNA. He claims they are positive for HPV DNA bound to the vaccine adjuvant
- Dr Lee supports that the HPV vaccine may have caused death due to a complex of vaccine constituents
- The Immunisation Advisory Centre at The University of Auckland **strongly question both his methods, interpretation and conclusions**

Testing of Gardasil samples - concerns

- The vaccine is be expected to contain traces of vaccine type HPV L1 gene DNA – consistent with manufacturing process.
- However:
  1. The tests used have never been validated – methods are not transparent
  2. DNA contamination can be tested by most laboratories – not ‘cutting edge’
  3. PCR extremely sensitive, nested PCR much more so. High risk of amplifying other DNA sequences
  4. Use of degenerate primers increases sensitivity but also increases risk of amplifying other DNA sequences (decreases specificity) wildcard. Given that the genetic code of the vaccine L1 gene is known this is a strange thing to do.

Testing of post mortem samples - concerns

- **There are no controls used (unvaccinated). This is a <u>vital</u> part of the scientific process**
- Inclusion of degenerate primers again
  - Over sensitive, increased risk of amplifying other irrelevant DNA (junk) + reduced specificity for vaccine type HPV L1 gene
- Why did Lee not include primers for the plasmid and promoter sequences which would provide more robust evidence for the presence of vaccine HPV DNA.
- Has not proven that the DNA is of vaccine origin (no plasmid, no yeast)
- The amount of residual DNA in the vaccine is miniscule
  - How can such a small amount of DNA detected dispersed through out body tissues?
- It is not biologically possible for the HPV DNA to integrate into the host genome (does not have the necessary sequences)

Extraordinary hypotheses

- Dr Lee supports hypotheses whereby HPV DNA has bound irreversibly to aluminium adjuvant (theory), been taken up by macrophage (theory) and caused an inflammatory response (theory) leading to death (theory).
- He contends that these complexes can be detected in post mortem tissue samples and that they were carried there by the macrophage
- He has little if any evidence for this hypothesis, which, if it were true, needs to be tested using rigorous scientific methods.
  1. Convincing proof of binding of vaccine DNA to the adjuvant
  2. Convincing proof of carriage by macrophage
  3. Convincing proof of deposition in tissues (especially brain)
  4. Convincing proof of inflammatory immune cascade including TNF

- No one else has replicated the findings
- The findings are not supported by existing extensive research on the immunology or epidemiology of HPV vaccine safety.

Immune response following vaccination with protein-based vaccine

- After injection immune cells such as macrophage take up vaccine (adjuvant and antigen) **at injection site**
- The cells become activated and migrate via the lymph to **local lymph node** (not spleen)
- Half life of a macrophage ~6 days
- These facts do not support the potential for presence of adjuvant/HPV DNA in either blood or spleen
- In addition, the immune activation on uptake of HPV vaccine does not include an increase in inflammatory factors (incl TNF) even in vaccinees with large injection site reactions at time of local inflammation.

The NZ case

- This type of death is often caused by underlying cardiac abnormalities.
  - Testing was refused by the family
- There was no evidence of inflammation in the autopsy results for the NZ case
  - including either the brain or the heart

Key question and answer

- Does Gardasil trigger death?
  - **No**
  - Large epidemiological studies and routine surveillance systems conducted globally directly investigating this find no difference between vaccinated and unvaccinated population (refs provided)
  - If it did then we would see a higher rate of cases in vaccinated people, and this has not been observed.
  - Data for over 100 million doses to date

Extraordinary claims require extraordinary evidence

Carl Sagan 1934-1996

Selected references on safety of Quadrivalent Human Papillomavirus Vaccine

- Food and Drug Administration. Clinical Review of Biologics License Application for Human Papillomavirus 6, 11, 16, 18 L1 Virus Like Particle Vaccine (S. cerevisiae) (STN 125126 GARDASIL), manufactured by Merck, In: Vaccines Clinical Trial Branch, Centre for Biologics Evaluation and Research, editor.: Food and Drug Administration.; 2006.
- Garland SM, Hernandez-Avila M, Wheeler CM, Perez G, Harper DM, Leodolter S, et al. Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases.[see comment]. N Engl J Med. 2007;356(19):1928-43.
- Villa LL, Costa RL, Petta CA, Andrade RP, Ault KA, Giuliano AR, et al. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. Lancet Oncology. 2005;6(5):271-8.
- Future II Study Group. Prophylactic efficacy of a quadrivalent human papillomavirus (HPV) vaccine in women with virological evidence of HPV infection.[see comment]. J Infect Dis. 2007;196(10):1438-46.
- Arnheim-Dahlström L, Pasternak B, Svanström H, Sparén P, Hviid A. Autoimmune, neurological, and venous thromboembolic adverse events after immunisation of adolescent girls with quadrivalent human papillomavirus vaccine in Denmark and Sweden: Cohort study. BMJ. 2013;347(f5906).
- Callréus T, Svanström H, Nielsen NM, Poulsen S, Valentiner-Branth P, Hviid A. Human papillomavirus immunisation of adolescent girls and anticipated reporting of immune-mediated adverse events. Vaccine. 2009;27(22):2954-8.
- Chao C, Klein NP, Velicer CM, Sy LS, Slezak JM, Takhar H, et al. Surveillance of autoimmune conditions following routine use of quadrivalent human papillomavirus vaccine. J Intern Med. 2012;271(2):193-203.
- Klein NP, Hansen J, Chao C, et al. Safety of quadrivalent human papillomavirus vaccine administered routinely to females. Arch Pediatr Adolesc Med. 2012;166(12):1140-8.
- Siegrist C-A, Lewis EM, Eskola J, Evans SJW, Black SB. Human papilloma virus immunization in adolescent and young adults: A cohort study to illustrate what events might be mistaken for adverse reactions. Pediatr Infect Dis J. 2007;26(11):979-84.
- Siegrist C-A. Autoimmune diseases after adolescent or adult immunization: What should we expect? CMAJ. 2007;177(11):1352-4.
- Slade BA, Leidel L, Vellozzi C, Woo EJ, Hua W, Sutherland A, et al. Postlicensure Safety Surveillance for Quadrivalent Human Papillomavirus Recombinant Vaccine. JAMA. 2009;302(7):750-7.

資料2

Aluminium adjuvanted vaccines: the position of the French Advisory Committee on Immunisation Practices

Pr Jean Beytout

University hospital of Clermont-Ferrand (France)

Member of the French Committee on Immunisation Practices. High Council of Public Health.

Aluminium adjuvanted vaccines
Long duration collective experience

- Aluminium salts have been added to vaccine antigens since 1926 . These vaccines have been used all over the world for more than eighty years and milliard of doses were inoculated.
- Tolerance is fair. Adverse effects are scarce : mainly local reaction (pain, inflammatory reaction), seldom general (fever); these reactions happen early.
- **No country or official body calls into question the validity of this addition or the safety of vaccines containing aluminium salts**.

Macrophagic Myofasciitis (MMF)

- MMF is defined by microscopic lesions found in muscles biopsies : infiltration of muscle tissue by PAS-positive macrophages.
- In patients suffering from musculoskeletal symptoms, Ghirardi, Authier and Cherin at the French Reference Center for Neuromuscular Diseases (GERMAAD), observed MMF in biopsy obtained from deltoïd muscle. Macrophages contained aluminium particles provided by inoculated vaccines.
- Since 1998, this team gathered more than 1000 observations of MMF. Biopsies were done 65 months in average between the inoculation of an adjuvanted vaccine **in the deltoïd**.

Gherardi RK, Authier FJ. Macrophagic myofasciitis: characterisation and pathophysiology. Lupus 2012; 21: 184-89.

Table 1 – Summary of reported clinical symptoms [12]

Symptoms	Percentage of patients
Myalgias	88–91
Arthralgias	57–68
Marked asthenia	55
Muscle weakness	45
Fever	20–32
Elevated CK levels	29–50
Increased ESR	37
Myopathic EMG	35
Demyelinating CNS disorder	9
Multiple sclerosis diagnosis	33
Chronic fatigue	50–93
Hashimoto's thyroiditis	2.7
Other autoimmune-related diseases (RA, Sjogren)	6.7

Latest aluminium vaccine received before diagnosis.

Vaccine	N	%
Vaccine against hepatitis B	289	69.3
Vaccine against tetanus	21	5.0
Revaxis®	11	2.6
Vaccine against hepatitis A	11	2.6
Vaccine against hepatitis B + Vaccine against hepatitis A	8	1.9
Vaccine against hepatitis B + Vaccine against tetanus	2	0.5
Vaccine against hepatitis B + Tetravac®	1	0.2
Vaccine against hepatitis A + Revaxis®	1	0.2
Infanrix®	1	0.2
Gardasil®	1	0.2
Tetravac®	1	0.2
HPV Vac	1	0.2
Not specified	**69**	**16.5**
Total	**417**	**100.0**

Macrophagic myofasciitis controversy

- An epidemiological study was set by the French Ministry of Health (2002 – 3):
  - MMF is an indisputable histological entity whose association with aluminium used as an adjuvant in vaccines is recognized.
  - But case-control study demonstrated there was no specific clinical entity could be demonstrated to be related with the use of aluminium adjuvanted vaccine.
- In 2004, the Global Advisory Committee on Vaccine Safety (WHO) reviewed the data of the case–control study performed in France and also concluded that " **the persistence of aluminium-containing macrophages at the site of a previous vaccination is not associated with specific clinical symptoms or disease ".**

Global Advisory Committee on Vaccine Safety. - Weekly Epidemiological Record . 1999; 41: 337-40. - WER. 2002; 77: 389-404. - WER. 2004; 79 : 3 – 20.

MMF suggested pathogenesis

Several successive hypothesis were challenged :

- 1st (1998): diffuse muscle disease,
- 2nd (1999): autoimmune process related with aluminium,
- 3rd (2001): Chronic fatigue syndrome,
- 4th (2009) : Direct cerebral toxicity due to aluminium nanoparticles,
- 5th (2011) : ASIA syndrome (autoimmune?),
- 6th (2013) : cerebral toxicity due to aluminium loaded monocytes
- …

Chronic Fatigue Syndrom (CFS)

1. **Unexplained, persistent fatigue** present for ≥ 6 months that is not substantially relieved by rest, is of new onset (not lifelong) and results in a significant reduction in previous levels of activity.
2. **Four or more of the following symptoms** are present for six months or more:
   - Impaired memory or concentration,
   - Postexertional malaise (extreme, prolonged exhaustion and sickness following physical or mental activity),
   - Unrefreshing sleep,
   - Muscle pain,
   - Multijoint pain without swelling or redness,
   - Headaches of a new type or severity,
   - Sore throat that's frequent or recurring,
   - Tender cervical or axillary lymph nodes.

CDC. Chronique fatigue syndrome. A toolkit for provider.

From MMF to CFS

The GERMMAD worked to demonstrate that MMF was associated with neuropsychogical problems linked to cerebral lesion.

Experimental studies were done:

- In mice, 1 year after IM injection of aluminium containing vaccine, Al deposits are detected in brain. Injected labelled materials can be followed up to brain, suggesting that macrophages loaded with nanoparticles are able to migrate and to access to cerebral area especially grey matter.
- In altered blood-brain barrier mice (BBB), they demonstrated a progressive diffusion into the cerebral grey matter suggesting an active process via macrophages containing nanoparticles that cross the blood-brain barrier : "Trojan horse" mechanism.
- In CCL2 deficient mice, substitution modulates the cerebral intake of aluminium labelled particles captured by monocytes.

Khan Z and al. Slow CCL2-dependent translocation of biopersistent particles from muscle to brain. BMC Medecine 2013 ; 11: 99

Pointed out uncertainties

- **Cerebral impairment does not explain all the symptoms which the MMF patients suffered.**
- Al-Rho particles are not representative of the aluminium used as a vaccine adjuvant.
- the experiment conducted in mice whose BBB integrity is deficient shows that increased permeability can amplify the phenomenon but does not prove that BBB is involved in the translocation.
- In the MCP-1 deficient mice loss experiment, it is not possible to ascertain whether the loss of function is linked to the primary recruitment of macrophages in the injected muscle and/or their translocation towards the brain and other organs.
- the presence of aluminium inclusions in the CNS does not signify the automatic existence of a "neurotoxicity".

The Haut Conseil de la santé publique

- **“Deems that the scientific data available today do not allow the safety of vaccines containing aluminium to be called into question with regard to their risk/benefit balance,**
- **Recommends the continuation of vaccinations according to the vaccine schedule in force,**
- **Warns of the consequences, in terms of the reappearance of infectious diseases, that could result from a decrease in vaccine coverage due to aluminium-containing vaccines being called into question without any scientific justification,**
- **Encourages the pursuit of research that aims to evaluate the safety of adjuvants that are available and in development”.**

Landon James Watt

From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Sunday, 23 February 2014 6:01 p.m.
To: SAHINOVIC, Isabelle; rpless2@gmail.com; Robert.Pless@phac-aspc.gc.ca; Helen Petousis-Harris; mew2@cdc.gov; ZUBER, Patrick Louis F.; jbeytout@chu-clermontferrand.fr
Cc: 阿部 圭史(abe-keishi); 難波江 功二(nabae-koji)
Subject: HPV vaccine conf call Follow-up

Dear all,

Thank you so much for your time and commitment. The conference call was very useful for us.

I talked to my boss and we agree that it is better not to have WHO GACVS presence during the public hearing session [redacted] and there is no need to hurry for a statement. We are hoping the statement to come out a week or two weeks later so that our expert committee can refer to it when they finalize the report in March (or a bit later) (if things go smoothely).

Thank you so much for your help.

I look forward to meeting and talking to you later.

Warm regards,

Koji Nabae

Landon James Watt

From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Thursday, 20 February 2014 2:48 a.m.
To: Helen Petousis-Harris; 'Robert Pless'; Robert Pless (Robert.Pless@phac-aspc.gc.ca); ZUBER, Patrick Louis F.; Wharton, Melinda (CDC/OID/NCIRD)
Cc: 難波江 功二(nabae-koji); 阿部 圭史(abe-keishi)
Subject: HPV vaccine Public Hearing meeting in Japan 26 Tokyo
Attachments: Annotated Agenda 26 Feb 2014.docx; [redacted]

Dear Helen, Rob, Melinda and Patrick,

Thank you so much for your assistance.

Attached please find a draft annotated agenda of the HPV vaccine safety meeting in Tokyo.

[redacted]

Melinda, we were thinking of inviting an expert from USCDC CISA group and asked for your support for this,
but given that there will be no presence of Dr Lucija Tomljenovic (vasculopathy), and time limitation,
we are now thiking of asking a WHO GACVS member (Rob?) to cover both MMF and DNA issues in
addition to Prof Beytou and Helen, if possible. May I ask for your suggestion whether we better still
ask CISA member (probably Dr. Philip LaRussa) to join?

Thank you so much for your valuable support. It has been extremely helpful for us.

Warmest regrads,

Koji Nabae
Deputy Director
Division of Tuberculosis and Infectious Disease Control
Ministry of Health, Labour & Welfare
Government of Japan-
Tel: [redacted]
Fax: +81-3-3581-6251
email: nabae-koji@mhlw.go.jp

From: Helen Petousis-Harris [mailto:h.petousis-harris@auckland.ac.nz]
Sent: Tuesday, February 18, 2014 5:19 AM
To: 'Robert Pless'
Cc: Robert Pless (Robert.Pless@phac-aspc.gc.ca); 難波江 功二(nabae-koji); ZUBER, Patrick Louis F.; Wharton, Melinda (CDC/OID/NCIRD)
Subject: RE: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

Dear Rob

Oh dear! I am so saddened to hear how extensive the impact of Lee, Shaw and Tomljenovic's activities has become.

I will certainly do anything I can to assist. To the best of my knowledge the rebuttal on our website is the only attempt to address this particular issue which Shaw and Lee presented at a coronal enquiry here. Placing the rebuttal in the public domain was the only means of providing the information to the crown representatives involved in that process at the 11th hour. Prof David Gorsky has written prolifically on some of the experiments in his science blog over the past few years so I assume he has also given the material some thought.

I do not know if I am expert on this but certainly have some experience in considering aluminium in vaccines and its role in inflammatory responses and local AEFI as part of my PhD some years ago. I assume you are referring to the VLP tightly bound to the adjuvant and the Shaw and Tomljenovic 'hypothesis' that it somehow finds its way to the brain carried by macrophage?

A phone call would probably be useful. It is a little after 9am in NZ.

Kind regards.

Helen

Helen Petousis-Harris. PhD, MRSNZ
Senior Lecturer, General Practice and Primary Health Care
Director of Immunsiation Research and Vaccinology
Immunisation Advisory Centre
University of Auckland
DDI +64 9 923 2078 Fax 9 3737030 Mob [redacted]
Building 734, Level 3, Tamaki Campus, Morrin Rd, Glen Innes
Private Bag 92019, Victoria St West, Auckland 1142, New Zealand

From: Robert Pless [mailto:rpless2@gmail.com]
Sent: Tuesday, 18 February 2014 6:20 a.m.
To: Helen Petousis-Harris
Cc: Robert Pless (Robert.Pless@phac-aspc.gc.ca); "難波江 功二(nabae-koji)"; ZUBER, Patrick Louis F.; Wharton, Melinda (CDC/OID/NCIRD)
Subject: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

Dear Dr. Petousis-Harris,

I am writing you with an urgent request outlined below, having read the Immunization Advisory Centre's Commentary on coronial inquiry expert witness testimony that was prepared in response to allegations by Chris Shaw and Sing Hang Lee. I am a current member of the WHO Global Advisory Committee on Vaccine Safety and am

writing in part on behalf of Dr. Koji Nabae of the Japanese Ministry of Health, for assistance. The GACVS has been looking at this issue from the global perspective and have released several statements over the last two years to address concerns around aluminum and autoimmune disease.

As you may be aware, there have been ongoing concerns in Japan regarding the HPV vaccine, where cases of chronic pain and complex regional pain syndrome allegedly linked to the vaccine have led to a partial suspension of their national vaccination program and there has been a great deal of public interest. An expert advisory group has met several times but have not reached a conclusion about the restart of the program. A meeting has recently been organized in Tokyo for February 26th, where Dr. Lee will present his findings. It is likely that Dr. Shaw's co-investigator, Lucija Tomljenovic will be present as well. There will be a second presentation on Macrophagic Myofasciitis and the HPV vaccine, a stretch of the MMF story first related to the hepatitis B vaccine.

We are seeking your advice on someone who may be able to address the more detailed questions around HPV DNA - specifically the hypotheses you have address in your statement regarding the alleged role of aluminum binding to DNA fragments and subsequent effects. While the issue of whether the fragments constitute "contamination" has been dealt with, your statement was the only one to address the more obscure alleged consequences of the presence of those fragments. The GACVS has not yet had a chance to delve into the DNA question.

While we appreciate the short notice, the meeting and even the date were very recently confirmed. That said, the ideal would be someone who would be available to travel to the meeting in Japan and address the issue as it arises in person. Please let me know if I can clarify anything by phone, and indeed if this is at all possible and who could be contacted and provided with further details.

Best regards,
Rob

==========

Robert Pless, MD, MSc
Medical Advisor
Health Security Infrastructure Branch
Public Health Agency of Canada
Ottawa, Ontario K1A 0K9
Tel./Mobile: [redacted]
Email: robert.pless@phac-aspc.gc.ca

Landon James Watt

From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Friday, 21 February 2014 11:05 p.m.
To: Robert Pless; Helen Petousis-Harris; ZUBER, Patrick Louis F.; jbeytout@chu-clermontferrand.fr; Wharton, Melinda (CDC/OID/NCIRD); Koji Nabae (k-nabae-@nifty.com); 阿部 圭史(abe-keishi); Robert Pless
Subject: RE: (FYI) HPV vaccine international sympo on 25 Feb in Tokyo
Attachments: GACVS Statement HPV Feb 2014 discussion draft.docx; Annotated Agenda 26 Feb 2014.docx; Participants List.docx

Dear Rob,

Thank you so much for the excellent work you and your colleagues have done.
It sounds very strong. It is indeed very helpful.

I made minor comments on the attached file.

===

For the conference call today, there will be 4 participants from Japan.

Koji Nabae (Ministry of Health, Labour and Welfare (MHLW)) Keishi Abe (MHLW) Ichiro Kurane (Chair of the public hearing session, Deputy Director General of National Institute of Infectious Diseases(NIID)) Dr Hiroshi Yoshikura (Former DG of NIID)

In case you wish to discuss GACVS statement only among GACVS members, please let me know so that we will join you later.

==

Attached please find the draft annotated agenda and participant list of the public heating meeting.

I look forward to talking to you soon.

Warm regards,

Koji
Deputy Director
Division of Tuberculosis and Infectious Disease Control Ministry of Health, Labour & Welfare Government of Japan-
Tel: [redacted]
Fax: +81-3-3581-6251
email: nabae-koji@mhlw.go.jp

-----Original Message-----
From: Robert Pless [mailto:rpless2@gmail.com]
Sent: Friday, February 21, 2014 4:19 PM
To: Helen Petousis-Harris; 難波江 功二(nabae-koji); ZUBER, Patrick Louis F.; jbeytout@chu-clermontferrand.fr; Wharton, Melinda (CDC/OID/NCIRD); Koji Nabae (k-nabae-@nifty.com); 阿部 圭史(abe-keishi); Robert Pless
Subject: Re: (FYI) HPV vaccine international sympo on 25 Feb in Tokyo

Dear all,

Attached please find a draft GACVS statement for review. We can discuss it tomorrow (actually in a few hours) and then it would go through vetting by the committee if the feeling remains that it should be posted in advance of the events of next week.

I propose the following topics for discussion on our call:

1. Introductions
2. Current situation in Japan with respect to the signal 3. Origins of the 2 meetings being held next week and potential outcomes 4. Planned and likely topics that may arise by the speakers (MMF, HPV DNA, …other) 5. Responses during the meeting on the 26th (invited experts, Ministry, Expert advisory group) 6. Format and timing of responses outside the meetings (GACVS statement, follow up statements?) 7. Other interventions?
8. Other issues

Please feel free to add/alter
Looking forward to getting together on the phone, Rob

Participant list

Date & Time: 10:00～11:00,26Feb,2014

Venue : Koku Kaikan,Shinbashi,Tokyo Japan（7F)

【Expert】

・**Dr.Ken Ishii**　National Institute of Biomedical Innovation
Adjuvant development project leader

・**Dr.Takashi Inamatsu**　Advisor of Tokyo Metropolitan Geriatric Hospital and Institute of gerontology

・**Dr.Kenji Okada**　Prof.Fukuoka Dental College pediatrics

・**Dr.Nobuhiko Okabe**　Director of Kawasaki Health Security Research Institute

○・**Dr.Ichiro Kurane**　Vice-director of The National Institute of Infectious Diseases

・**Dr.Tetsuo Nakayama**　Prof. Kitasato Institute for Life Sciences Graduate School of Infection Control Immunology section, Infection control research

・**Dr.Mariko Momoi**　Vice-director of International University of Health and Welfare

・**Dr.Hiroshi Yoshikura**　Former Director of The National Institute of Infectious Diseases/Former Prof. Tokyo university school of Medicine

○ : Chairman

【Presenters】

・**Dr.Harumi Sakai**　Former Prof.Tokai University school of medicine

・**Dr. Sin Hang Lee**【US】　Milford Hospital pathologist

・**Dr. Francois-Jerome Authier**【ＦＲ】　Henry mon Dole Hospital doctor

【Designated speakers(Experts)】

・**Dr. Jean Beytout**　Head of the Infectious and Tropical Diseases Ward at the Clermont teaching hospital / Member of the anti-infectious agents working party

・**Dr. Helen Petousis-Harris**【ＮＺ】　Director of Immunisation Research and Vaccinology, Immunisation Advisory Centre Senior Lecturer, Dept. General Practice and Primary Health Care, University of Auckland

・**Dr. Robert Pless**【ＣＡ】　Medical Advisor Health Security Infrastructure Branch Public Health Agency of Canada / Member of ＷＨＯ・GACVS

※Dr.Helen Petousis-Harris and Dr. Robert Pless will participate in Video meeting.

Landon James Watt

From: Robert Pless <rpless2@gmail.com>
Sent: Friday, 21 February 2014 8:19 p.m.
To: Helen Petousis-Harris; nabae-koji@mhlw.go.jp; ZUBER, Patrick Louis F.; jbeytout@chu-clermontferrand.fr; Wharton, Melinda (CDC/OID/NCIRD); Koji Nabae (k-nabae-@nifty.com); "阿部 圭史(abe-keishi)"; Robert Pless
Subject: Re: (FYI) HPV vaccine international sympo on 25 Feb in Tokyo
Attachments: GACVS Statement HPV Feb 2014 discussion draft.docx

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8. Other issues

Please feel free to add/alter
Looking forward to getting together on the phone, Rob

Landon James Watt

From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Tuesday, 25 February 2014 1:56 p.m.
To: Helen Petousis-Harris
Subject: RE: Doc and Video Conf
Attachments: NZ Public hearing session on HPV safety.pptx

Fantastic!! Very strong and convincing. Many many thanks!
It think there is no need for further explanation since your slides tell all the story.

One thing I came up to my mind,

- In addition, the immune activation on uptake of HPV vaccine does not include an increase in inflammatory factors (incl TNF) even in vaccinees with large injection site reactions at time of local inflammation.

In our previous meeting, one expert presented his studies on mice,
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Grateful for your confirmation!!

Best regards,

Koji

From: Helen Petousis-Harris [mailto:h.petousis-harris@auckland.ac.nz]
Sent: Tuesday, February 25, 2014 8:03 AM
To: 難波江 功二(nabae-koji)
Subject: RE: Doc and Video Conf

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We look forward to talking to you again soon.

Warm regards,

Koji Nabae

Public hearing session on HPV safety.
Tokyo, Japan 26 February 2014

Helen Petousis-Harris. PhD
Director of Immunisation Research and Vaccinology
Immunisation Advisory Centre
The University of Auckland
Convenor, NZ Expert Advisory Group on Vaccine Safety

THE UNIVERSITY OF AUCKLAND
FACULTY OF MEDICAL AND HEALTH SCIENCES

Overview

- Dr Lee claims to have tested vials of Gardasil vaccine for the presence of vaccine derived HPV L1 gene DNA sequences and to have detected it.
- Dr Lee claims to have tested post mortem samples from a single person for the presence of HPV DNA. He claims they are positive for HPV DNA bound to the vaccine adjuvant
- Dr Lee supports that the HPV vaccine may have caused death due to a complex of vaccine constituents
- The Immunisation Advisory Centre at The University of Auckland **strongly questions both his methods, interpretation and conclusions**

Testing of Gardasil samples - concerns

- The vaccine is be expected to contain traces of vaccine type HPV L1 gene DNA – consistent with manufacturing process.
- However:
  1. The tests used have never been validated – methods are not transparent
  2. DNA contamination can be tested by most laboratories – not ‘cutting edge’
  3. PCR extremely sensitive, nested PCR much more so. High risk of amplifying other DNA sequences
  4. Use of degenerate primers increases sensitivity but also increases risk of amplifying other DNA sequences (decreases specificity) wildcard given that the genetic code of the vaccine L1 gene is known this is a strange thing to do.

Testing of post mortem samples - concerns

- **There are no controls used (unvaccinated). This is a <u>vital</u> part of the scientific process**
- Inclusion of degenerate primers again
  - Over sensitive, increased risk of amplifying other irrelevant DNA (junk) + reduced specificity for vaccine type HPV L1 gene
- Why did Lee not include primers for the plasmid and promoter sequences which would provide more robust evidence for the presence of vaccine HPV DNA.
- Has not proven that the DNA is of vaccine origin (no plasmid, no yeast)
- The amount of residual DNA in the vaccine is miniscule
  - How can such a small amount of DNA detected dispersed through out body tissues?
- It is not biologically possible for the HPV DNA to integrate into the host genome (does not have the necessary sequences)

Extraordinary hypotheses

- Dr Lee supports hypotheses whereby HPV DNA has bound irreversibly to aluminium adjuvant (theory), been taken up by macrophage (theory) and caused an inflammatory response (theory) leading to death (theory).
- He contends that these complexes can be detected in post mortem tissue samples and that they were carried there by the macrophage
- He has little if any evidence for this hypothesis, which, if it were true, needs to be tested using rigorous scientific methods.
  1. Convincing proof of binding of vaccine DNA to the adjuvant
  2. Convincing proof of carriage by macrophage
  3. Convincing proof of deposition in tissues (especially brain)
  4. Convincing proof of inflammatory immune cascade including TNF

- No one else has replicated the findings
- The findings are not supported by existing extensive research on the immunology or epidemiology of HPV vaccine safety.

Immune response following vaccination with protein-based vaccine

- After injection immune cells such as macrophage take up vaccine (adjuvant and antigen) **at injection site**
- The cells become activated and migrate via the lymph to **local lymph node** (not spleen)
- Half life of a macrophage ~6 days
- These facts do not support the potential for presence of adjuvant/HPV DNA in either blood or spleen
- In addition, the immune activation on uptake of HPV vaccine does not include an increase in inflammatory factors (incl TNF) even in vaccinees with large injection site reactions at time of local inflammation.

The NZ case

- This type of death is often caused by underlying cardiac abnormalities.
  - Testing was refused by the family
- There was no evidence of inflammation in the autopsy results for the NZ case
  - including either the brain or the heart

Key question and answer

- Does Gardasil trigger death?
  - **No**
  - Large epidemiological studies and routine surveillance systems conducted globally directly investigating this find no difference between vaccinated and unvaccinated population (refs provided)
  - If it did then we would see a higher rate of cases in vaccinated people, and this has not been observed.
  - Data for over 100 million doses to date

Extraordinary claims require extraordinary evidence

Carl Sagan 1934-1996

Selected references on safety of Quadrivalent Human Papillomavirus Vaccine

- Food and Drug Administration. Clinical Review of Biologics License Application for Human Papillomavirus 6, 11, 16, 18 L1 Virus Like Particle Vaccine (S. cerevisiae) (STN 125126 GARDASIL), manufactured by Merck, In: Vaccines Clinical Trial Branch, Centre for Biologics Evaluation and Research, editor.: Food and Drug Administration.; 2006.
- Garland SM, Hernandez-Avila M, Wheeler CM, Perez G, Harper DM, Leodolter S, et al. Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases.[see comment]. N Engl J Med. 2007;356(19):1928-43.
- Villa LL, Costa RL, Petta CA, Andrade RP, Ault KA, Giuliano AR, et al. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. Lancet Oncology. 2005;6(5):271-8.
- Future II Study Group. Prophylactic efficacy of a quadrivalent human papillomavirus (HPV) vaccine in women with virological evidence of HPV infection.[see comment]. J Infect Dis. 2007;196(10):1438-46.
- Arnheim-Dahlström L, Pasternak B, Svanström H, Sparén P, Hviid A. Autoimmune, neurological, and venous thromboembolic adverse events after immunisation of adolescent girls with quadrivalent human papillomavirus vaccine in Denmark and Sweden: Cohort study. BMJ. 2013;347(f5906).
- Callréus T, Svanström H, Nielsen NM, Poulsen S, Valentiner-Branth P, Hviid A. Human papillomavirus immunisation of adolescent girls and anticipated reporting of immune-mediated adverse events. Vaccine. 2009;27(22):2954-8.
- Chao C, Klein NP, Velicer CM, Sy LS, Slezak JM, Takhar H, et al. Surveillance of autoimmune conditions following routine use of quadrivalent human papillomavirus vaccine. J Intern Med. 2012;271(2):193-203.
- Klein NP, Hansen J, Chao C, et al. Safety of quadrivalent human papillomavirus vaccine administered routinely to females. Arch Pediatr Adolesc Med. 2012;166(12):1140-8.
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- Siegrist C-A. Autoimmune diseases after adolescent or adult immunization: What should we expect? CMAJ. 2007;177(11):1352-4.
- Slade BA, Leidel L, Vellozzi C, Woo EJ, Hua W, Sutherland A, et al. Postlicensure Safety Surveillance for Quadrivalent Human Papillomavirus Recombinant Vaccine. JAMA. 2009;302(7):750-7.

Landon James Watt

From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Tuesday, 25 February 2014 3:10 p.m.
To: Helen Petousis-Harris
Subject: RE: Doc and Video Conf

Hi helen,

Please try with the following site.

Meeting Number: 862 540 421
https://mhlw-web.webex.com/mw0307l/mywebex/default.do?service=1&siteurl=mhlw-web-en&nomenu=true&main_url=%2Fmc0806l%2Fmeetingcenter%2Fmeetinginfo%2Fmeetinginfo.do%3Fsiteurl%3Dmhlw-web-en%26confID%3D1619492336%26Action%3DWMI%26MTID%3Dm1919d8b290c2b43a2d0c4d8eebba5bab%26FrameSet%3D2%26Host%3Dcc3c242909564b46%26UID%3D0

Password (if required): 100%KOJI

From: Helen Petousis-Harris [mailto:h.petousis-harris@auckland.ac.nz]
Sent: Tuesday, February 25, 2014 11:07 AM
To: 難波江 功二(nabae-koji)
Subject: RE: Doc and Video Conf

Hi Koji
I have finished the teleconference so free to figure out the technology
Kind regards
Helen

From: 難波江 功二(nabae-koji) [mailto:nabae-koji@mhlw.go.jp]
Sent: Tuesday, 25 February 2014 2:12 p.m.
To: Helen Petousis-Harris
Subject: RE: Doc and Video Conf

Thanks!!!

From: Helen Petousis-Harris [mailto:h.petousis-harris@auckland.ac.nz]
Sent: Tuesday, February 25, 2014 10:11 AM
To: 難波江 功二(nabae-koji)
Subject: RE: Doc and Video Conf

…yes, this was measured in human serum the day after vaccination – when the innate immune response and macrophages are at their busiest.

From: 難波江 功二(nabae-koji) [mailto:nabae-koji@mhlw.go.jp]
Sent: Tuesday, 25 February 2014 2:06 p.m.
To: Helen Petousis-Harris
Subject: RE: Doc and Video Conf

Great!! I understand this is in human serum. We will set the slides as I sent in my previous mail (change red color to black in page 2).
Thanks!!

Koji

From: Helen Petousis-Harris [mailto:h.petousis-harris@auckland.ac.nz]
Sent: Tuesday, February 25, 2014 10:02 AM
To: 難波江 功二(nabae-koji)
Subject: RE: Doc and Video Conf

Great!

Actually that is my own work, We have conducted a clinical trial using Gardasil vaccine. We specifically examined the reactogenicity of the vaccine and associations with 27 cytokines inlc TNF and IL1, all the main players. There was no elevation of any cytokine associated with reactogencity. I have it on a list to publish and it had been peer reviewed in a PhD thesis which is available in the University Library and the data is available for scrutiny.

From: 難波江 功二(nabae-koji) [mailto:nabae-koji@mhlw.go.jp]
Sent: Tuesday, 25 February 2014 1:56 p.m.
To: Helen Petousis-Harris
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One thing I came up to my mind,

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Sent: Tuesday, 25 February 2014 2:00 p.m.
To: Helen Petousis-Harris
Subject: RE: Doc and Video Conf

Sorry, Helen, our tech person is late in coming (he said he just woke up since he was working till early morning…) We will contact you after 3pm.
sorry

koji

From: Helen Petousis-Harris [mailto:h.petousis-harris@auckland.ac.nz]
Sent: Tuesday, February 25, 2014 8:06 AM
To: 難波江 功二(nabae-koji)
Subject: RE: Doc and Video Conf

Re the testing

I have organised a short teleconference with our vaccine safety group at 2.30 nz time. Hopefully we can do the testing before that (2.00-2.30) and follow up any issues after 3pm. It will be good to get comments from the safety group, they are very wise ☺.

Look forward to talking with you
Kind regards
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From: ZUBER, Patrick Louis F. <zuberp@who.int>
Sent: Thursday, 27 February 2014 4:07 a.m.
To: 難波江 功二(nabae-koji); Robert Pless; SAHINOVIC, Isabelle; Robert Pless; mew2@cdc.gov; Helen Petousis-Harris; jbeytout@chu-clermontferrand.fr; LAMBACH, Philipp
Cc: 阿部 圭史(abe-keishi); ONDARI, Clive
Subject: RE: HPV mtgs in Japan

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With best wishes,

Patrick

From: 難波江 功二(nabae-koji) [mailto:nabae-koji@mhlw.go.jp]
Sent: 26 February 2014 15:31
To: Robert Pless; SAHINOVIC, Isabelle; Robert Pless; mew2@cdc.gov; h.petousis-harris@auckland.ac.nz; ZUBER, Patrick Louis F.; jbeytout@chu-clermontferrand.fr; LAMBACH, Philipp
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From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Tuesday, 4 March 2014 2:21 a.m.
To: ZUBER, Patrick Louis F.; Robert Pless; SAHINOVIC, Isabelle; Robert Pless; mew2@cdc.gov; Helen Petousis-Harris; jbeytout@chu-clermontferrand.fr; LAMBACH, Philipp
Cc: 阿部 圭史(abe-keishi); ONDARI, Clive
Subject: RE: HPV mtgs in Japan
Attachments: HPV meeting summary_eng_02262014.pdf

Dear Colleagues,

Attached please find a brief summary of the HPV meetings that took place on 26 Feb.

Many thanks!

Koji

From: ZUBER, Patrick Louis F. [mailto:zuberp@who.int]
Sent: Thursday, February 27, 2014 12:07 AM
To: 難波江 功二(nabae-koji); Robert Pless; SAHINOVIC, Isabelle; Robert Pless; mew2@cdc.gov; h.petousis-harris@auckland.ac.nz; jbeytout@chu-clermontferrand.fr; LAMBACH, Philipp
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From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Thursday, 27 February 2014 5:15 a.m.
To: ZUBER, Patrick Louis F.; Robert Pless; SAHINOVIC, Isabelle; Robert Pless; mew2@cdc.gov; Helen Petousis-Harris; jbeytout@chu-clermontferrand.fr; LAMBACH, Philipp
Cc: 阿部 圭史(abe-keishi); ONDARI, Clive
Subject: RE: HPV mtgs in Japan

Great!

The meeting documents have been posted on the following website.
http://www.mhlw.go.jp/stf/shingi/0000038484.html

cheers,

Koji

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Sent: Thursday, 27 February 2014 6:03 a.m.
To: ZUBER, Patrick Louis F.; Robert Pless; SAHINOVIC, Isabelle; Robert Pless; mew2@cdc.gov; Helen Petousis-Harris; jbeytout@chu-clermontferrand.fr; LAMBACH, Philipp
Cc: 阿部 圭史(abe-keishi); ONDARI, Clive
Subject: RE: HPV mtgs in Japan

Additional TV coverage
http://www.fnn-news.com/news/headlines/articles/CONN00263818.html

Koji

From: 難波江 功二(nabae-koji)
Sent: Thursday, February 27, 2014 1:15 AM
To: 'ZUBER, Patrick Louis F.'; Robert Pless; SAHINOVIC, Isabelle; Robert Pless; mew2@cdc.gov; h.petousis-harris@auckland.ac.nz; jbeytout@chu-clermontferrand.fr; LAMBACH, Philipp
Cc: 阿部 圭史(abe-keishi); ONDARI, Clive
Subject: RE: HPV mtgs in Japan

Great!

The meeting documents have been posted on the following website.
http://www.mhlw.go.jp/stf/shingi/0000038484.html

cheers,

Koji

From: ZUBER, Patrick Louis F. [mailto:zuberp@who.int]
Sent: Thursday, February 27, 2014 12:07 AM
To: 難波江 功二(nabae-koji); Robert Pless; SAHINOVIC, Isabelle; Robert Pless; mew2@cdc.gov; h.petousis-harris@auckland.ac.nz; jbeytout@chu-clermontferrand.fr; LAMBACH, Philipp
Cc: 阿部 圭史(abe-keishi); ONDARI, Clive
Subject: RE: HPV mtgs in Japan

Many thanks Koji for the prompt feed-back,

We will assess with GACVS chairs the most desirable course of action taking into account your assessment and the fact the committee's final conclusion will come within a month or two.

With best wishes,

Patrick

From: 難波江 功二(nabae-koji) [mailto:nabae-koji@mhlw.go.jp]
Sent: 26 February 2014 15:31
To: Robert Pless; SAHINOVIC, Isabelle; Robert Pless; mew2@cdc.gov; h.petousis-harris@auckland.ac.nz; ZUBER, Patrick Louis F.; jbeytout@chu-clermontferrand.fr; LAMBACH, Philipp
Cc: 阿部 圭史(abe-keishi); 難波江 功二(nabae-koji)
Subject: HPV mtgs in Japan

Dear colleagues,

The two meetings on HPV vaccine in Japan today went very well.

I wish to express our sincere appreciation to all of you, particularly Prof Beytout who came all the way to Japan (in economy class!) and made an excellent and very convincing presentation (attached file) and Helen, despite the technical difficulty, generated a straightforward and punchy argument (attached file).

The media has already picked up the events. The tone is very neutral and some reports that these arguments were dismissed for lack of scientific evidence.

http://www.tv-tokyo.co.jp/mv/mplus/news/post_61006
http://www3.nhk.or.jp/news/html/20140226/k10015558551000.html

And I thank colleagues in WHO GACVS (Philipp, Patrick, Isabelle, Rob and Melinda) for your enormous support and encouragement. We really hope that our committee would come to a conclusion in a month or two and we can move forward with our immunization program.

I will send you a summary of the meeting today once ready. In the meantime, I attach an internal report of the symposium that took place yesterday (not for wider circulation, please keep it only among GACVS members).

Millions of thanks again and I look forward to talking to you soon.

Warmest regards,

Koji

Landon James Watt

From: Robert Pless <rpless2@gmail.com>
Sent: Monday, 24 February 2014 12:48 p.m.
To: "難波江 功二(nabae-koji)"
Cc: SAHINOVIC, Isabelle; Robert Pless; Helen Petousis-Harris; mew2@cdc.gov; ZUBER, Patrick Louis F.; jbeytout@chu-clermontferrand.fr; "阿部 圭史(abe-keishi)"
Subject: Re: HPV vaccine conf call Follow-up

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Best wishes for the coming week.
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These are all my thoughts – of course the GACVS secretariat and Chair would
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Thank you so much for your help.

I look forward to meeting and talking to you later.

Warm regards,

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From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Wednesday, 26 February 2014 4:41 a.m.
To: Robert Pless
Cc: SAHINOVIC, Isabelle; Robert Pless; Helen Petousis-Harris; mew2@cdc.gov; ZUBER, Patrick Louis F.; jbeytout@chu-clermontferrand.fr; 阿部 圭史(abe-keishi)
Subject: RE: HPV vaccine conf call Follow-up

Dear Helen and Robert,

Many thanks for your kind and warm remarks. We are very much encouraged.
We are hoping that there will be major media coverage over today's symposium.
Here is the brief summary we received from a participant.

- Symposium: there were about 60 people and 5 TV cameras.
- Press conference: there were about 30 people and 2 TV cameras.
- Symposium was originally planned as 3 hours but became 5 hours because of longer presentation than plan and consecutive interpretation.
- It was not very well organized e.g. Dr. Sakai chided Mr. Fukushima since he blended his opinion into his translation.
- Their arguments were those we expected based on their publications.
- Key words they repeatedly mentioned were Aluminum, Autoimmune disease, MMF, DNA.

I will keep you posted on tomorrow's development.

Warm regards,

Koji

From: Robert Pless [mailto:rpless2@gmail.com]
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To: 難波江 功二(nabae-koji)
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FYI, the following was circulated by SANEVAX. Koji

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Breaking News from Japan: International Symposium on the Adverse Reactions Experienced by those Vaccinated with Human Papillomavirus Vaccines
By Norma Erickson
Sanevax
February 24, 2014

Tuesday, February 25, 2:30 p.m. Tokyo time marks the beginning of an International Symposium on the adverse reactions experienced by girls who have been vaccinated by Human Papillomavirus vaccines. This symposium was organized on behalf of The Researchers' Organization Sounding a Warning concerning the Adverse Reactions induced by Human Papillomavirus Vaccines, through the collaborative efforts of Dr. Harumi Sakai, former Professor at the Tokai University School of Medicine, Dr. Shohei Matsuzaki, Professor Emeritus at the Tokai University School of Medicine, Mutsuo Fukushima, Kyoto News International Department, and SaneVax Inc.

This event will give a voice to the thousands of young women and families around the world who have suffered debilitating side effects, sometimes death, after using HPV vaccines. Many of these families have been told these events are psychosomatic or coincidental.

In fact on January 20, 2014, the Japanese government's advisory council released an official report quite typical of those issued in other countries in which they dismissed all of the symptoms that have shown up in the bodies of vaccinated girls as the consequences of psychogenetic "psychosomatic reactions" – in other words, the consequences of mental reactions of girls who the council suggested in a sophisticated manner may have been spoiled by mothers who do not know how to give discipline to their girls.

According to Kyoto News Reporter, Mutsuo Fukushima, the key proponent of this theory is Dr. Yutaka Ohno of Keio University, who has stated publicly:

"It is impossible to find physical causes for the alleged and presumed adverse reactions at those vaccinated girls, so we cannot help concluding that their so-called adverse reactions are the mere consequences of psychosomatic reactions. The government should provide counselling to the girls so that they may be freed from their psychosomatic reactions."

The organizers of this symposium, along with countless others, find this callous lack of concern for the victims of adverse reactions to HPV vaccines appalling. They want the world to know there are several biologically plausible mechanisms of action via which Gardasil and Cervarix could precipitate these events. They want the world to know what their research shows in the hope of halting HPV vaccination campaigns until these mechanisms of action are identified, and quantified, so those most at risk can be eliminated from any future HPV vaccination programs. Above all, they want the world to know there are those who will not give up working until the devastation following in the wake of mass HPV vaccination programs is stopped.

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    1. Complete information is available here.
2. **Press Conference** for reporters from newspaper companies and TV broadcasters – open to the public – February 25th from 5:45 to 7:30 p.m. – same venue as above
    1. Dr. Lee, Professor Authier, Lucija Tomljenovic PhD, Dr. Sasaki, Dr. Shiozawa, Dr. Uhide Kiyoshi, Dr. Hama and Mutsuo Fukushima will be available to answer questions from the public and the press.
3. **Government-Sponsored Public Hearing** of the Health Ministry's Advisory Council for the Deliberations on the Reported Adverse Events of HPV Vaccines, the advisory panel consisting of 15 scientists – February 26th, 10:00 to 11:30 a.m. (Evidence to be presented by scientists

and medical professionals from the United States, Canada, France and Japan regarding potential mechanisms of action between HPV vaccines and serious adverse events.)

4. **Briefing on HPV Matters to Influential Lawmakers** of the Ruling Liberal Democratic Party (LDP) – February 26th from 12:00 to 1:30 p.m. – at the room of Chairman Eriko Yamatani of the LDP Policy Deliberations Committee at the House of Councilors in the upper chamber of Japan's bicameral parliament.
5. **Press Conference** – February 26th beginning at 5:45p.m. In the press room of the Japanese Health Ministry.

Symposium Participants:

- Organizer: Harumi Sakai, MD, former Professor, Tokai University School of Medicine
- Co-organizer: Shohei Matsuzaki, MD, Professor Emeritus, Tokai University School of Medicine
- Interpreter: Mutsuo Fukushima, Reporter, Kyodo News, International Department
- Sin Hang Lee, MD, former Yale University Associate Professor, Pathologist at Milford Hospital, Director of Milford Medical Laboratory, Inc.
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How and why was this symposium organized?

Early in September 2013, the Secretary General of the Nationwide Liaison Association of Cervical Cancer Vaccine Victims and Parents in Japan, and Mr. (Francis) Mutsuo Fukushima, journalist with the Kyodo News, informed the SaneVax team that there were rumors of a delegation from the Japanese Ministry of Health planning to visit London in October to have discussions on the HPV vaccines with officials in the UK Department of Health and the Medicines and Healthcare Products Regulatory Agency (MHRA). It was thought that this delegation may be willing to speak with scientific and medical experts who were independent of pharmaceutical industry ties. Knowing what this delegation would hear from the official sources, the SaneVax Team felt it would be critical for this delegation to speak with medical and scientific professionals who had no ties to the pharmaceutical industry.

Prior to official confirmation of this meeting, Norma Erickson, President of SaneVax Inc. and Freda Birrell, Secretary provided their new contacts with information relating to many cases of young girls who had also been harmed by the HPV vaccines, Cervarix and Gardasil, in other parts of the world. The adverse events being reported in Japan were almost identical to those being reported in every country where these vaccines were administered. This fact alone seemed to indicate that the adverse events should not be regarded as a coincidence. The entire SaneVax Team thought it critical to demonstrate that this was a global problem. The young women of Japan were not the only ones suffering after HPV vaccinations. The more information sent to Japanese contacts, the more everyone agreed that independent experts had to be heard.

Following many discussions, SaneVax obtained confirmation from Mr. Tetsuya Miyamoto, Director of the Office of Vaccination Policy at the Ministry of Health, Labor and Welfare's Health Policy Bureau and a qualified medical doctor in his own right, that he was leading a team embarking on a fact finding mission regarding HPV vaccines. He indicated that he and his team would be willing to meet with independent scientists and a doctor from London on Monday, 7th October 2013 at the Double Tree by Hilton Hotel in London.

This meeting was scheduled to begin at 2 pm and last for 2 hours. His team consisted of first class medical doctors from two of the six university hospitals in Japan which had been entrusted with official research efforts to shed light on potential cause-and-effect relationships between HPV vaccines and adverse effects being experienced by young girls in Japan.

Those attending this very important meeting on behalf of SaneVax were: Dr. Sin Hang Lee, MD, Pathologist, Milford Hospital, Director, Milford Medical Laboratory Inc., and former associate professor of pathology at Yale University; Professor Francois Jerome Authier, MD, PhD, Reference Center for Neuromuscular Disorders, Henri Mondor Hospital, Paris and Dr. Damien Downing, MB BS, MIBiol from London who is a pioneer of Ecological Medicine. Also in attendance at this meeting were

Mrs. Freda Birrell, Secretary of SaneVax Inc. and her husband David Birrell, VAERS Research Analyst for SaneVax Inc.

Our team in London was treated with the greatest of respect by Mr. Miyamoto and his fellow doctors. They listened attentively to all that was said and watched diligently the excellent power point presentations – stopping many times to ask very important questions. A Japanese interpreter was also in attendance.

At that time, we understood that there would be the opportunity for a further meeting organized by SaneVax to be held in Washington, DC when the Japanese delegation visited officials from the FDA and the CDC. The Japanese delegation did visit Washington, later than expected because of the breakdown in the U.S. government administration. There was no opportunity for a second meeting with SaneVax representatives to take place.

Therefore, the London meeting became very important as it has proven to be the catalyst for the historic events now scheduled to take place in Japan on the 25th and 26th February 2014.

For the first time in the history of public immunization campaigns, government officials are willing to listen to the voices of truly independent scientists and medical professionals regarding vaccination policy and immunization practices. This is something that should have happened decades ago. Perhaps the meetings in Japan will herald the beginning of a new medical paradigm.

At the very least, the meetings will provide a voice for thousands of young people and their families who are having to cope with the sometimes debilitating effects of HPV vaccination use.

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Dear all,

Thank you so much for your time and commitment. The conference call was very useful for us.

I talked to my boss and we agree that it is better not to have WHO GACVS presence during the public hearing session [redacted] and there is no need to hurry for a statement. We are hoping the statement to come out a week or two weeks later so that our expert committee can refer to it when they finalize the report in March (or a bit later) (if things go smoothely).

Thank you so much for your help.

I look forward to meeting and talking to you later.

Warm regards,

Koji Nabae

Landon James Watt

From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Tuesday, 25 February 2014 9:09 p.m.
To: Robert Pless; SAHINOVIC, Isabelle; Robert Pless; Helen Petousis-Harris; mew2 @cdc.gov; ZUBER, Patrick Louis F.; jbeytout@chu-clermontferrand.fr
Cc: 阿部 圭史(abe-keishi); 難波江 功二(nabae-koji)
Subject: RE: HPV vaccine conf call Follow-up

Dear Colleagues,

FYI, the following was circulated by SANEVAX. Koji

====

Breaking News from Japan: International Symposium on the Adverse Reactions Experienced by those Vaccinated with Human Papillomavirus Vaccines
By Norma Erickson
Sanevax
February 24, 2014

Tuesday, February 25, 2:30 p.m. Tokyo time marks the beginning of an International Symposium on the adverse reactions experienced by girls who have been vaccinated by Human Papillomavirus vaccines. This symposium was organized on behalf of The Researchers' Organization Sounding a Warning concerning the Adverse Reactions induced by Human Papillomavirus Vaccines, through the collaborative efforts of Dr. Harumi Sakai, former Professor at the Tokai University School of Medicine, Dr. Shohei Matsuzaki, Professor Emeritus at the Tokai University School of Medicine, Mutsuo Fukushima, Kyoto News International Department, and SaneVax Inc.

This event will give a voice to the thousands of young women and families around the world who have suffered debilitating side effects, sometimes death, after using HPV vaccines. Many of these families have been told these events are psychosomatic or coincidental.

In fact on January 20, 2014, the Japanese government's advisory council released an official report quite typical of those issued in other countries in which they dismissed all of the symptoms that have shown up in the bodies of vaccinated girls as the consequences of psychogenetic "psychosomatic reactions" – in other words, the consequences of mental reactions of girls who the council suggested in a sophisticated manner may have been spoiled by mothers who do not know how to give discipline to their girls.

According to Kyoto News Reporter, Mutsuo Fukushima, the key proponent of this theory is Dr. Yutaka Ohno of Keio University, who has stated publicly:

"It is impossible to find physical causes for the alleged and presumed adverse reactions at those vaccinated girls, so we cannot help concluding that their so-called adverse reactions are the mere consequences of psychosomatic reactions. The government should provide counselling to the girls so that they may be freed from their psychosomatic reactions."

The organizers of this symposium, along with countless others, find this callous lack of concern for the victims of adverse reactions to HPV vaccines appalling. They want the world to know there are several biologically plausible mechanisms of action via which Gardasil and Cervarix could precipitate these events. They want the world to know what their research shows in the hope of halting HPV vaccination campaigns until these mechanisms of action are identified, and quantified, so those most at risk can be eliminated from any future HPV vaccination programs. Above all, they want the world to know there are those who will not give up working until the devastation following in the wake of mass HPV vaccination programs is stopped.

Symposium Calendar of Events:

1. **International Symposium** on the Adverse Reactions experienced by girls who have been vaccinated with Human Papillomavirus Vaccines (Gardasil and Cervarix) – February 25th from 2:30 to 5:30 p.m. – Tokai University Extension Center, 35th Floor, Kasumigaseki Building, Tokyo, Japan
    1. Complete information is available here.

2. **Press Conference** for reporters from newspaper companies and TV broadcasters – open to the public – February 25th from 5:45 to 7:30 p.m. – same venue as above
    1. Dr. Lee, Professor Authier, Lucija Tomljenovic PhD, Dr. Sasaki, Dr. Shiozawa, Dr. Uhide Kiyoshi, Dr. Hama and Mutsuo Fukushima will be available to answer questions from the public and the press.
3. **Government-Sponsored Public Hearing** of the Health Ministry's Advisory Council for the Deliberations on the Reported Adverse Events of HPV Vaccines, the advisory panel consisting of 15 scientists – February 26th, 10:00 to 11:30 a.m. (Evidence to be presented by scientists and medical professionals from the United States, Canada, France and Japan regarding potential mechanisms of action between HPV vaccines and serious adverse events.)
4. **Briefing on HPV Matters to Influential Lawmakers** of the Ruling Liberal Democratic Party (LDP) – February 26th from 12:00 to 1:30 p.m. – at the room of Chairman Eriko Yamatani of the LDP Policy Deliberations Committee at the House of Councilors in the upper chamber of Japan's bicameral parliament.
5. **Press Conference** – February 26th beginning at 5:45p.m. In the press room of the Japanese Health Ministry.

Symposium Participants:

- Organizer: Harumi Sakai, MD, former Professor, Tokai University School of Medicine
- Co-organizer: Shohei Matsuzaki, MD, Professor Emeritus, Tokai University School of Medicine
- Interpreter: Mutsuo Fukushima, Reporter, Kyodo News, International Department
- Sin Hang Lee, MD, former Yale University Associate Professor, Pathologist at Milford Hospital, Director of Milford Medical Laboratory, Inc.
- François-Jerome Authier, MD, Universite Paris XII, Systeme Hospital Henri Mondor de Paris
- Lucija Tomljenovic, PhD, Research Associate, British Columbia University, Canada
- Mirna Hajjar, MD, Department of Neurology, Hartford Hospital, Hartford Connecticut
- Masayuki Sasaki, MD, National Center of Neurology and Psychiatry, Director of Child Neurology
- Shunichi Shiozawa, MD, Professor, Kyushu University Hospital
- Uhide Kiyoshi, MD, Assistant Professor, Kanazawa University
- Rokuro Hama, MD, Director, Japan Institute of Pharmacovigilance (non-profit organization)

How and why was this symposium organized?

Early in September 2013, the Secretary General of the Nationwide Liaison Association of Cervical Cancer Vaccine Victims and Parents in Japan, and Mr. (Francis) Mutsuo Fukushima, journalist with the Kyodo News, informed the SaneVax team that there were rumors of a delegation from the Japanese Ministry of Health planning to visit London in October to have discussions on the HPV vaccines with officials in the UK Department of Health and the Medicines and Healthcare Products Regulatory Agency (MHRA). It was thought that this delegation may be willing to speak with scientific and medical experts who were independent of pharmaceutical industry ties. Knowing what this delegation would hear from the official sources, the SaneVax Team felt it would be critical for this delegation to speak with medical and scientific professionals who had no ties to the pharmaceutical industry.

Prior to official confirmation of this meeting, Norma Erickson, President of SaneVax Inc. and Freda Birrell, Secretary provided their new contacts with information relating to many cases of young girls who had also been harmed by the HPV vaccines, Cervarix and Gardasil, in other parts of the world. The adverse events being reported in Japan were almost identical to those being reported in every country where these vaccines were administered. This fact alone seemed to indicate that the adverse events should not be regarded as a coincidence. The entire SaneVax Team thought it critical to demonstrate that this was a global problem. The young women of Japan were not the only ones suffering after HPV vaccinations. The more information sent to Japanese contacts, the more everyone agreed that independent experts had to be heard.

Following many discussions, SaneVax obtained confirmation from Mr. Tetsuya Miyamoto, Director of the Office of Vaccination Policy at the Ministry of Health, Labor and Welfare's Health Policy Bureau and a qualified medical doctor in his own right, that he was leading a team embarking on a fact finding mission regarding HPV vaccines. He indicated that he and his team would be willing to meet with independent scientists and a doctor from London on Monday, 7th October 2013 at the Double Tree by Hilton Hotel in London.

This meeting was scheduled to begin at 2 pm and last for 2 hours. His team consisted of first class medical doctors from two of the six university hospitals in Japan which had been entrusted with official research efforts to shed light on potential cause-and-effect relationships between HPV vaccines and adverse effects being experienced by young girls in Japan.

Those attending this very important meeting on behalf of SaneVax were: Dr. Sin Hang Lee, MD, Pathologist, Milford Hospital, Director, Milford Medical Laboratory Inc., and former associate professor of pathology at Yale University; Professor Francois Jerome Authier, MD, PhD, Reference Center for Neuromuscular Disorders, Henri Mondor Hospital, Paris and Dr. Damien Downing, MB BS, MIBiol from London who is a pioneer of Ecological

Medicine. Also in attendance at this meeting were Mrs. Freda Birrell, Secretary of SaneVax Inc. and her husband David Birrell, VAERS Research Analyst for SaneVax Inc.

Our team in London was treated with the greatest of respect by Mr. Miyamoto and his fellow doctors. They listened attentively to all that was said and watched diligently the excellent power point presentations – stopping many times to ask very important questions. A Japanese interpreter was also in attendance.

At that time, we understood that there would be the opportunity for a further meeting organized by SaneVax to be held in Washington, DC when the Japanese delegation visited officials from the FDA and the CDC. The Japanese delegation did visit Washington, later than expected because of the breakdown in the U.S. government administration. There was no opportunity for a second meeting with SaneVax representatives to take place.

Therefore, the London meeting became very important as it has proven to be the catalyst for the historic events now scheduled to take place in Japan on the 25th and 26th February 2014.

For the first time in the history of public immunization campaigns, government officials are willing to listen to the voices of truly independent scientists and medical professionals regarding vaccination policy and immunization practices. This is something that should have happened decades ago. Perhaps the meetings in Japan will herald the beginning of a new medical paradigm.

At the very least, the meetings will provide a voice for thousands of young people and their families who are having to cope with the sometimes debilitating effects of HPV vaccination use.

The SaneVax Team would like to express their sincere appreciation to those who organized this symposium for allowing us the opportunity to assist in the planning and coordination. It has been an honor to be a part of such a historic event.
This may be the beginning of the end to the devastating health changes experienced by some families after HPV vaccinations.

From: Robert Pless [mailto:rpless2@gmail.com]
Sent: Monday, February 24, 2014 8:48 AM
To: 難波江 功二(nabae-koji)
Cc: SAHINOVIC, Isabelle; Robert Pless; h.petousis-harris@auckland.ac.nz; mew2@cdc.gov; ZUBER, Patrick Louis F.; jbeytout@chu-clermontferrand.fr; 阿部 圭史(abe-keishi)
Subject: Re: HPV vaccine conf call Follow-up

Dear Koji,

This is an excellent compromise - and will give GACVS some additional time both to process the information presented at both the anti-vaccine conference and the public meeting, as well as anything that may have come out of discussion at the expert advisory committee meeting. GACVS could then adjust the statement as needed in order to address some additional points. We might be able to use the time also to convene a discussion with Committee members to provide additional input into the statement.

Best wishes for the coming week.
Rob

These are all my thoughts - of course the GACVS secretariat and Chair would
On Feb 23, 2014, at 12:00 AM, 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp> wrote:

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Thank you so much for your help.

I look forward to meeting and talking to you later.

Warm regards,

Koji Nabae

Landon James Watt

From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Monday, 24 February 2014 4:34 p.m.
To: Helen Petousis-Harris
Subject: RE: HPV vaccine conf call Follow-up
Attachments: Annotated Agenda 26 Feb 2014.docx; (revised)AI adjuvanted Vacc Present Tokyo JB.pptx; [redacted]

Thanks Helen,

For videoconference, we use WebEx

http://www.webex.com.au/?DCMP=OTC-FromGP

In case your computer does not have this software, please download the free trial version.

We will send you URL and password to join the conference.
I hope we can do a trial this afternoon.

==
For the conference itself, attached please find a revised annotated agenda.
We expect you to make a five-minute presentation (with no more than 10 slides)
[redacted]

FYI, attached please find Dr Beytout's presentation.
==

Warmest regards,

Koji Nabae
Deputy Director
Division of Tuberculosis and Infectious Disease Control
Ministry of Health, Labour & Welfare
Government of Japan-
Tel: +81-3-3595-2257
Mobile: +[redacted]
Fax: +81-3-3581-6251
email: nabae-koji@mhlw.go.jp

From: Helen Petousis-Harris [mailto:h.petousis-harris@auckland.ac.nz]
Sent: Monday, February 24, 2014 11:38 AM
To: 難波江 功二(nabae-koji)
Subject: RE: HPV vaccine conf call Follow-up

Dear Koji

Jut to let you know I will do my best to get you something as soon as possible. I have just returned and will focus on this now.

Could you let me know some further details for the video conferencing? I need to tell the technology person what is required. He asked the following.

Are you doing it from your PC
Do you have the IP address for Tokyo,
Are you calling them
Are you calling a bridge
Do they need your IP address

Kind regards
Helen

From: 難波江 功二(nabae-koji) [mailto:nabae-koji@mhlw.go.jp]
Sent: Sunday, 23 February 2014 6:01 p.m.
To: SAHINOVIC, Isabelle; rpless2@gmail.com; Robert.Pless@phac-aspc.gc.ca; Helen Petousis-Harris; mew2@cdc.gov; ZUBER, Patrick Louis F.; jbeytout@chu-clermontferrand.fr
Cc: 阿部 圭史(abe-keishi); 難波江 功二(nabae-koji)
Subject: HPV vaccine conf call Follow-up

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Thank you so much for your help.

I look forward to meeting and talking to you later.

Warm regards,

Koji Nabae

Aluminium adjuvanted vaccines: the position of the French Advisory Committee on Immunisation Practices

Pr Jean Beytout

University hospital of Clermont-Ferrand (France)

Member of the French Committee on Immunisation Practices. High Council of Public Health.

Aluminium adjuvanted vaccines Long duration collective experience

- Aluminium salts have been added to vaccine antigens since 1926 . These vaccines have been used all over the world for more than eighty years and milliard of doses were inoculated.
- Tolerance is fair. Adverse effects are scarce : mainly local reaction (pain, inflammatory reaction), seldom general (fever); these reactions happen early.
- **No country or official body calls into question the validity of this addition or the safety of vaccines containing aluminium salts**.

Macrophagic Myofasciitis (MMF)

- MMF is defined by microscopic lesions found in muscles biopsies : infiltration of muscle tissue by PAS-positive macrophages.
- In patients suffering from musculoskeletal symptoms, Ghirardi, Authier and Cherin at the French Reference Center for Neuromuscular Diseases (GERMAAD), observed MMF in biopsy obtained from deltoïd muscle. Macrophages contained aluminium particles provided by inoculated vaccines.
- Since 1998, this team gathered more than 1000 observations of MMF. Biopsies were done 65 months in average between the inoculation of an adjuvanted vaccine **in the deltoïd**.

Gherardi RK, Authier FJ. Macrophagic myofasciitis: characterisation and pathophysiology. Lupus 2012; 21: 184-89.

Table 1 – Summary of reported clinical symptoms [12]

Symptoms	Percentage of patients
Myalgias	88–91
Arthralgias	57–68
Marked asthenia	55
Muscle weakness	45
Fever	20–32
Elevated CK levels	29–50
Increased ESR	37
Myopathic EMG	35
Demyelinating CNS disorder	9
Multiple sclerosis diagnosis	33
Chronic fatigue	50–93
Hashimoto's thyroiditis	2.7
Other autoimmune-related diseases (RA, Sjogren)	6.7

Latest aluminium vaccine received before diagnosis.

Vaccine	N	%
Vaccine against hepatitis B	289	69.3
Vaccine against tetanus	21	5.0
Revaxis®	11	2.6
Vaccine against hepatitis A	11	2.6
Vaccine against hepatitis B + Vaccine against hepatitis A	8	1.9
Vaccine against hepatitis B + Vaccine against tetanus	2	0.5
Vaccine against hepatitis B + Tetravac®	1	0.2
Vaccine against hepatitis A + Revaxis®	1	0.2
Infanrix®	1	0.2
Gardasil®	1	0.2
Tetravac®	1	0.2
HPV Vac	1	0.2
Not specified	**69**	**16.5**
Total	**417**	**100.0**

Macrophagic myofasciitis controversy

- An epidemiological study was set by the French Ministry of Health (2002 – 3):
  - MMF is an indisputable histological entity whose association with aluminium used as an adjuvant in vaccines is recognized.
  - But case-control study demonstrated there was no specific clinical entity could be demonstrated to be related with the use of aluminium adjuvanted vaccine.
- In 2004, the Global Advisory Committee on Vaccine Safety (WHO) reviewed the data of the case–control study performed in France and also concluded that " **the persistence of aluminium-containing macrophages at the site of a previous vaccination is not associated with specific clinical symptoms or disease ".**

Global Advisory Committee on Vaccine Safety. - Weekly Epidemiological Record . 1999; 41: 337-40. - WER. 2002; 77: 389-404. - WER. 2004; 79 : 3 – 20.

MMF suggested pathogenesis

Several successive hypothesis were challenged :

- 1st (1998): diffuse muscle disease,
- 2nd (1999): autoimmune process related with aluminium,
- 3rd (2001): Chronic fatigue syndrome,
- 4th (2009) : Direct cerebral toxicity due to aluminium nanoparticles,
- 5th (2011) : ASIA syndrome (autoimmune?),
- 6th (2013) : cerebral toxicity due to aluminium loaded monocytes
- …

Chronic Fatigue Syndrom (CFS)

1. **Unexplained, persistent fatigue** present for ≥ 6 months that is not substantially relieved by rest, is of new onset (not lifelong) and results in a significant reduction in previous levels of activity.
2. **Four or more of the following symptoms** are present for six months or more:
   - Impaired memory or concentration,
   - Postexertional malaise (extreme, prolonged exhaustion and sickness following physical or mental activity),
   - Unrefreshing sleep,
   - Muscle pain,
   - Multijoint pain without swelling or redness,
   - Headaches of a new type or severity,
   - Sore throat that's frequent or recurring,
   - Tender cervical or axillary lymph nodes.

CDC. Chronique fatigue syndrome. A toolkit for provider.

From MMF to CFS

The GERMMAD worked to demonstrate that MMF was associated with neuropsychogical problems linked to cerebral lesion.

Experimental studies were done:

- In mice, 1 year after IM injection of aluminium containing vaccine, Al deposits are detected in brain. Injected labelled materials can be followed up to brain, suggesting that macrophages loaded with nanoparticles are able to migrate and to access to cerebral area especially grey matter.
- In altered blood-brain barrier mice (BBB), they demonstrated a progressive diffusion into the cerebral grey matter suggesting an active process via macrophages containing nanoparticles that cross the blood-brain barrier : “Trojan horse” mechanism.
- In CCL2 deficient mice, substitution modulates the cerebral intake of aluminium labelled particles captured by monocytes.

Khan Z and al. Slow CCL2-dependent translocation of biopersistent particles from muscle to brain. BMC Medecine 2013 ; 11: 99

Pointed out uncertainties

- **Cerebral impairment does not explain all the symptoms which the MMF patients suffered.**
- Al-Rho particles are not representative of the aluminium used as a vaccine adjuvant.
- the experiment conducted in mice whose BBB integrity is deficient shows that increased permeability can amplify the phenomenon but does not prove that BBB is involved in the translocation.
- In the MCP-1 deficient mice loss experiment, it is not possible to ascertain whether the loss of function is linked to the primary recruitment of macrophages in the injected muscle and/or their translocation towards the brain and other organs.
- the presence of aluminium inclusions in the CNS does not signify the automatic existence of a "neurotoxicity".

The Haut Conseil de la santé publique

- **“Deems that the scientific data available today do not allow the safety of vaccines containing aluminium to be called into question with regard to their risk/benefit balance,**
- **Recommends the continuation of vaccinations according to the vaccine schedule in force,**
- **Warns of the consequences, in terms of the reappearance of infectious diseases, that could result from a decrease in vaccine coverage due to aluminium-containing vaccines being called into question without any scientific justification,**
- **Encourages the pursuit of research that aims to evaluate the safety of adjuvants that are available and in development”.**

Landon James Watt

From: Robert Pless <Robert.Pless@phac-aspc.gc.ca>
Sent: Thursday, 20 February 2014 3:29 a.m.
To: 難波江 功二; ZUBER, Patrick Louis F.
Cc: 阿部 圭史; Helen Petousis-Harris; Wharton, Melinda (CDC/OID/NCIRD); 難波江 功二; 'Robert Pless'
Subject: Re: HPV vaccine Public Hearing meeting in Japan 26 Tokyo

Dear Koji,

[redacted]

[redacted]

I think it would be important to get together and discuss.

Patrick: can your team set up a conference call (if others agree)? I am also wondering about the review of the draft statement as I have not heard back. [redacted]

There may be a time window when we are all awake!
Regards,
Rob

難波江 功二(nabae-koji) ---2014-02-19 08:48:11 AM---Dear Helen, Rob, Melinda and Patrick, Thank you so much for your assistance.

From: **難波江 功二**(nabae-koji) <nabae-koji@mhlw.go.jp>
To: Helen Petousis-Harris <h.petousis-harris@auckland.ac.nz>, '"Robert Pless"' <rpless2@gmail.com>, "Robert Pless (Robert.Pless@phac-aspc.gc.ca)" <robert.pless@phac-aspc.gc.ca>, "ZUBER, Patrick Louis F." <zuberp@who.int>, "Wharton, Melinda (CDC/OID/NCIRD)" <mew2@cdc.gov>
Cc: **難波江 功二**(nabae-koji) <nabae-koji@mhlw.go.jp>, **阿部 圭史**(abe-keishi) <abe-keishi@mhlw.go.jp>
Date: 2014-02-19 08:48 AM
Subject: HPV vaccine Public Hearing meeting in Japan 26 Tokyo

---

Dear Helen, Rob, Melinda and Patrick,

Thank you so much for your assistance.

Attached please find a draft annotated agenda of the HPV vaccine safety meeting in Tokyo.

[redacted]

[redacted]

[redacted]

Melinda, we were thinking of inviting an expert from USCDC CISA group and asked for your support for this,
but given that there will be no presence of Dr Lucija Tomljenovic (vasculopathy), and time limitation,
we are now thiking of asking a WHO GACVS member (Rob?) to cover both MMF and DNA issues in
addition to Prof Beytou and Helen, if possible. May I ask for your suggestion whether we better still
ask CISA member (probably Dr. Philip LaRussa) to join?

Thank you so much for your valuable support. It has been extremely helpful for us.

Warmest regrads,

Koji Nabae
Deputy Director
Division of Tuberculosis and Infectious Disease Control
Ministry of Health, Labour & Welfare
Government of Japan-
Tel: [redacted]
Fax: +81-3-3581-6251
email: nabae-koji@mhlw.go.jp

From: Helen Petousis-Harris [mailto:h.petousis-harris@auckland.ac.nz]
Sent: Tuesday, February 18, 2014 5:19 AM
To: 'Robert Pless'
Cc: Robert Pless (Robert.Pless@phac-aspc.gc.ca); 難波江 功二(nabae-koji); ZUBER, Patrick Louis F.; Wharton, Melinda (CDC/OID/NCIRD)
Subject: RE: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

Dear Rob

Oh dear! I am so saddened to hear how extensive the impact of Lee, Shaw and Tomljenovic's activities has become.

I will certainly do anything I can to assist. To the best of my knowledge the rebuttal on our website is the only attempt to address this particular issue which Shaw and Lee presented at a coronal enquiry here. Placing the rebuttal in the public domain was the only means of providing the information to the crown representatives involved in that process at the 11th hour. Prof David Gorsky has written prolifically on some of the experiments in his science blog over the past few years so I assume he has also given the material some thought.

I do not know if I am expert on this but certainly have some experience in considering aluminium in vaccines and its role in inflammatory responses and local AEFI as part of my PhD some years ago. I assume you are referring to the VLP tightly bound to the adjuvant and the Shaw and Tomljenovic 'hypothesis' that it somehow finds its way to the brain carried by macrophage?

A phone call would probably be useful. It is a little after 9am in NZ.

Kind regards.

Helen

Helen Petousis-Harris. PhD, MRSNZ
Senior Lecturer, General Practice and Primary Health Care
Director of Immunsiation Research and Vaccinology
Immunisation Advisory Centre
University of Auckland
DDI +64 9 923 2078 Fax 9 3737030 Mob [redacted]
Building 734, Level 3, Tamaki Campus, Morrin Rd, Glen Innes
Private Bag 92019, Victoria St West, Auckland 1142, New Zealand

From: Robert Pless [mailto:rpless2@gmail.com]
Sent: Tuesday, 18 February 2014 6:20 a.m.
To: Helen Petousis-Harris
Cc: Robert Pless (Robert.Pless@phac-aspc.gc.ca); "難波江 功二(nabae-koji)"; ZUBER, Patrick Louis F.; Wharton, Melinda (CDC/OID/NCIRD)
Subject: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

Dear Dr. Petousis-Harris,

I am writing you with an urgent request outlined below, having read the Immunization Advisory Centre's Commentary on coronial inquiry expert witness testimony that was prepared in response to allegations by Chris Shaw and Sing Hang Lee. I am a current member of the WHO Global Advisory Committee on Vaccine Safety and am writing in part on behalf of Dr. Koji Nabae of the Japanese Ministry of Health, for assistance. The GACVS has been looking at this issue from the global perspective and have released several statements over the last two years to address concerns around aluminum and autoimmune disease.

As you may be aware, there have been ongoing concerns in Japan regarding the HPV vaccine, where cases of chronic pain and complex regional pain syndrome allegedly linked to the vaccine have led to a partial suspension of their national vaccination program and there has been a great deal of public interest. An expert advisory group has met several times but have not reached a conclusion about the restart of the program. A meeting has recently been organized in Tokyo for February 26th, where Dr. Lee will present his findings. It is likely that Dr. Shaw's co-investigator, Lucija Tomljenovic will be present as well. There will be a second presentation on Macrophagic Myofasciitis and the HPV vaccine, a stretch of the MMF story first related to the hepatitis B vaccine.

We are seeking your advice on someone who may be able to address the more detailed questions around HPV DNA - specifically the hypotheses you have address in your statement regarding the alleged role of aluminum binding to DNA fragments and subsequent effects. While the issue of whether the fragments constitute "contamination" has been dealt with, your statement was the only one to address the more obscure alleged consequences of the presence of those fragments. The GACVS has not yet had a chance to delve into the DNA question.

While we appreciate the short notice, the meeting and even the date were very recently confirmed. That said, the ideal would be someone who would be available to travel to the meeting in Japan and address the issue as it arises in person. Please let me know if I can clarify anything by phone, and indeed if this is at all possible and who could be contacted and provided with further details.

Best regards,
Rob

==========
Robert Pless, MD, MSc
Medical Advisor
Health Security Infrastructure Branch
Public Health Agency of Canada

Ottawa, Ontario K1A 0K9
Tel./Mobile: [redacted]
Email: robert.pless@phac-aspc.gc.ca [redacted]

Landon James Watt

From: ZUBER, Patrick Louis F. <zuberp@who.int>
Sent: Thursday, 20 February 2014 9:25 p.m.
To: Helen Petousis-Harris
Cc: nabae-koji@mhlw.go.jp; Robert Pless; 阿部 圭史(abe-keishi) ; Wharton, Melinda (CDC/OID/NCIRD); Robert Pless
Subject: Re: HPV vaccine Public Hearing meeting in Japan 26 Tokyo

There was a typo

I meant Friday 21

Sorry for the confusion

Patrick

On 20 Feb 2014, at 05:05, "Helen Petousis-Harris" <h.petousis-harris@auckland.ac.nz> wrote:

> Dear All
>
> I can see it will be very difficult to get a time so quickly to suit all.
>
> While it will be 1am in NZ, I will be at a wedding and therefore very likely to still be up so do not change it on my account.
>
> [redacted]
>
> Kind regards
> Helen

From: 難波江 功二(nabae-koji) [mailto:nabae-koji@mhlw.go.jp]
Sent: Thursday, 20 February 2014 1:30 p.m.
To: ZUBER, Patrick Louis F.; Robert Pless
Cc: 阿部 圭史(abe-keishi); Helen Petousis-Harris; Wharton, Melinda (CDC/OID/NCIRD); 'Robert Pless'
Subject: RE: HPV vaccine Public Hearing meeting in Japan 26 Tokyo

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OK Helen,

We will provide the bridge for NZ and glad if you can join! Sorry it was a time slot challenge.

Patrick

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I can see it will be very difficult to get a time so quickly to suit all.

While it will be 1am in NZ, I will be at a wedding and therefore very likely to still be up so do not change it on my account.

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Kind regards
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Dear all,

I am Keishi Abe from MHLW in Japan.
Koji and I can join it on Friday 20 Feb at 1PM in Geneva (7am on the East Coast, 9pm in Japan and 1am on Sat in NZ).

Thank you for your assistance,
Keishi

****
Keishi ABE, M.D.

Section Chief
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Health Service Bureau
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Cc: Robert Pless (Robert.Pless@phac-aspc.gc.ca); "難波江 功二(nabae-koji)"; ZUBER, Patrick Louis F.; Wharton, Melinda (CDC/OID/NCIRD)
Subject: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

Dear Dr. Petousis-Harris,

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Medical Advisor
Health Security Infrastructure Branch
Public Health Agency of Canada
Ottawa, Ontario K1A 0K9
Tel./Mobile: [redacted]
Email: robert.pless@phac-aspc.gc.ca [redacted]

Landon James Watt

From: 阿部 圭史(abe-keishi) <abe-keishi@mhlw.go.jp>
Sent: Saturday, 22 February 2014 12:49 a.m.
To: SAHINOVIC, Isabelle; rpless2@gmail.com; Robert.Pless@phac-aspc.gc.ca; Helen Petousis-Harris; 難波江 功二(nabae-koji); mew2@cdc.gov; ZUBER, Patrick Louis F.; jbeytout@chu-clermontferrand.fr; 渡邊 周介(watanabe-shuusuke)
Subject: RE: Teleconference - the HPV vaccine Public Hearing meeting in Japan 26 Tokyo
Attachments: Participants on the Conference Call.docx

Dear all,

The conference time start soon.
Please find the attached the agenda and participants of it.

Keishi
-----Original Appointment-----
From: SAHINOVIC, Isabelle [mailto:SAHINOVICI@who.int]
Sent: Thursday, February 20, 2014 5:55 PM
To: rpless2@gmail.com; Robert.Pless@phac-aspc.gc.ca; h.petousis-harris@auckland.ac.nz; 難波江 功二(nabae-koji); 阿部 圭史(abe-keishi); mew2@cdc.gov; ZUBER, Patrick Louis F.
Subject: Teleconference - the HPV vaccine Public Hearing meeting in Japan 26 Tokyo
When: 2014年2月21日金曜日 13:00-14:00 (UTC+01:00) アムステルダム、ベルリンw「xD戦襯鵝▲蹇璽沍mストックホルムw「xDE・璽・・錫鮠 **Where:** Participant PIN: 16981746 Web Login: 118357443

Dear participants to the HPV vaccine Public Hearing meeting in Japan 26 Tokyo

(Friday 21 Feb at 1PM in Geneva (7am on the East Coast, 9pm in Japan and 1am on Sat in NZ).

Join the AUDIO conference using your landline or mobile phone

1. Dial the appropriate number from the list attached:

   (From an iPhone or a Blackberry, simply tap on the number to access the conference automatically)

2. Enter the participant PIN code: **16981746** followed by **#**

If you need ASSISTANCE:

- During your conference: **Dial *0 on your phone keypad** to speak to an Arkadin Live Assistance operator.
- At other times, please click here to contact the Arkadin Customer Service Team.

<< File: International list GE011013 (2).pdf >>

Participants on the Conference Call
regarding HPV vaccine Public Hearing Session in Japan
on February 21, 2014

Date & Time: 1PM in Geneva
(7am on the US East Coast, 9pm in Japan and 1am on Sat in NZ)

Participants:

1. Dr. Jean Beytout
   (Clermont teaching hospital, France,)

2. Dr. Robert Pless
   (Canada Public Health Agency, on behalf of GACVS, WHO)

3. Dr. Helen Petousis-Harris
   (Auckland University, New Zealand)

4. Dr. Melinda Warton
   (USCDC and GACVS, WHO)

5. Dr. Patrick Zuber
   Dr. Isabelle Sahinovic
   (WHO GACVS, Geneva)

6. Dr. Hiroshi Yoshikura
   (Former DG of NIID, Japan)

7. Dr. Ichiro Kurane
   (Chair of the session and Deputy DG of NIID, Japan)

8. Dr. Koji Nabae
   Dr. Keishi Abe
   Dr. Shu Watanabe
   (MHLW, Japan)

Agenda;

1. Introductions

2. Current situation in Japan with respect to the signal

3. Origins of the 2 meetings being held next week and potential outcomes

4. Planned and likely topics that may arise by the speakers (MMF, HPV DNA, …other)

5. Responses during the meeting on the 26th (invited experts, Ministry, Expert advisory group)

6. Format and timing of responses outside the meetings (GACVS statement, follow up statements?)

7. Other interventions?

8. Other issues

Landon James Watt

From: Robert Pless <rpless2@gmail.com>
Sent: Tuesday, 18 February 2014 12:29 p.m.
To: Helen Petousis-Harris
Subject: Re: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

Helen,

Many thanks for the speedy reply. I would be glad to chat of course, and am quite happy to work with the time difference as it suits you, so please let me know of a good time to reach you.

I delayed responding a bit in the hope that I hear a bit more about the way the meeting might unfold, but in the absence of that, it might at least be good to say hello and exchange information.
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I re-read David Gorsky's piece. I had forgotten how detailed he was about the entire hypothesis so I failed to resurrect it this weekend while doing more reading.

Looking forward to getting in touch,
Regards,
Rob

On Feb 17, 2014, at 3:18 PM, Helen Petousis-Harris <h.petousis-harris@auckland.ac.nz> wrote:

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A phone call would probably be useful. It is a little after 9am in NZ.

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Helen

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Senior Lecturer, General Practice and Primary Health Care
Director of Immunsiation Research and Vaccinology
Immunisation Advisory Centre
University of Auckland
DDI +64 9 923 2078 Fax 9 3737030 Mob [redacted]
Building 734, Level 3, Tamaki Campus, Morrin Rd, Glen Innes

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Ottawa, Ontario K1A 0K9
Tel./Mobile: [redacted]
Email: robert.pless@phac-aspc.gc.ca

Landon James Watt

From: Robert Pless <rpless2@gmail.com>
Sent: Wednesday, 19 February 2014 12:32 p.m.
To: Helen Petousis-Harris
Subject: Re: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

That would be great. My number is +[redacted]

Thanks,
Rob

Sent from my iPad

On Feb 18, 2014, at 18:13, Helen Petousis-Harris <h.petousis-harris@auckland.ac.nz> wrote:

> This afternoon is fine by me. Can I call you using my cell? Last time we tried skype the delay made conversation frustrating.
>
> Can you provide me with a number and I will call you after I have gone to find some lunch?
>
> Kind regards
> Helen

From: Robert Pless [mailto:rpless2@gmail.com]
Sent: Wednesday, 19 February 2014 12:02 p.m.
To: Helen Petousis-Harris
Subject: Re: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

Helen,

I just got in from work - was at an all day workshop so could not escape. If you are still available during your afternoon, I am fine this evening to chat. I spoke with an old colleague who was on our advisory committee for causality assessment in the 1990's (!). He is a neurologist and while I realize you had done the homework on the DNA issue, I asked for his perspective as well on some of the demyelination issues. He also co-authored a paper with Chris Shaw.

If there is a good time for me to try a call, let me know. I may use Skype if that is ok, as I have never tried my office cell for an international call!

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Landon James Watt

From: Robert Pless <Robert.Pless@phac-aspc.gc.ca>
Sent: Wednesday, 19 February 2014 3:56 a.m.
To: michael.gold@adelaide.edu.au
Cc: 難波江 功二; Helen Petousis-Harris; Wharton, Melinda (CDC/OID/NCIRD); 'Robert Pless'; ZUBER, Patrick Louis F.
Subject: RE: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

Mike,

I had meant to c.c. you but pressed "send" too quickly.

All: just wanted to inform you that I c.c'd Mike Gold - apologies for the duplicate email.

Rob

Robert Pless---2014-02-18 09:42:30 AM---Dear Koji and Helen, I am glad it worked out - and very grateful indeed to Helen for being so gracio

From: Robert Pless/HC-SC/GC/CA
To: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Cc: Helen Petousis-Harris <h.petousis-harris@auckland.ac.nz>, "Wharton, Melinda (CDC/OID/NCIRD)" <mew2@cdc.gov>, '"Robert Pless"' <rpless2@gmail.com>, "ZUBER, Patrick Louis F." <zuberp@who.int>
Date: 2014-02-18 09:42 AM
Subject: RE: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

---

Dear Koji and Helen,

I am glad it worked out - and very grateful indeed to Helen for being so gracious with this.
Mike Gold and I have also exchanged emails and as GACVS prepares a statement, sharing the key messages and making sure they are correct and consistent would be crucial and a decision on whether a GACVS member really needs to attend the meeting can be finalized.

Best regards,
Rob

難波江 功二(nabae-koji) ---2014-02-18 07:12:53 AM---Dear Rob, Thank you so much for introducing me to Helen. I had a chance to talk to Helen over the p

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Date: 2014-02-18 07:12 AM
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Dear Rob,

Thank you so much for introducing me to Helen. I had a chance to talk to Helen over the phone today.

Dear Helen,

It was so nice talking to you over the phone, and thank you so much for agreeing on participating in our meeting at least via skype and, if possible, in person.

We really look forward to having your inputs during the meeting.

Warm regards,

Koji Nabae
Deputy Director
Division of Tuberculosis and Infectious Disease Control
Ministry of Health, Labour & Welfare
Government of Japan-
Tel: [redacted]
Fax: +81-3-3581-6251
email: nabae-koji@mhlw.go.jp

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Sent: Tuesday, February 18, 2014 5:19 AM
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Senior Lecturer, General Practice and Primary Health Care
Director of Immunsiation Research and Vaccinology
Immunisation Advisory Centre
University of Auckland
DDI +64 9 923 2078 Fax 9 3737030 Mob [redacted]
Building 734, Level 3, Tamaki Campus, Morrin Rd, Glen Innes
Private Bag 92019, Victoria St West, Auckland 1142, New Zealand

From: Robert Pless [mailto:rpless2@gmail.com]
Sent: Tuesday, 18 February 2014 6:20 a.m.
To: Helen Petousis-Harris
Cc: Robert Pless (Robert.Pless@phac-aspc.gc.ca); "難波江 功二(nabae-koji)"; ZUBER, Patrick Louis F.; Wharton, Melinda (CDC/OID/NCIRD)
Subject: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

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Best regards,
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Tel./Mobile: [redacted]
Email: robert.pless@phac-aspc.gc.ca

Landon James Watt

From: Robert Pless <Robert.Pless@phac-aspc.gc.ca>
Sent: Wednesday, 19 February 2014 3:43 a.m.
To: 難波江 功二
Cc: Helen Petousis-Harris; Wharton, Melinda (CDC/OID/NCIRD); 'Robert Pless'; ZUBER, Patrick Louis F.
Subject: RE: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

Dear Koji and Helen,

I am glad it worked out - and very grateful indeed to Helen for being so gracious with this.
Mike Gold and I have also exchanged emails and as GACVS prepares a statement, sharing the key messages and making sure they are correct and consistent would be crucial and a decision on whether a GACVS member really needs to attend the meeting can be finalized.

Best regards,
Rob

難波江 功二(nabae-koji) ---2014-02-18 07:12:53 AM---Dear Rob, Thank you so much for introducing me to Helen. I had a chance to talk to Helen over the p

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Date: 2014-02-18 07:12 AM
Subject: RE: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

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Warm regards,

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A phone call would probably be useful. It is a little after 9am in NZ.

Kind regards.

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From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Wednesday, 19 February 2014 1:13 a.m.
To: Helen Petousis-Harris; 'Robert Pless'
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Email: robert.pless@phac-aspc.gc.ca

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From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Tuesday, 18 February 2014 6:25 p.m.
To: Helen Petousis-Harris
Subject: RE: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony
Attachments: Rapport HCP Aluminium_en-US.pdf; Dr Sin Hang Lee.zip

Dear Helen,

Thank you for your response.

The details of our meeting is as follows;

Date and Time: **Feb 26th (Wed) AM10:00-11:00 in Tokyo. (Feb 26th 14:00-15:00 NZ time)**

- There will be around 15-20 Japanese experts (basically listening to the presentation and Q&A).
- It is open public (around 100 audience), with English/Japanese simultaneous interpretation.
- The meeting is a hearing session and will not make any decisions.

With this limited time, there will be following two foreign speakers who would raise concerns about HPV vaccine safety.

1. Detection of human HPV L1 gene DNA fragments in postmortem blood and spleen after Gardasil vaccination
Dr. Sin hang Lee, former associate Prof. at Yale Univ. director of Milford Molecular Biology, Connecticut (attached file)

2. HPV vaccine causes Macrophagic myofasciitis (MMF)
Dr. Jerome Francois Autheir, Hospital Henri Mondor de l'Assistance Publique Hospitauz de Paris

Prof Beytout from France who was one the members of the report writing team on "aluminum and vaccine" (attached file)
will be present in person and present on the report.

We are also contacting CDC and WHO experts and hopefully there will be one or two more experts joining via skype besides you.

Please feel free to contact me should you have any inquiries.

Many thanks!!

Warm regards,

Koji Nabae
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From: Helen Petousis-Harris [mailto:h.petousis-harris@auckland.ac.nz]
Sent: Tuesday, February 18, 2014 12:57 PM
To: 難波江 功二(nabae-koji)
Subject: RE: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

Dear Koji

I have been thinking about the possibility of coming to Tokyo but think it may be tricky as I have very little time between now and then and have two other things I need to do quite a bit of preparation for, one I need to be in London for a couple of days later.

I will certainly be able to give a presentation via video conferencing or similar. I could send you some material and perhaps also prepare a written statement directed at the most pertinent points that could be useful for circulation.

Could you tell me about the audience so I can pitch the information appropriately?

Look forward to receiving the material from you.

Kind regards
Helen

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Sent: Tuesday, 18 February 2014 3:29 p.m.
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Dear Helen,

It was so nice talking to you and thank you so much for agreeing on joining our meeting.
It is indeed very helpful.

Just quickly, **I understand you are unable to travel to Japan in such a short notice next week.**
Is this correct!? We are happy to invite you (we will cover the travel cost) in case you happen
to be able to travel!!

Grateful for your response at your earliest opportunity.

Warm regards,

Koji Nabae
Deputy Director
Division of Tuberculosis and Infectious Disease Control
Ministry of Health, Labour & Welfare
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Haut
Conseil de la
Santé
Publique [French High Council for Public Health]

Aluminium and Vaccines

Collection
Opinions and Reports

Haut
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Santé
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Aluminium and Vaccines

For more than ten years, there has been a debate in France about the safety of aluminium used as an adjuvant in most vaccines in all countries around the world for more than sixty years now.

At the request of the Direction générale de la santé [French General Directorate of Health], the Haut Conseil de la Santé Publique (HCSP) carried out a critical review of the literature on aluminium in vaccines and a risk-benefit analysis of aluminium as an adjuvant in vaccines.

Updated pharmacovigilance data, the mechanisms of action of adjuvants, alternatives to aluminium salt-based adjuvants as well as toxicological data on aluminium are also included in this report.

The HCSP considers that the scientific data available at this time do not call the safety of aluminium-containing vaccines into question in terms of their risk-benefit ratio. It recommends that vaccinations be continued according to the vaccine schedule that is currently in effect and warns about the consequences, in terms of the reappearance of infectious diseases that could result from a decrease in vaccine coverage due to aluminium-containing vaccines being called into question without any scientific justification.

Additionally, the HSCP supports the continuation of research that aims to assess the safety of adjuvants that are available and under development.

Haut Conseil de la santé publique
14 avenue Duquesne
75350 Paris 07 SP
www.hcsp.fr

Haut
Conseil de la
Santé
Publique

Aluminium and Vaccines

Report

11 July 2013

This report was adopted by the Commission spécialisée Maladies transmissibles [Specialized Transmissible Diseases Commission] on 11 July 2013 after consultation of the Comité technique des vaccinations [Technical Vaccination Committee].

SUMMARY

WORKING GROUP

Composition

Brigitte AUTRAN, HCSP-CTV

Jean BEYTOUT, HCSP-CTV

Daniel FLORET, HCSP-CTV

Alexis JACQUET, ANSM (Agence Nationale de Sécurité du Médicament et des Produits de Santé [French National Agency for Medicines and Health Product Safety])

Jean-Louis KOECK, Service de santé des Armées [Armed Forces Health Department]

Daniel LEVY-BRUHL, InVS (Institut de Veille Sanitaire [French Institute for Public Health Surveillance])

Isabelle MORER, ANSM

Henri PARTOUCHE, HCSP-CTV

Corinne LE GOASTER, SG-HCSP

Odile LAUNAY, HCSP-CTV

Didier TORNY, HCSP-CTV

Individual interviewed

Prof. Romain GHERARDI, Hôpital Universitaire Henri Mondor [Henri Mondor University Hospital], Créteil

Public disclosures of interest

The working group members submitted a public disclosure of interest.

The Haut Conseil de la santé publique received a referral relating to the presence of aluminium in certain vaccines from the Direction générale de la santé on 12 July 2012.

The Haut Conseil de la santé publique (HCSP) is asked to provide information on:

- a critical review of the literature on aluminium in vaccines and macrophagic myofasciitis (MMF), particularly the studies conducted by Professor Gherardi's team;
- a risk-benefit analysis of aluminium as an adjuvant in vaccines in connection with the Agence nationale de sécurité du médicament et des produits de santé (ANSM) and, if possible, in comparison to other adjuvants.

1 - Genesis and chronology of a controversy

For several years, there has been a debate in France relating to the safety of aluminium used as an adjuvant in most vaccines in all countries around the world for more than sixty years now. The work of only one team in the world, a French team that has published on this topic since 1998, is the source of this controversy.

While the presence of aluminium granulomas in muscles that are injected with vaccines has been known about since 1982 [1], Germmad (Groupe de recherche sur les maladies musculaires acquises et dysimmunitaires [Research Group on Acquired Muscular and Immune Dysfunction Diseases]) alerted the Institut de veille sanitaire (InVS) in 1997 following the identification of a new histological entity, named "macrophagic myofasciitis," which was found in muscle biopsies. It was reported that the presence of this lesion often seemed to be associated with diffuse muscle pain and fatigue.

In 1998, Gherardi *et al.* report in the Lancet [2] about a series of 18 adult patients presenting with poorly defined musculoskeletal symptoms (myalgia, arthralgia, muscle weakness, elevated CPK (creatine phosphokinase) and EMG (electromyography) changes) and general symptoms (asthenia and fever). The muscle biopsy carried out in the deltoid of the non-dominant arm reveals the presence of unusual anatomical lesions that the authors name "macrophagic myofasciitis" (MMF). This "new entity" does not have a known etiology. Under a hypothesis of Whipple's disease, ten of these patients received treatment combining antibiotics and corticosteroids, and eight improved.

According to the authors [3], the origin of this work goes back to 1996, when a new research group, the Germmad, was created as a branch of the Association française contre les myopathies (AFM [French Myopathy Association]). Two patients were studied who presented with a myopathy, the histological aspect of which had never been observed before. Consequently, the principal French myology centers were asked to revisit their atypical muscle biopsy slides from the last 30 years. Thus, by December 1998, 35 observations had been collected, with the first one being observed in Bordeaux in 1993.

The hypothesis that was quickly advanced by the Germmad was that the granulomas observed in the muscles were linked to the presence of aluminium and the link was established with the abnormal persistence of vaccine aluminium in the muscle [4]. The vaccines called into question are primarily the hepatitis B vaccines that were widely used in France in the mid-1990s.

The InVS's exploratory investigation of the first 53 cases confirmed the Germmad hypothesis, according to which the observed histological lesion was linked to the

injection of vaccines containing aluminium hydroxide. However, in the absence of a control group, it was not possible to draw any conclusions about an association between the presence of this histological lesion and a particular symptomatology [5].

In September 1999 and then in June 2000, the data collected by the Germmad and the InVS were presented to the World Health Organization's (WHO) Global Advisory Committee on Vaccine Safety (GACVS). International experts in the field of myology, vaccinology, immunology and pharmacovigilance were involved in this Committee's work. The GACVS also concluded that there was a very likely causal link between the administration of a vaccine containing aluminium hydroxide and the presence of the histological lesion characterizing MMF [6], but considered that the available data did not allow for a conclusion to be drawn on the existence of an association between the histological lesion and a specific pathology. It issued recommendations concerning the necessity of continuing the studies seeking to characterize MMF and to search for a statistical association between the presence of MMF as a histological entity and the occurrence of MMF as a specific clinical entity.

Thus, a study of control cases was conducted by the Agence française de sécurité sanitiare des produits de santé (Afssaps [French Agency for the Safety of Health Products]), which the InVS contributed to by providing its epidemiological expertise. The principal conclusion in the study was that "it did not permit, under any circumstances, the conclusion that there is an association between the presence of aluminium in macrophages and the occurrence of a disease". This led the scientific committee of the Afssaps to conclude in May 2004 that "no specific clinical syndrome is found to be associated with vaccination with vaccines containing an aluminium adjuvant".

After examining all the new available data concerning MMF, and especially the French data, the GACVS also concluded, in December 2003, that the "persistence of macrophages containing aluminium at the point of vaccination is not associated with a disease or with particular clinical symptoms" [7].

In 2001, the same authors suggest that MMF is also associated with damage to the central nervous system [8] in 14 out of 92 patients in their cohort. This central nervous system damage is described as likely or certain multiple sclerosis in the seven cases described. MMF is then connected to chronic fatigue syndrome [9].

The next step is the bringing to light of cognitive problems in patients suffering from MMF via two studies [10, 11] conducted in patients that belonged to the cohort that was monitored by this team (without any description of the selection criteria). This was done by way of neuropsychological tests that are difficult to interpret. The neurological disorders are interpreted as being the result of the migration of aluminium nanoparticles across the blood-brain barrier and their deposit in the brain.

In 2011, MMF is incorporated into a larger syndrome, the ASIA syndrome (Autoimmune/Autoinflammatory Syndrome Induced by Adjuvants), which includes MMF, Gulf War syndrome and problems induced by silicone implants, described by Shoenfeld *et al.* [12].

The most recent step aims to show aluminium's mode of migration and penetration into the brain by the "Trojan horse" mechanism, which involves monocytes that phagocyte the aluminium nanoparticles and transport them into various organs, including the brain, by making them cross the blood-brain barrier. These hypotheses rely on an experimental study in mice [13] which has still to be confirmed.

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2 - Macrophagic myofasciitis (MMF). Literature review

For the past fifteen years, a controversy has arisen, primarily in France, relating to the safety of aluminium when used as an adjuvant. The source of this controversy is the work of only one French team that has published on this topic since 1998, even though this adjuvant has been used around the world in most inactivated or subunit vaccines for over eighty years.

2.1 - Macrophagic myofasciitis: a primarily French ailment

The review of the literature available on 23 April 2013 was carried out based on a PubMed bibliographical search with the keyword “macrophagic myofasciitis,”, i.e., 72 references including 22 from the same team (with R. Gherardi and/or F. Authier and/or P. Cherin as the author or co-author).
Gherardi and Authier’s most recent review, published in Lupus 2012 [1], documents **1,000 cases** of MMF recorded in France by the E3M association (Association d’entraide aux malades de myofasciite à macrophages [Macrophagic Mayofasciitis Assistance Association] (Victims of Vaccine Aluminium)), including **457 who were monitored by the Centre de référence des maladies neuromusculaires de Créteil [Créteil Referral Center for Neuromuscular Diseases]**.
In contrast and as highlighted by the authors, 14 years after the original publication by Gherardi *et al.* [2], other countries (the United States, Germany, Portugal, Spain, the United Kingdom, Ireland, Korea and Australia) have only reported sporadic cases.

2.2 - Analysis of publications by the team of Gherardi and Authier

As indicated above, this team is the only one to have published on “series” of patients suffering from MMF. Overall, this team’s successive publications refer to an increasing number of cases recorded by their center over the years and present data from a limited number of their cohort’s patients in each publication. The authors do not specify the criteria used to select these patients (although they usually mention “successive patients”), nor do they specify whether the patients included in the various publications overlap or not.

- In 1998, MMF was described as an emerging disease in a publication by Gherardi *et al.* that appeared in the Lancet [2]. In fact, in 1982 R.E. Mrak [3] reported on the presence of unusual granulomatous lesions in the biopsy of the quadriceps muscle of an eight-month-old infant who had congenital myopathy. This child died quickly from respiratory complications of the disease and the autopsy did not find any similar lesions in the other muscles. The authors were able to experimentally reproduce this type of lesion in animals with IM (intramuscular) injections of diphtheria-tetanus and pertussis vaccines and showed that the granules present in the histiocytes were made of aluminium salt.
  The 1998 Lancet publication referred to observations of 18 adult patients compiled between May 1993 and December 1997, including 14 that could be used. These patients were seen for polymyositis (10), rheumatic pain (4), muscular dystrophy (1) or suspected mitochondrial disease (3). Four were hospital staff and 3 had histories of connective tissue diseases (systemic lupus erythematous (SLE), rheumatoid polyarthritis (RP) and Hashimoto’s thyroiditis). These patients’ clinical pictures consisted of:
  - myalgia in 12 out of 14 cases;
  - arthralgia 9/14;

- muscle weakness 6/14;
- general symptoms 6/14: asthenia and fever;
- changes in the electromyogram: 4/12;
- an increase of CPK (creatine phosphokinase) 6/14.

All these patients had a biopsy of the deltoid muscle within a time frame ranging from 3 to 48 months after the onset of the clinical symptoms. This biopsy revealed unusual lesions that were described as MMF. The lesion consisted of centripetal infiltration, from the fascias to the subjacent muscle, of cohesive, non-epithelioid, PAS+ (Periodic Acid-Schiff) macrophages with large cytoplasms of fine granularity associated with the presence of certain CD8+ (cluster of differentiation 8) T (thymus) lymphocytes and minimal myocytic pain.

Ten of these patients were treated with a combination of antibiotics (under the hypothesis of Whipple's disease) and corticosteroids: eight improved and the other two stabilized.

- This team published a second publication in the Revue de médecine interne [Review of Internal Medicine] in 1999 [4]. In this paper, the authors report that in 1996, as part of the creation of the new research group Germmad, a branch of the AFM, two patients with myopathy were observed who had a histological aspect that had never been seen before. The principal French myology centers were asked to revisit their atypical muscle biopsy slides from the last 30 years. Thus, by December 1998, 35 observations had been compiled, the first one having been observed in Bordeaux in 1993.

  The analysis covers 22 cases having undergone a deltoid muscle biopsy with the presence of the MMF lesions described above. These patients are adults (with an average age of 43 years) with a M/F *gender ratio* of 1.3, including five hospital workers. They were seen for polymyositis (11), rhizomelic pseudopolyarthritis (RPP) (5), mitochondrial cytopathy (4), muscular dystrophy or myopathy (2). Five presented with an immune dysfunction pathology (sarcoidosis, SLE, RP, thyroiditis and Sjögrens syndrome). Additionally, one of these patients had cancer, another had myelodysplasia and kidney cancer was discovered in one patient during the investigations.

  The symptoms presented by these patients were:

  - myalgia: 91% primarily affecting the lower limbs;
  - arthralgia: 68%;
  - asthenia: 55%;
  - fever: 32%;
  - unexplained dyspnea or cough: 14%;
  - for the additional exams: 6/17 had a myopathic electromyogram (EMG); 50% had an increase of CPK, 37% had an increase in the erythrocyte sedimentation rate (ESR) and 30% had an increase of C reactive protein (CRP).

  The progression of these 22 patients was as follows:

  - four were not treated: three got worse and one remained stable;
  - eight received corticosteroids with a complete response in four cases and a partial response in four cases;
  - seven received antibiotics and corticosteroids with a clear improvement;
  - three received antibiotics alone, two of whom improved.

- In 2000, the same team published two other articles [5-6]: the first references 65 recorded cases and the second references 70 cases, but the analysis in these two publications seems to cover the same 22 cases as those reported in

the 1999 publication [4].

- Starting in 2001, links between MMF and vaccination, on the one hand, and the idea of symptoms stemming from damage to the central nervous symptom appear in this team's publications:
- The article by Cherin *et al.* [7] published in 2001 reports three adult cases with muscle weakness linked to inflammatory inclusion-body myositis, proven by biopsy. A new biopsy, justified by an aggravation, confirms the initial diagnosis but reveals signs associated with MMF. In one case, the initial biopsy was done in the quadriceps, while the biopsy showing the MMF lesions was done in the deltoid, with vaccinations (against the hepatitis B virus (HBV) and against the hepatitis virus (HAV)) performed between the two biopsies. In one case, the two biopsies were performed in the deltoid and there was no vaccination (between the two?) and in the third case the vaccination (HBV) was performed before the onset of the disease, with the first biopsy performed in the quadriceps and the second one in the deltoid.

- The article by Authier *et al.* [8] published in 2001 indicates that among the 92 cases of diagnosed MMF, 14 have symptoms of damage to the central nervous system. Only seven of these cases are presented in the article: five have certain muscular dystrophy (MS) and two probably have MS. These patients all had a vaccine containing aluminium (4 HBV, 1 tetanus+HBV and 2 tetanus) 3 to 78 months (33 months on average) before the biopsy. The biopsies were conducted due to pain that is not unusual in patients suffering from MS (back, muscle or joint pain) [9] in the midst of the controversy regarding the hepatitis B/MS link.
- The Gherardi *et al.* [10] article published in 2001 reports more than 130 cases of MMF recorded in France (38 additional cases compared to the previous publication from several months earlier!). The presented study consists of an ultrastructural and chemical muscular study conducted in 40 patients suffering from MMF compared to 40 patients suffering from dermatomyositis and 40 suffering from congenital muscular dystrophy. This study shows that the inclusions detected in the patients suffering from MMF are of aluminium. In this same study, the incidence of myalgia in 46 patients suffering from MMF is compared to that of 1,252 patients who were biopsied without an MMF diagnosis: the incidence is 85% in the MMF patients compared to 45% in the control group. This result can be easily explained because myalgia was the primary indication for conducting a biopsy in the patients with an MMF diagnosis.

- Vaccination status is studied in 50 MMF patients: all received vaccines containing aluminium in the last eight years: 84% received the HBV vaccine, 19% received the HAV vaccine and 58% received the tetanus vaccine (which is not surprising for a vaccine recommended to be given every 10 years). There is no control group. The proportion of individuals vaccinated against hepatitis B may appear elevated. Certain MMF series showed a high percentage of hospital workers: these individuals have easier access to specialized facilities where muscle biopsies are performed. Furthermore, the time period when the patients were recruited is the same period when the controversy surrounding the side effects of vaccination against hepatitis B was at its height. It can be

assumed that the vaccinated individuals were more likely to have visited for non-specific pathologies that they thought were linked to the vaccine and the idea of a vaccination could have influenced the decision to biopsy a muscle in which the probability of finding aluminium was high.
Finally, this article reports on the experimental reproduction of MMF lesions by intramuscular (IM) injection of the GenHevac B® vaccine in rats.

- In a brief 2003 note [11], Authier *et al.* compare the symptomatology of a series of 30 patients with MMF ("unselected") to the criteria for the definition of chronic fatigue syndrome from the *Centers for Disease Control and Prevention* (CDC) and Oxford. Fatigue is found in 93% of patients, which is disabling in 87%. The authors consider that 53% of the patients meet the criteria for the definition of chronic fatigue syndrome (47% for the CDC definition, 40% for the Oxford definition and 37% for both); 19/20 had received a vaccine containing aluminium before the onset of symptoms.
- Finally, in 2012, a general review of MMF is published by Gherardi and Authier in the Lupus review [1]. The authors estimate that 1,000 MMF cases have been documented in France. They estimate that the frequency of this disease in France is linked to three causes:
  - the transition from the subcutaneous (SC) route to the intramuscular route (IM) for the administration of vaccines in the 1990s (without asking whether other countries have different practices, which they do not);
  - the magnitude of the vaccination campaign against hepatitis B in certain adults at that time (real);
  - their team's choice to perform muscle biopsies in the deltoid (whereas most countries recommend avoiding biopsies in muscles into which vaccines are injected...).

  In this article, the authors state that the MMF lesion is universally recognized by referencing their own publications. They also consider, without scientific reasoning, that the persistence of this lesion beyond 18 months is pathological (Authier specifies two years in an expert report for the Cour administrative d'Appel [French Administrative Appeals Court]). They estimate that this time period exposes children to a risk of coincidence (whereas this lesion has rarely been described in children, cf. *infra*), whilst in adults, the percentage of false positives only represents 5 to 10% of the biopsies of patients who were completely asymptomatic (hence, we can ask why they were biopsied) or who were examined for a genetic disease. Here also, this estimate is not supported by any references.
  The authors report on the review of 457 records of adults who were monitored in their center between 1994 and 2011 (270 examined and biopsied in their center and 187 referred to another center after diagnosis). It should be noted that 70% are women while the M/F *gender ratio* was 1.3 in the previous publications. The median age is 45 years old. These patients received between 1 and 17 injections of vaccines containing aluminium (hepatitis B vaccine in 85% of them) in the ten years preceding the diagnosis. The average length of time between vaccination and the first symptom of the disease is 7 months and the length of time between vaccination and the appearance of myalgia is 11 months (although the authors do not consider the persistence of aluminium in the muscle to be pathological until it exceeds 18 months...). The

average length of time between the vaccination and the biopsy is 65 months.
The clinical symptoms presented by these patients are the following: diffuse myalgia for more than six months in 89% of cases. This myalgia starts in the lower parts of the body, then ascends, affecting the paravertebral muscles, and then becomes generalized. Arthralgia is no longer mentioned. The second major symptom is fatigue, which progresses for at least six months in 77% of cases. Attention and memory problems are also reported in 51% of cases. This point will be specifically addressed below, but it is noted here that the neurological symptoms reported in these patients are very polymorphic (cf. *supra*) and that the comparison to lesions caused by acute or chronic aluminium poisoning is highly debatable. Dyspnea is noted in 50% of cases. Sleeping problems and headaches are also cited. A decrease in muscle strength is rare. About 50% of patients have a myopathic reaction in the EMG or an increase of CPK. The authors mention that 15% to 20% of patients developed an autoimmune disease whereas it would be more precise to say that these patients with a proven autoimmune disease (cf. *supra*) had a muscle biopsy because they complained of muscle pain. It is not ruled out that a previous history of vaccination played a role in the decision to perform a deltoid biopsy.
It should be noted that fever or the existence of an inflammatory biological syndrome are no longer mentioned in this review. Similarly, the beneficial effect of corticotherapy ± antibiotic therapy is no longer reported.
It is possible to compare the clinical symptoms reported in this review to the summary carried out by Israeli *et al.* [12] based on prior publications by the same authors (Table 1).

Table 1 – Summary of reported clinical symptoms [12]

Symptoms	Percentage of patients
Myalgias	88–91
Arthralgias	57–68
Marked asthenia	55
Muscle weakness	45
Fever	20–32
Elevated CK levels	29–50
Increased ESR	37
Myopathic EMG	35
Demyelinating CNS disorder	9
Multiple sclerosis diagnosis	33
Chronic fatigue	50–93
Hashimoto's thyroiditis	2.7
Other autoimmune-related diseases (RA, Sjogren)	6.7

Furthermore, the article published in the Lupus review [1] includes MMF in chronic fatigue syndrome, and in a larger context including Gulf War syndrome, which is attributed to the aluminium in anthrax vaccines (but which has not been demonstrated), and to a larger syndrome, "adjuvant disease" (ASIA), which has been described more recently by Shoenfeld *et al.*[13].
Finally, this review touches on the physiopathological aspects of MMF based on arguments which merit commentary:

- The predisposing genetic factors rely on:

- One mother-son familial case [14] of debatable interpretation given the pathology presented by the mother who had successive diagnoses of inflammatory myositis (biopsy), algodystrophy and then MMF, and the pathology of the 11-year-old child who complained of fatigue and myalgia;
- the observation of cases in two identical twins with symptoms that appeared several months after a vaccination against hepatitis B performed at the age of 64 and who had an identical HLA (*Human Leukocyte Antigen*) (which is not very surprising), notably DRB1*01 [15];
- the HLA-DRB1*01 group is a contributing factor that is regularly cited in articles. Beyond the previous observation, this relies on a study of ten patients [16] from the Marseilles region, including at least six who have a symptomatology that is very different from that found in the reported cases of MMF: this HLA group is carried by 66% of the MMF patients compared to 17% of controls. It should be stressed that no confirmatory study has been published;
- experimental studies in rats [17]: the authors cause MMF lesions by injecting hepatitis B vaccine into Sprague-Dawley (SD) rats (who have balanced Th1/Th2 immunity) and Lewis rats (who have Th1-oriented immunity). By comparing the surface area of MMF lesions and its progression over time, they believe that they show that the muscle's aluminium clearance is different in the two groups: in reality, an examination of the graphs shows that the SD rats have lesions with initial surface areas that are much higher and that the reduction of the surface areas of these lesions (one can ask about the viability of this determination) appears to take place over time without a clear speed difference between the two groups.

➢ An overload of aluminium: this relies on the case [18] of a patient diagnosed with MMF three years after the beginning of a symptomatology that is labeled as chronic disease syndrome. Problems began several months after having received five vaccine injections containing aluminium. An overload of aluminium is demonstrated via urinary excretion that is "significantly higher than what is normal at this age," the values of which are not provided.

➢ The addition of silicone to drinking water increases the excretion of aluminium for several months before returning to low values. There is no clinical improvement. This unique case goes against prior data showing that patients suffering from MMF do not have an aluminium overload.

➢ Migration of aluminium into the brain. Nanomaterials are referenced, which appears to be erroneous: the aluminium hydroxide aggregates that are deposited in the muscles are crystals whose dissociation is only possible at pH 2, which is not a physiological pH. It is therefore not possible that aluminium adjuvants release aluminium nanoparticles in a physiological state [19]. Furthermore, after an injection of aluminium, the visceral deposit is low and occurs primarily in the bones, and the brain is the organ that retains it the least [20]. Finally, the "Trojan horse" mechanism (migration into the brain via monocytes loaded with aluminium nanoparticles that cross the blood-brain barrier) is suggested based on recent works. The cited references [21-22] are presentations at conferences in 2010 and 2011. An experimental study in mice has

just been published by this same team in support of this theory [23].

The article, “Slow CCL2-dependent translocation of biopersistent particles from muscle to brain” recently published by Z. Khan *et al.* [23], evaluated the role of chemokine CCL2 and macrophages in the biodistribution of nanoparticles after IM injection, focusing on the question of aluminium salts in the particular forms that are present particularly in vaccines and the mechanism of their cerebral biodistribution. According to the authors, the clinical symptoms associated with aluminium salts are typical of ASIA “autoimmune/autoinflammatory syndrome,” which is also observed in patients exposed to silicone gel (7). Again according to the authors, the persistence of macrophages that are loaded with alum more than 12 years after injection is associated with MMF.

A preliminary study that only reported on additional data had shown a specific and significant increase in the blood levels of the proinflammatory chemokine CCL2 (C-C Motif Ligand 2) or MCP-1 (Monocyte Chemotactic Protein-1) in 44 patients suffering from MMF, who had an average age of 44 and who were 2/3 women, compared to 10 healthy subjects without inflammatory symptoms. This increase, frequently found in infectious and inflammatory diseases and atherosclerosis, led the authors to test the hypothesis of a predisposing genetic factor linked to polymorphism of the CCL2 gene in the physiopathology of MMF. To do this, they conducted a study comparing 516 controls to 252 patients suffering from an “MMF syndrome” defined by: 1) the onset of clinical signs prior to a vaccination containing alum, 2) diffuse arthromyalgia and/or cognitive or sleeping problems for more than 6 months, 3) histological lesions of the deltoid typical of MMF in a biopsy performed more than 18 months after vaccination, 4) without any other etiopathogenic factor. The MMF group, with an average age of 45 years and composed of 2/3 women, had received, on average, 5.2 (1-17) vaccinations in the previous 10 years, with the average length of time since the last vaccination being 5.5 years. The analysis of two CCL2 polymorphisms did not show any significant difference between the MMF group and the controls. One of the alleles (---927G) was observed in 20% of patients compared with 16% of controls (non-significant difference) and the haplotype carrying this allele was 1.28 more frequent in the MMF group compared to the controls (p=0.088). The analysis of these genetic polymorphisms in the initial group of 44 patients suffering from MMF (it is not specified whether these patients are included in the 252) does not establish any associations between these CCL2 alleles and the CCL2 serum levels. These non-significant results lead the authors to test the role of CCL2 in the cerebral biodistribution of aluminium salts in mice after IM injection.

To test this hypothesis in mice, they evaluate whether various preparations containing various aluminium salts injected into the muscle are absorbed by dendritic cells that then migrate to distant organs via a mechanism linked to phagocytosis and to the action of the chemokines CCL2 or MCP-1. The authors use a model with adult C57Bl/6 mice, mdx mice with a permeability anomaly of the blood-brain barrier, mice deficient in CCL2CX3 or CR1-GFP+ mice. In the 1st series of experiments, the IM injection of a quantity of anti-HBV vaccine (of

unknown origin) containing 0.5 mg aluminium, meant to reproduce 5 human vaccine doses, induces an inflammatory reaction with infiltration of macrophages loaded with aluminium on D4, followed by a 50% local decrease of the aluminium level from D4 to D21, and accumulation of aluminium in the muscle, spleen and brain up to 6 and 12 months. A 2nd series of experiments uses IM injections of 500-nm fluorescent latex beads that migrate to the drainage lymph node in 1 H. They are captured and concentrated by inflammatory macrophages on D4 then their level decreases locally, followed by blood transit and accumulation in the spleen and the brain from D21 to D90-180. These macrophages loaded with beads are mostly detected in the gray matter in a diffuse manner from M3 to M12. This accumulation is interrupted by exeresis of the drainage lymph nodes or IV (intravenous) injection while the IM injection of these particles in the mdx mice increases cerebral accumulation, showing that intracerebral transit requires an IM injection and transit into the drainage lymph node, and is facilitated by the porosity of the blood-brain barrier (BBB). A 3rd series of experiments uses the fluorescent particles of recovered aluminium that are meant to "mimic" the aluminium salt-based adjuvants. The same IM injections induce the same effects as earlier, amplified after SC injections, whereas the IM injections in mice deficient in CCL2 reduce tissue distribution by 80%, the recombinant CCL2 IM injection in normal mice increases the tissue distribution and the intracerebral CCL2 injection increases cerebral accumulation, which suggests a cerebral penetration mechanism that is dependent on CCL2. From this test on a very small cohort of mice (generally three to six mice per point), the authors draw the following conclusions: 1) macrophages have a transportation role, which in fact has already been largely demonstrated and which is the basis of the principal of induction of any immune response, vaccinal or not, and 2) CCL2 has a chemotactic effect in the cerebral distribution of these macrophages, which is also known.

The authors acknowledge that this cerebral redistribution is nevertheless small, on a scale of 1 out of 107 cells, which is compatible with the generally good tolerance of alum, but suggesting that the immaturity of the blood-brain barrier, "over-immunization," genetic factors or age facilitate it. They suggest that the regulatory authorities should reinvestigate the effect of increasing doses of alum salt-based adjuvants. However, it should be pointed out that in no cases did these studies, obtained with microparticles having no relation to real aluminium salt-based adjuvants, actually demonstrate these suppositions.

- The cognitive problems associated with MMF merit particular attention. Starting with the initial publications, the team that distinguished MMF has worked to demonstrate that this muscle disease was associated with neurological problems linked to central lesion (cf. *supra*). This led to the inclusion of and emphasis on patients with neurological diseases that could be labeled, including multiple sclerosis. The data, on which the authors rely to describe this central neurological damage, draw on two studies [24-25] that were financed (in addition to the last study) by the Association française de lutte contre les myopathies (AFM) but above all by Entraide aux maladies de la myofasciïte à macrophages, E3M [Macrophagic Myofasciitis Assistance

Association], which is a patient organization whose goals are clearly to have MMF recognized as the consequence of an adverse effect of vaccination and to obtain compensation for the "victims" from the State:

- The first study demonstrated [24] that this disease is associated with cognitive problems via a battery of neuropsychological tests carried out on a cohort of 25 patients suffering from MMF. These include deterioration of visual and verbal memory, and problems with executive functions: attention, memory, planning and deterioration of the left ear's auditory capacity (?). This study relies on the idea of a migration of nanoparticles and of their crossing the blood-brain barrier (whereas vaccine aluminium deposited in the muscle does not seem to be capable of forming nanoparticles: cf. supra). The battery of tests applied to these patients (on which we cannot comment) was chosen afterwards, based on a retrospective study of 22 MMF patients. Then 11 patients, described as non-selected, were compared to 11 patients with an inflammatory disease causing chronic pain. Fourteen patients, described as non-selected, were subsequently added to the 11 initial MMF patients, together forming a "prospective" cohort of 25 patients. The reproducibility of these tests is not known and it is not specified whether they were carried out by the same person. The bases on which the normality thresholds were established were not indicated. Furthermore, it is regrettable that the control group is limited to patients suffering from inflammatory diseases. If this design is supposed to answer the question of whether or not the cognitive problems observed are the consequence of chronic pain and depression, it does not answer the question of the specificity of cognitive disorders. These patients were free of central neurological problems at the time of the biopsy. The MRIs (Magnetic Resonance Imaging) of these patients were normal for 16 of them (60%), showed non-specific anomalies in the form of hyperdensities in T2 of white matter in seven of them, sequelae of a cerebral ischemic stroke in 1 and a carotid aneurysm in 1. One of the patients died unexpectedly whilst sleep without an autopsy being carried out. The authors consider that there is no correlation between the MRIs and the observed anomalies. Finally, it should be noted that, in the discussion, the authors indicate that 60% to 80% of patients suffering from chronic fatigue syndrome had cognitive problems.

- The second study [25] concerns the monitoring of 30 MMF patients who, over an unspecified period, had several neuropsychological evaluations. This is a retrospective study including patients "monitored in the Créteil center" without this study's inclusion criteria being provided. There is no control group. The comments made for the previous article concerning the reproducibility of the tests and the individuals who carried them out are also valid for this article. The authors indicate that during an initial evaluation 60% of patients appear depressed whereas after another test only 37% do, which calls its reproducibility into question. The included patients appear to be very different from the initial cohort because the MRIs are normal in only 48% of cases; cortical atrophy is seen in 5/25 patients and callosum atrophy is noted in three patients. The SPECT (Single Photon Emission Computed Tomography) scan is abnormal in 89% of included patients. According to the authors, there is no correlation between the MRI anomalies and the cognitive performance which is

altered in all the patients for at least one test. Over the successive tests, the cognitive functions improve in 20% and remain stable or slightly worsen in the others.

2.3 - Macrophagic myofasciitis and children

Infants receive numerous vaccines, particularly during the first year of their lives. The vaccines against diphtheria, tetanus, poliomyelitis, whooping cough, *Haemophilus* and hepatitis B require three or four doses of vaccine for prime vaccination depending on the country. The vaccine against invasive pneumococcal infections requires three or four doses. In Europe, most countries vaccinate against meningococcal C with a vaccine scheme that depends on the age at which the vaccination is given. It consists of either three injections (for vaccination before the age of one) or one sole dose (for vaccination after the age of one). All these vaccines have an aluminium adjuvant. Thus, infants are heavily exposed to aluminium adjuvants, especially when the doses given are compared to body mass. Based on the hypothesis that vaccine aluminium is responsible for the genesis of MMF, as described by Gherardi and Authier, the study of the MMF cases reported in children is of capital importance. The Créteil team's publications do not mention pediatric cases, which makes sense in that children are not cared for in that type of facility. The investigation conducted by the InVS in 2001 [26] reports two cases in children, without providing any details. Additionally, a familial case involving a mother and her 11-year-old child was published in France [14].
Outside of France, 33 cases were found in the published literature in the form of case reports or short series. They come from Saudi Arabia (8 cases), Brazil (3 cases), the United States (6 cases), Spain (7 cases), Italy (1 case), Israel (6 cases) and Germany (2 cases).

- Müller HD *et al.* [27] report two cases of children presenting with congenital muscular dystrophy (merosinopathy and dystrophinopathy). Their muscle biopsies revealed MMF lesions. The authors consider that, as in the other pediatric cases reported in the literature, MMF is a coincidence and that this type of disease will disappear when noninvasive genetic tests replace the muscle biopsy.

- Lach B *et al.* [28] report eight cases of children aged from seven months to six years old and in whom a typical MMF lesion was observed in the quadriceps biopsy performed within two months to one year following the last administration of a vaccine containing aluminium. In five cases, the muscle biopsy allowed for a diagnosis other than MMF (infantile spinal amyotrophy: two cases; Duchenne's disease: one case; phosphoglycerate kinase deficit: one case; cytochrome c oxidase deficit: one case). The authors consider that there is no correlation between the histological lesion and the clinical signs, and that MMF is a localized impression of a vaccination containing an aluminium adjuvant more than it is an inflammatory muscle disease.

- Kalil RK *et al.* [29] report three cases of children aged from 13 months to three and a half years old, vaccinated normally in whom the muscle biopsy revealed MMF lesions. These children had muscle hypotonia in two cases and myotonia in one case. The authors consider that the clinical history does not favor a relationship between the histological lesions and the systemic symptoms, and that there is probably just a coincidence between these two phenomena.

- Gruis KL *et al.* [30] report four cases of children examined for a motor delay, hypotonia and growth delay without an established diagnosis for whom the muscle biopsy of the quadriceps revealed MMF lesions. No central nervous damage was demonstrated. These children progressed favorably.
- Rivas E *et al*. [31] report seven cases of children aged from 5 to 33 months (m=17.3 months), vaccinated against hepatitis B at the normal customary ages (0, 2 and 6 months) as well as against tetanus (2, 4, 6 and 18 months) in whom MMF lesions were revealed in the quadriceps biopsy performed between less than two months after the last vaccination (six times) and 15 months after (one time). Six of seven children presented cerebral abnormalities in their MRIs, one had an abnormal EMG and two had elevated CPK. The final diagnosis was: West's syndrome (two cases), Leigh's disease (one case) and mitochondrial myopathy (two cases). Two cases remained undiagnosed (hypotonia and motor delay for one; hypotonia and bilateral ptosis for the other). The authors consider that, in the majority of cases, MMF constitutes a transitory reaction to the administration of a vaccine containing aluminium and that the histological lesions observed in their patients do not clearly correlate with clinical disorders.
- Di Muzio A *et al.* [32] report the first (and apparently only) Italian case. It involves an infant examined at the age of seven months old for irritability, hypotonia and motor delay. He had very elevated CPK. At 3, 4 and 10 months, he received the DTCaHib and Engerix B® vaccines in the quadriceps and the biopsy was performed at 12 months. The child subsequently received degressive corticosteroid treatment over one year and gradually improved. At 29 months, the examination of the child was completely normal and his CPK normalized.
- Nevo *et al.* [33] report six observations in Israel of children examined for psychomotor development disorders combined with hypotonia. These children are of Arab origin and 5/6 are from consanguineous marriages with family histories of children who passed away at an early age. The described disorders present with microcephaly (three cases) and epilepsy (two cases including one case of myoclonic epilepsy). In one case (born at 1,200 g), the mother had noted a cessation of fetal movement several weeks before birth. In one case, the biopsy showed an enzyme anomaly in the respiratory chain. One case of a deceased patient showed serious encephalic malformations in the autopsy. The biopsies were carried out in the quadriceps at times ranging from 2 to 21 months relative to the last vaccine injection. The authors conclude that genetic factors are probably responsible for expressivity differences in the disease, but at least three of their patients have symptoms that have nothing to do with MMF.
- Lacson AG *et al.* [34] report two cases of MMF. The first case concerns a child with digestive disorders since birth in connection with chronic intestinal pseudoobstruction. This disease (otherwise well known and linked to smooth muscle motor disorders) is combined with dysautonomia signs (mydriasis and abnormal pupillary reflexes) that led to a biopsy of the quadriceps muscle at the age of five, in which he received vaccines at the ages of two, four and six months. This child improved under antibiotic, antireflux and parenteral feeding treatment. The second patient presented with a developmental delay combined with hypotonia (he was born by cesarean due to fetal distress). The quadriceps biopsy was done at the age of one. He had been vaccinated in the

same manner. This child gradually improved. The authors support epidemiological studies on this entity in the United States while at the same time reckoning that the lesion observed in their patients did not seem to have contributed to their patient's clinical symptoms.

2.4 - Adult macrophagic myofasciitis outside of France

Apart from in the French team's articles, no series of adult MMF patients is found in the international literature. Several review articles from a limited number of authors substantiate the theories of Gherardi and Authier [12,35-39] or broaden the framework to include an ASIA adjuvant disease [13,37-39]. Others, while recognizing the MMF entity, do not give a formal opinion on its significance [40].
In total, ten cases of adult MMF have been published. They come from the United States (two cases), Korea (one case), Germany (two cases), Australia (one case), Ireland (one case), Portugal (one case) and the United Kingdom (two cases).

- The American cases involve: a patient who actually had histiocytosis whose muscle biopsy revealed MMF lesions [41]; a patient presenting with multiple autoimmune diseases (hemolytic anemia, diabetes, celiac disease and vitiligo) that started at the age of 33, in a familial context [42]. Examined at 35 years old for fatigue, myalgia, arthralgia and decreased muscle strength, the MMF lesions were discovered by a biopsy of the deltoid, in which the patient had received an anthrax vaccine five years earlier. The corticosteroid treatment and immunoglobulin infusion were ineffective. The authors believe that the existence of multiple autoimmune diseases could have contributed to the genesis of this extreme fatigue and to the failure of the treatment. □
- The Korean case [43] involves a 59-year-old man examined for localized muscle induration with unilateral myofasciitis lesions. The muscle biopsy revealed MMF lesions. No link to vaccination was shown and the authors consider that explanations other than vaccination should be sought.
- The German cases involve:
    - a 67-year-old man presenting with progressive muscle pain and generalized muscle weakness for the previous six weeks [44]. The muscle biopsy revealed MMF lesions but the ultrastructural study did not show the presence of aluminium. The vaccination history is not mentioned but the antibody dosage is compatible with a tetanus vaccination. The patient recovered completely with treatment that included corticosteroids and azathioprine over a two-year period (*Nota bene*: this article is in German. Only the abstract was consulted).
    - a 62-year-old woman [45] presenting with progressive muscle weakness for the previous ten years [44]. The muscle biopsy revealed MMF lesions that the authors deem to be unrelated to the symptomatology.
- The Australian case [46] involves a 32-year-old man in whom elevated CPK was fortuitously discovered during examinations for gastroesophageal reflux disease (GERD). It should be noted that his father passed away from neuromuscular disease and that one brother had muscle fasciculations. The muscle biopsy was carried out even though the patient did not manifest any signs of muscle disease. The patient was vaccinated against hepatitis A three years previously, against polio and had received a hepatitis A booster two

years before the biopsy that revealed signs of MMF. The authors consider that this observation is a coincidence in a patient presenting with elevated CPK of unknown cause.

- The Irish case [47] involves a 50-year-old patient presenting with pain in the left chest and in the upper part of the body with elevated CPK. His biopsy of the left deltoid revealed MMF lesions. The patient's vaccination history dated back more than ten years before the first clinical signs. Corticosteroid therapy improved the clinical signs and decreased the CPK. However, the signs reappeared when the treatment was stopped.
- The Portuguese case [48] involves a 47-year-old woman examined for diffuse mechanical arthralgia, lower back pain, asthenia and fatigue for the previous four years. A muscle biopsy revealed MMF lesions. The authors state that no link could be established with a vaccination, even though the patient was vaccinated against tetanus and whooping cough three years previously. Corticosteroid therapy led to a slight improvement.
- The cases from the United Kingdom involve:
  - one 54-year-old woman presenting with a clinical picture including generalized fatigue with muscle weakness [49] for the previous six months, preceded by lymph node swelling and arthralgia. A muscle biopsy revealed MMF lesions with inclusions corresponding to aluminium particles. This patient was vaccinated against hepatitis B 11 years earlier.
  - the preceding article's authors reference a case presented in a poster at the *Centenary Meeting of the British Neuropathological Society* (London, January 2001) [50]. It involves a 32-year-old man examined for progressive pain with muscle rigidity in the thighs and shoulders that was triggered by muscle activity. The deltoid biopsy revealed MMF lesions. No reference was made to a prior vaccination.

2.5 - Summary and discussion

Macrophagic myofasciitis is an indisputable histological entity whose association with aluminium used as an adjuvant in vaccines is recognized. Moreover, this fact has been known since 1982 [3].
The question is how to interpret this lesion.

- It is currently most often admitted that this lesion only represents a "vaccine tattoo" [51] linked to the persistence of aluminium in the muscle that was injected with the vaccine [52]. This prompted neuropathology specialists to recommend against the use of the deltoid for muscle biopsies, to the extent that the probability of finding stigmas of the prior injections was high given that this muscle was used for the injection of vaccines in adults.

  It must be stressed that questions have still to be answered on this subject:

  - what is the normal duration of persistence of these lesions and after what period of time has the aluminium in the muscle been absorbed?
  - does the persistence of these lesions beyond a certain time frame constitute a pathological characteristic? An experimental study in 24 monkeys reproduced MMF histological lesions [52] (without any clinical symptoms), which were still present 12 months after the injection;
  - are there factors, particularly genetic factors, as suggested by the WHO

[53], that might cause certain individuals to eliminate aluminium from their muscles more slowly than others?

All these questions have still to be answered.

- The theory of a causal link between the MMF histological lesion and the occurrence of a systemic disease is supported by the French team on the basis of both clinical and experimental studies conducted over a period of fifteen years. Two types of explanations have been advanced to attribute systemic symptoms to aluminium from vaccines:
  - first, this group has suggested that the persistence of the aluminium adjuvant was responsible for immune stimulation that exceeds its goal and triggers an autoimmune process. The number of patients suffering from autoimmune disease, particularly MS, in the initial publications, suggest a recruitment bias for this type of patient starting from when they complained about muscle pain, which is relatively commonplace. The supporters of the ASIA concept still defend this theory [12, 13, 36, 37].
  - second, the group developed the concept and focused on the migration of aluminium into the brain with accumulation and central neurological toxicity.
- As highlighted in the analysis of the Créteil team's scientific output, numerous comments can be made on their work:
  - the patient cohort progressively filled out but the inclusion criteria of the patients taken from the cohort in the successive studies were not specified;
  - the clinical symptomatology of the systemic symptoms of MMF has varied over time (see supra). Myalgia is an essential sign of the disease (it would be surprising if the opposite were true to the extent that this symptom has, according to the evidence, been the most frequent indication criterion for the muscle biopsy). The second symptom is fatigue, which helps bring MMF closer to chronic fatigue syndrome, a syndrome with an imprecise definition and which many specialists are raising questions about. Arthralgia, initially dominant, has practically disappeared from the latest publications. On the contrary, dyspnea has become very important. It is surprising to see that the M/F gender ratio was 1.3 in the first publications whereas women represent 70% of cases in the revue Lupus publication [1]. The patients of the Créteil cohort were recruited for the most part in the midst of the period of controversy regarding the association between multiple sclerosis and hepatitis B vaccination. One can ask to what extent vaccine histories influenced the decision to biopsy the deltoid. This would explain, in particular, the fact that 85% of patients had histories of hepatitis B vaccination whereas the population exposed to tetanus vaccines is larger than the one exposed to hepatitis B vaccination. It should also be noted that the first publications (in the autoimmunity period) reported the interesting success of corticosteroid treatments (whether or not combined with antibiotics). This idea no longer appears in the most recent publications;
  - the description of cognitive disorders associated with MMF is certainly marred by the significant biases that were already highlighted.

- the patients presented in the Créteil team's publications are in fact very dissimilar. This leads to a significant confusion bias and a recruitment bias: as indicated above, some of them have other labeled diseases, notably autoimmune diseases. Many have disorders that are difficult to describe and that are close to chronic fatigue syndrome, a syndrome that is difficult to classify. The highlighting of abnormal muscle fixation in these MMF patients' fascias [55] gives this ailment a note of organicity. Nevertheless, it should be noted that this study covers 12 patients. It was published in 2000 and since then, the practice of this examination has never been mentioned again.

In total, this patient cohort raises questions. Is it possible to access the source files?

- The most important and highly publicized part of the physiopathology concerns the migration of aluminium from the injection site. It has been clearly demonstrated that aluminium injected via an IM pathway is mostly eliminated quickly and that a small portion binds in the organs [20], primarily bone, while the brain is the least affected organ. The toxicity of aluminium for the brain is not disputed, but it occurs in acute poisoning or in significant and prolonged exposures [19]. It should be noted that researchers close to the Créteil team have also developed an argument stating that aluminium is involved in Alzheimer's disease, which has not been demonstrated at this time [19]. The Créteil team advances the "Trojan horse" hypothesis involving penetration of aluminium-loaded monocytes/macrophages across the blood-brain barrier by relying on recently published experimental studies. These studies were conducted in mice with particles covered in aluminium that are different than the aluminium present in adjuvants [23]. This study, conducted in an experimental context that is not transposable to humans and to the vaccine situation, clarifies a transportation mechanism (already known) of particles that are internalized by the macrophages towards the brain. It does not prove that this applies to vaccine aluminium nor does it prove that this transportation could have consequences in terms of induced diseases.
- Finally, the Créteil team first described a histological lesion, then clinical symptoms observed in individuals who had this histological lesion in a biopsy of the deltoid and, going back to the vaccine history, attribute the presence of aluminium in the muscle and the MMF lesion (which is not contested) to the vaccination. But they also attribute responsibility for the clinical symptoms presented by these patients to this same vaccination. This fact has not been demonstrated. Given that the MMF lesion is localized around the injection site, which nobody contests, the biopsy could very well miss the lesion.
- Thus, within the population of individuals vaccinated with aluminium-containing vaccines (i.e., almost everyone), we do not know the proportion of individuals who actually retained aluminium in their muscle or, among them, the proportion of subjects who developed MMF lesions. Furthermore, given that the creation of an "aluminium granuloma" is almost a physiological fact after the administration of a vaccine containing it, in order to show the link between MMF and clinical symptomatology, it must be proven that the individuals manifesting clinical signs had significantly higher chances of having MMF lesions than those who did not. This type of proof is confronted with the ethical impossibility of performing muscle biopsies on asymptomatic individuals (although biopsies could be performed during orthopedic operations) and with

the lack of noninvasive means for diagnosing an MMF lesion. Furthermore, research on this subject has been very limited [54].

New case-control-type epidemiological studies are nevertheless no longer possible on this subject due to the very high chance of bias linked to the publicity surrounding MMF. In particular, there is a possible diagnosis bias. The subjects who are vaccinated and who present with a symptomatology that could potentially be connected to an MMF clinical entity have a higher tendency to consult and to have a muscle biopsy offered to them. Another possible information bias is of concern: vaccinated subjects are now likely to report the symptoms, which are described as being part of the supposed MMF picture, in the studies. In new case-control studies, such biases could lead to incorrect conclusions on the existence of an association between MMF histological lesions and the existence of a specific MMF clinical entity.

- Finally, two facts of capital importance have not been explained at this time:
  - Children and especially infants are by far the population that is most exposed to aluminium vaccines. Thus, it appears from the analysis of the literature, and even according to the authors of the reported cases, that the observation of MMF lesions in the muscle biopsy constitutes an epiphenomenon in a context of neurological and genetic diseases that are often thoroughly authenticated.
  - Why is a disease that has more than 1,000 identified patients within several years in France so rare in other countries? The analysis of the few published cases in fact leads one to think that the MMF histological lesion is yet again merely a coincidence. It is true that biopsies of the deltoid are avoided in other countries for the reasons mentioned above. The Créteil team's publications were largely disseminated and one would suppose that biopsies of the deltoid would be performed in other countries if MMF were considered to be a recognized entity. Finally, if it is true that a larger number of adults have been vaccinated against hepatitis B in France than in other countries, accepting this fact as an explanation supposes that there is an explanation for the lesion being exclusive to adults. Furthermore, other vaccines containing aluminium adjuvants (tetanus for example) are largely used in foreign countries, some much more than in France and for longer (vaccine against meningococcal C).
- Ultimately, there is currently no convincing publication concerning MMF in the international literature. Only Shoenfeld's Israeli team, which is actually trying to broaden the framework, devotes publications to this ailment [12, 13, 36, 37]. Some authors cite MMF as being a possible side-effect of vaccines [39]. A limited number of publications consider this fact to be established and content themselves with reproducing the writings of the French team [35, 38, 39].

In conclusion

The review of the literature does not allow one to conclude that macrophagic myofasciitis (histological lesions linked to the deposit of aluminium from vaccines in the muscle) is associated with one or more systemic symptoms.

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3 - Current assessment of event reports of macrophagic myofasciitis sent to the national pharmacovigilance system

In order to search for a possible connection between the MMF histological lesion and specific clinical symptoms, a case-control study was requested, starting in 1999, by the Global Advisory Committee on Vaccine Safety of the World Health Organization (WHO) and decided on in February 2002 by the Afssaps. A case-control study, finalized in October 2003, did not allow for the conclusion that there is an association between the presence of aluminium in the macrophages and the occurrence of clinical symptoms.

In May 2004, based on the level of knowledge available for MMF, the Scientific Committee of the Afssaps notably made the following recommendations:

- the association between the MMF histological entity at the muscle site typically chosen for vaccination and the administration of vaccines containing aluminium adjuvant is highly likely;
- it is not possible to consider that there is an association between the MMF histological entity and a specific clinical syndrome;
- no specific clinical syndrome was found to be associated to vaccination with vaccines containing an aluminium adjuvant;
- the risk-benefit balance of vaccines containing an aluminium adjuvant should not be called into question.

In February 2002, a pharmacovigilance investigation initiated by the Afssaps was placed under the responsibility of the Centre régional de pharmacovigilance [Regional Pharmacovigilance Center] (CRPV) of Lorraine (CHU Nancy (Centre Hospitalier Universitaire Nancy [Nancy University Hospital Center])). As part of the national monitoring of MMF cases, the updated assessment of pharmacovigilance data collected and analyzed to date takes into account all of the MMF event reports to the national pharmacovigilance network, to laboratories, to patient organizations (E3M or “Entraide aux malades atteints de myofasciite à macrophages” and REVAHB “Réseau vaccin Hépatite B” [Hepatitis B Vaccine Network]), and to the anatomical pathology referral departments of the CHUs of Henri Mondor (Créteil), Pitié-Salpêtrière (Paris), Bordeaux and Marseille.

On 30 April 2013, based on the 379 anatomical pathology reports available out of the 496 cases collected by the Lorraine CRPV, the MMF histopathological diagnosis was confirmed for 267 patients, considered as “doubtful” for 81 patients and eliminated for 31 patients. Among the 117 files without an anatomical pathology report, 69 cases were included in the study based on confirmation using the concept of a “positive biopsy”.
Of the 496 cases collected in total, 311 patients are registered with patient organizations. Overall, 417 files with an MMF diagnosis were included in the descriptive statistical analysis.
The characteristics that emerge from the global investigation of the 417 files in question in terms of vaccine histories, symptomatology, paraclinical and intercurrent-disease aspects are similar to those from the prior assessments: they are mostly women (2/3 of cases) (Fig. 1).

Gender ratio

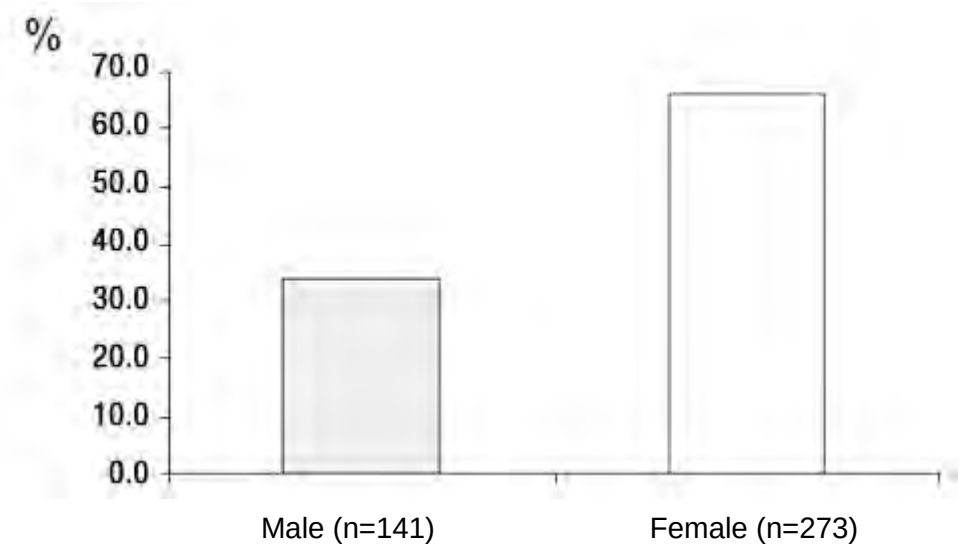

Fig. 1 – Distribution of patients by gender (n=414)

Before the onset of the first clinical signs, more than 69% of patients were vaccinated during the campaign against the hepatitis B virus conducted in the1990s (69.3%) (Table 1, Fig. 2).

Table 2 – Latest vaccine product(s) containing aluminium administered before the appearance of the first clinical signs.

Vaccine	N	%
Vaccine against hepatitis B	289	69.3
Vaccine against tetanus	21	5.0
Revaxis®	11	2.6
Vaccine against hepatitis A	11	2.6
Vaccine against hepatitis B + Vaccine against hepatitis A	8	1.9
Vaccine against hepatitis B + Vaccine against tetanus	2	0.5
Vaccine against hepatitis B + Tetravac®	1	0.2
Vaccine against hepatitis A + Revaxis®	1	0.2
Infanrix®	1	0.2
Gardasil®	1	0.2
Tetravac®	1	0.2
HPV Vac	1	0.2
Not specified	**69**	**16.5**
Total	**417**	**100.0**

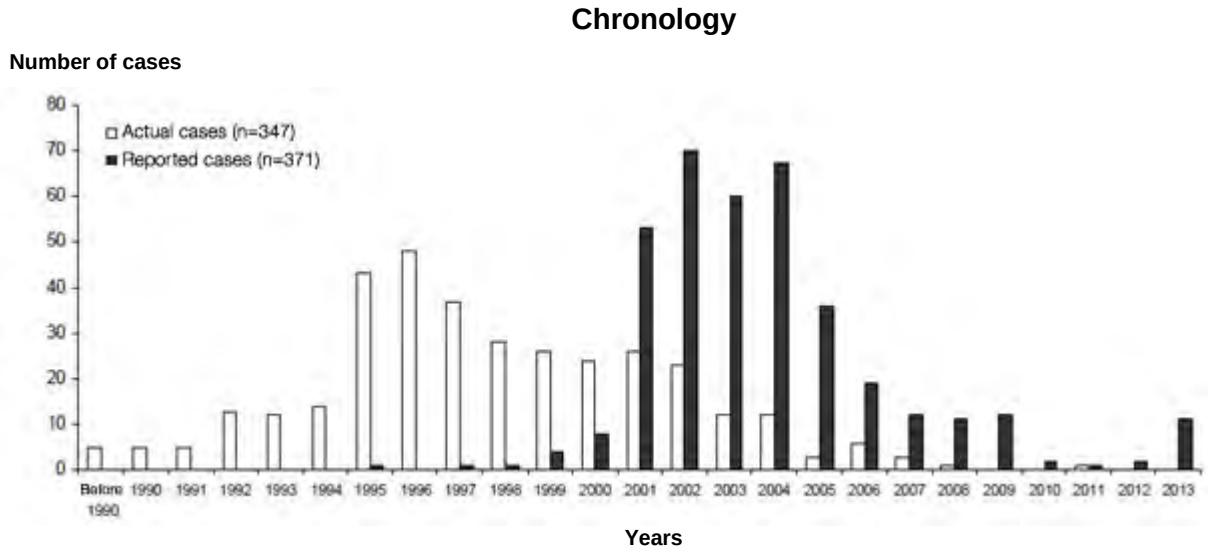

Fig. 2 – Chronology of the start of clinical signs and the date of cases reported to the CRPV networks and to the laboratories

The average age of the patients at the last vaccination (containing aluminium) is 40 (Fig. 3).

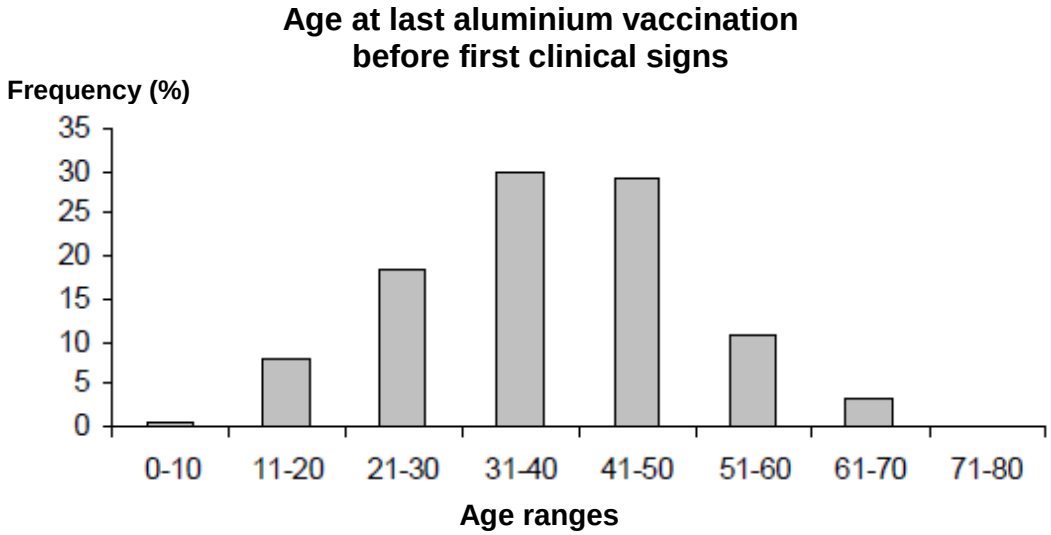

Fig. 3 – Distribution of patients as a function of age at the last aluminium vaccination preceding the first clinical signs (n=310)

The patients describe the occurrence, on average one and a half years after the last vaccination, of a functional clinical picture primarily comprising an arthromyalgia syndrome, marked asthenia, sleeping disorders and varied functional symptomatology with no objective signs or specific additional examination outside of the biopsy, which itself is typical [see Appendix, p. 30]. An intercurrent disease identified in more than half of the cases is likely to actively participate in the observed symptomatology, with the clinical picture most often justifying a muscle biopsy.

In conclusion, the analysis of all the pharmacovigilance data available from the event reporting does not provide any new elements that are likely to change the conclusions issued at the end of the prior assessments (http://ansm.sante.fr). In fact, all the elements collected and analyzed to date allow for the conclusion that the aluminium contained in vaccines may persist for years in the form of a focalized histological muscle lesion.

The MMF biopsies are essentially (but not exclusively) reported in France. This French specificity can be explained by the following arguments [1, 2]:

- in France, muscle biopsies for diagnosis purposes are performed in the deltoid whereas other countries favor another muscle;
- in the 1990s, millions of adults were vaccinated as part of a national vaccination campaign against the hepatitis B virus. However, this same vaccine was administered to adults in other countries without the identification of any MMF cases. Furthermore, the significant increase in the level of reporting since 1998 could be linked to an increase in the screening rate. (Fig. 2);
- during this vaccination campaign against hepatitis B, the route of administration was modified by substituting the subcutaneous route by the intramuscular route.

At the present time, there are no convincing epidemiological arguments to support the relationship between vaccination and the existence of a disease connected to the lesion.

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Special thanks to the CRPV of the CHU of Lorraine.

Appendix – Clinical symptoms described at least once during the disease by the clinician or the patient (n=417)

Clinical signs	Medical event described at least once by the Clinician or the Patient (n=417)	
	n	%
Myalgia	324	77.7
Asthenia	294	70.5
Arthralgia	212	50.8
Diffuse pain	180	43.2
Memory problems	155	37.2
Muscle weakness	142	34.1
Sleeping problems	131	31.4
Mood disorder, Anxiety	99	23.7
Sensory disorders	89	21.3
Ophthalmological disorders	87	20.9
Headaches	76	18.2
Equilibrium disorders	74	17.7
Cramps	70	16.8
Gastrointestinal disorders	55	13.2
Respiratory disorders	38	9.1
Burning sensation	38	9.1
Fever	33	7.9
Urinary disorders	30	7.2
Speech disorders	28	6.7
Tremors	27	6.5
Cardiovascular signs	26	6.2
Dermatological signs	24	5.8
Weight loss	22	5.3
Amyotrophy	17	4.1
Dysphagia	17	4.1
Abdominal pain	15	3.6
Weight gain	14	3.4
Hair loss	6	1.4
Dry mouth	5	1.2

4 - Adjuvants - Immunological aspects

The development of immunity adjuvants associated with vaccines was justified by the need to amplify the specific immune response to subunit or inactivated vaccines incapable of inducing on their own protective, effective and long-lasting immunity, mediated by antibodies and memory T and B lymphocytes. In fact, one of the reasons for the major efficacy of attenuated living vaccines comes from their capacity to simultaneously activate several innate immunity receptors that are necessary to induce these specific immune responses [1, 2]. Unlike these living vaccines that retain the main pathogenic capacities that activate innate immunity, the subunit vaccines composed only of proteins and, to a lesser degree, inactivated vaccines, do not have these properties. In order to overcome these limitations, adjuvants were traditionally used to increase the amplitude and durability of the vaccine response that is conventionally measured in antibody titers or the protective effectiveness [3].

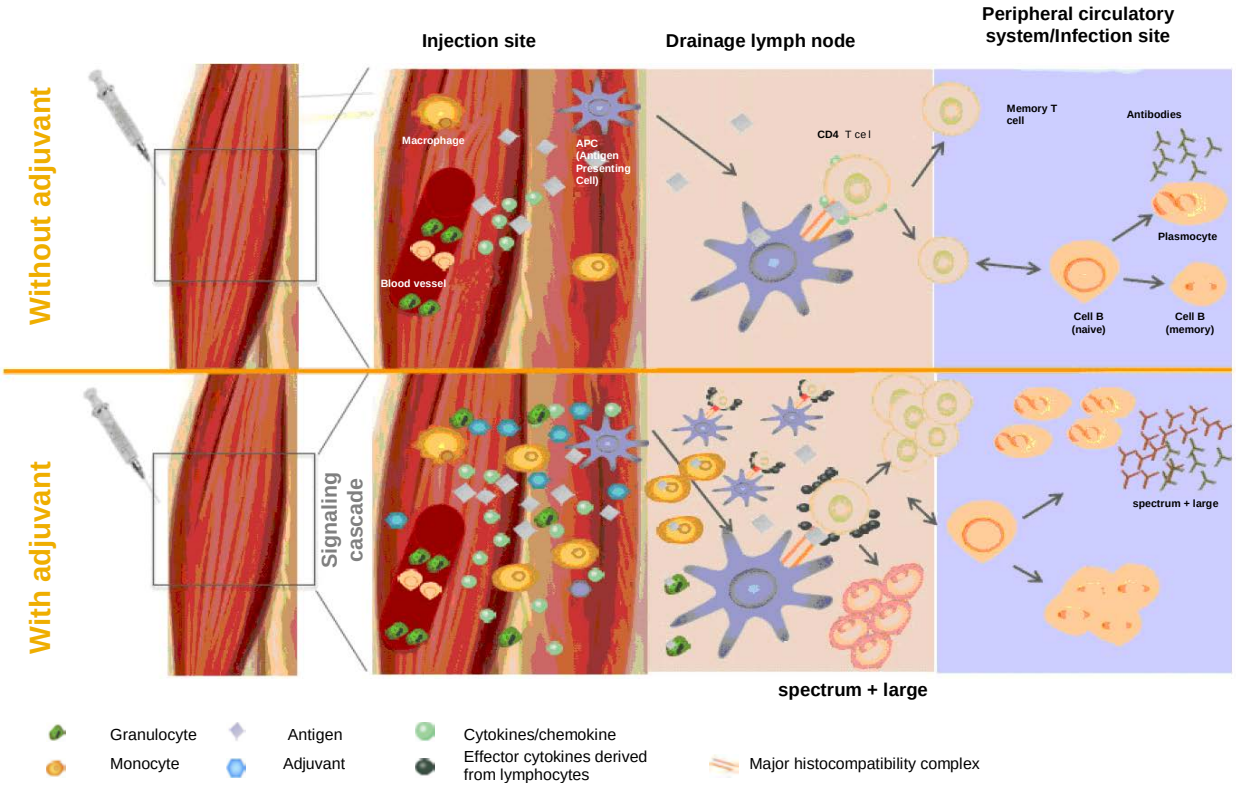
4.1 - Objectives and mechanisms of action of adjuvants

Most of these adjuvants were developed according to empirical methods starting in the 1920s with aluminium salts, but it is only recently that the elucidation of the induction mechanisms of innate immunity, illustrated by the 2011 Nobel Prizes for Medicine, particularly J. Hoffmann and B. Beutler [4,5], allowed for an understanding of these adjuvants' mechanisms of action. Thus, receptors named PRR (Pattern Recognition Receptors) receptors were identified in macrophages and other cells with antigens. They are capable of detecting certain molecules belonging to the infectious agents, PAMPs (Pathogen-Associated Molecular Patterns). Thus, the TLRs (Toll-Like-Receptors) recognize lipids, lipoproteins, nucleic acids or proteins. The diphtheric or tetanic anatoxin subunit vaccines, or HbS antigens lack these PAMPs, which are partially denatured during the inactivation process of the inactivated vaccines, unlike living vaccines which retain the majority of the PAMPs of their pathogens of origin. In the macrophages and antigen-presenting cells, all these molecules and activation routes create an intracellular complex, inflammasome, that activates the production of certain key cytokines in the initiation of specific immune responses such as interleukin-1.

By activating these PRRs, adjuvants compensate for the absence of these PAMPs in the subunit or inactivated vaccines [3,6-9] by triggering this first wave of innate immunity that is necessary to develop an effective and long-lasting immune response. Adjuvants are also capable of guiding the type of immune response to be triggered against a given pathogen: for example, the Th2 auxiliary CD4 response that induces the antibody response against extracellular pathogens or the Th1 auxiliary CD4 response and the CD8 response against intracellular pathogens.

Thus, there are several immunological reasons for including an adjuvant in vaccines:

1) to increase the amplitude of the immune response, on the one hand in the general population in order to increase the proportion of protected subjects, and on the other hand in populations with immune systems that are fragile (particularly elderly subjects) or suppressed by diseases or treatments [1,6-12];
2) to reduce the quantity of vaccine antigens and the number of necessary injections [1,6-9,13] in order to be able quickly immunize very large populations, for instance in a flu pandemic situation.



N. Garçon *et al.*, Perspectives in vaccinology 2011 Didierlaurent *et al.* JI, 2009 Morel *et al.*, Vaccine, 2011

Fig. 4 – Adjuvants: general mode of action based on current evidence[1]

4.2 - Aluminium salt-based adjuvants

The aluminium salt-based adjuvants used since the 1920s are precipitates of aluminium phosphate or hydroxide in which vaccine antigens are adsorbed [14-17]. The particle composition of these compounds favors the prolonged deposit of vaccine antigens in the lymphatic tissues and their internalization by antigen-presenting cells. Therefore, they prolong the persistence of the antigen at the injection site and the drainage lymph node tissues. This increased persistence of the antigen is an essential element in the induction of the Th2 route. It is certainly a key mechanism for the increase of vaccine immunogenicity by aluminium salts. These could also stimulate the Nalp3 route [15] which induces the production of IL-1 and IL-18 cytokines. Thus, aluminium salts have a significant capacity to induce elevated and durable specific antibody levels by their ability to stimulate Th2-type auxiliary CD4 T lymphocytes that activate memory B lymphocytes and plasmocytes. This type of immune response is particularly effective against toxins and extracellular pathogens, but less so against intracellular pathogens.

All experts recognize that very widespread and eighty-year-old use and the large volume of data concerning aluminium salts show excellent tolerance of aluminium salts. This makes them the adjuvant of choice to increase the effectiveness of vaccines directed against pathogens that require high levels of antibodies for their prevention [1,3,6-9,18]. However, the aluminium-based adjuvants have certain limitations.

[1] Garçon N, *et al.* Vaccine adjuvants. Perspectives in Vaccinology, 2011; (1): 89-113. Available at http://www.sciencedirect.com/science/journal/22107622 (accessed on 08/07/2013).

They do not cause a sufficient increase in the production of antibodies directed against small peptides or certain vaccines like typhoid and the influenza virus and do not induce the cellular immunity required against other pathogens like mycobacteria. There is, therefore, a need to develop new adjuvants.

4.3 - Alternatives to aluminium salt-based adjuvants

4.3.1 - Phospholipidic adjuvants

Phospholipidic-type emulsions seek to activate T lymphocytes in a more comprehensive manner. The synergy between MPL (monophosphoryl lipid A [MPL]), a detoxified derivative of the LPS (lipopolysaccharide) of Gram-negative bacteria *Salmonella minnesota* [19] adsorbed in aluminium hydroxide, was behind the development of *Adjuvant System 04* (ASO4) [20]. In fact, MPL mainly activates TLR4-type Toll-Rs [21, 22] and the production of proinflammatory cytokines, such as TNF-a and IL-6 which, in turn, stimulate the maturation of APCs while suppressing tolerance induction. MPL principally induces an auxiliary Th1-type CD4 response that is required against intracellular pathogens, a property that aluminium salts do not have. ASO4 is associated with two vaccines, which have a marketing authorization (MA), against the oncogenic viruses HPV-16 and -18, where it associated with viral pseudoparticles made of the L1 protein of these HPVs [23], and the HBV virus where it is associated with the HBs antigen to increase the immunogenicity of this vaccine in kidney failure patients on dialysis [10,11].
AS03 has demonstrated its ability to increase the production of protective antibodies against avian flu or against seasonal flu in elderly individuals, suggesting its utility for the prevention of other infections that require antibody induction [24]. Its widespread use during the A(H1N1)v pandemic of 2009 also demonstrated its safety [18,25]. Nevertheless, in some countries (Finland, Switzerland, France and the United Kingdom) a significant increase in narcolepsy cases was observed in individuals who received this vaccine without the cause and effect relationship being clearly established, particularly the responsibility of the adjuvant.
ASO1, from the same family, is the adjuvant of a vaccine against malaria that is currently in development.

4.3.2 - Calcium phosphate adjuvants

An alternative to aluminium-based adjuvants was proposed in the form of calcium phosphate during the 1970s in conjunction with the search for alternative solutions to potential aluminium allergies. The Pasteur-Production firm developed calcium-phosphate-based adjuvants for the DTP-Polio-Whoop. product range. The figure showed that in the presence of Ag, they did not induce an increase of specific IgEs and induced fewer allergic reactions than aluminium [26]. These Ca^3Po^4 adjuvants are also prepared faster with a high absorption capacity, allowing for slower salting-out and longer immunological stimulation and thus increased antibody levels. Additionally, the French experience showed that higher post-vaccination antibody levels and better local and systemic tolerance than after IM injection with aluminium (except for Pertussis). This experience of the Institut Pasteur (Pasteur Institute) seemed particularly interesting for allergic subjects [27]. Nevertheless, this vaccine strategy of the Institut Pasteur was not taken up by the other firms and, in 1995, articles reported contradictory data showing that these adjuvants induce equal or lower antibody levels, except as a booster, than the aluminium base-salt adjuvants [28-30]. Additionally, their absorption capacity was 50% lower than that of aluminium. Its adjuvanticity in mice depended on the antigen: for tetanus, it was higher than or

equal to that of aluminium as a primary vaccination. For diphtheria, it was lower than that of aluminium. Its adjuvanticity in guinea pigs was higher than that of aluminium.

The local tolerance of these diverse classes of adjuvant was compared in guinea pigs. This work showed that Ca and aluminium induce a more significant infiltration of inflammatory PN if these adjuvants are in the form of a suspension compared to being in gel form. This is equally true for both calcium and aluminium. This inflammation lasts about eight weeks for the alum gel and four weeks for the Ca gel. Furthermore, an inflammatory granulomatous reaction of the conjunctival area was brought to light after injection of calcium phosphate gel for about eight weeks. The tetanus immunogenicity obtained was higher after aluminium than it was after Ca [31]. However, the problem of the animal model possibly not reflecting the effects in humans must be highlighted [32].

In total, these old and contradictory data do not allow for any conclusions to be drawn about better tolerance or about good adjuvanticity of calcium-phosphate-based adjuvants. These were definitively abandoned during the merger of the Pasteur-Production firm with the Institut Mérieux (Mérieux Institute).

4.3.3 - Virosomes

Aluminium adjuvant does not allow a Th1-type immune response to be induced and it may persist at the injection site. The virosome is a construct that resembles a natural virus, capable of presenting an antigen in the context of class I or II major histocompatibility complexes and, ultimately, favors the development of a complete, humoral and cellular immune response.

The virosome is both an adjuvant and a transportation system of the vaccine antigen. It takes the form of a viral pseudoparticle that comes from an enveloped virus. Lacking a capsid and a viral genome, it does not have replicative or infectious capability. The virosome is obtained from a liposome, an artificial vesicle formed by lipid bilayers. The virosome is made up of a viral membrane that contains lipids of the host cell from which it is derived and, contrary to the liposome, viral proteins. These allow for fusioning with the target cells. The presentation of the antigen to the immune system and the stimulation thereof are very similar to natural immune mechanisms.

Currently, the virosomal system is used as an adjuvant in two vaccines with a European MA and marketed outside of France by the Crucell company: the Inflexal® V vaccine against seasonal flu and the Epaxal® vaccine against hepatitis A [33,34]. For these two vaccines, the virosome that is used is derived from an influenza virus. It consists of the envelope and the glycoproteins of the virus. The influenza virus's hemagglutinin molecules are incorporated in the double membrane of lecithin-and-cephalin-based phospholipidic liposomes, thus forming virosomes. In the body, these virosomes actively bind to corresponding receptors located, on the one hand, on macrophages that phagocyte them and, on the other hand, on B lymphocytes whose proliferation is thereby activated. In the macrophages' endosomes, the hemagglutinin fusion peptide of the influenza virus is activated so that the phagocyted liposome can immediately fuse with the endosome's membrane.

The manufacturer emphasizes the completely biodegradable and nontoxic characteristics of the virosome, as well as the absence of an immunological response directed against the virosome itself.

- ***Epaxal® vaccine***

Hepatitis A viruses, obtained from human diploid cell cultures and then inactivated, are bound to virosomes by a specific process [35]. The Epaxal® vaccine is effective and well tolerated [36]. In a study conducted in children aged from 1 to 16 years old, a 0.25 mL dose of Epaxal® vaccine leads to a humoral response that is as effective as a 0.5 mL of vaccine containing an aluminium adjuvant (Havrix® 720 IU) [37].

Another study, conducted in more than 500 adult travelers, shows that local adverse effects are two times less frequent after vaccination with the Epaxal® vaccine than after vaccination with a vaccine containing aluminium hydroxide as an adjuvant [38].

- ***Inflexal® V vaccine***

This vaccine against seasonal flu is the only marketed product that contains an adjuvant (the virosome) and can be given starting from the age of six months old. Inflexal® V has been used for fifteen years. It is effective and well tolerated [39].

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5 - Aluminium toxicity

5.1 - Composition, structure and physicochemistry of aluminium adjuvants

Today, the two principal adjuvants used in vaccines are aluminium hydroxide and aluminium hydroxyphosphate. Recourse to aluminium hydroxide is much more common if one refers to the vaccines administered in the French vaccine schedule.

5.1.1 - Physicochemical properties of adjuvants

Physicochemical methods help determine the structure of the adjuvants as well as their behavior in relation to the pH of the environment. As stated above, the aluminium adjuvants that are used are aluminium hydroxide: $Al(OH)_3$, AlOOH and aluminium hydroxyphosphate: $Al(OH)_x(PO4)_y$.

The physicochemical characterization of AlOOH is based on:

→ Measurement of the BET (Brunauer–Emmett–Teller) gas absorption titration;
→ Determination of the isoelectric point (zeta potential);
→ Determination of the size of formed aggregates (laser diffraction);
→ Structural crystallinity (x-ray diffraction).

There are different marketed AlOOH adjuvants like: Alhydrogel, Rehydragel HPA, Rehydragel LV, etc. The AlOOH used is the Boehmite-type, with a crystalline structure. The surface is composed of hydroxyl groups and the total surface charge at pH = 7.4 is positive. The zero charge point is 11.4. The protein-binding capacity is above 0.5 mg BSA/mg AlOOH. AlOOH's crystalline structure takes the form of octahedral aluminium layers that are organized in the form of platelets that ultimately form aggregates on the scale of µm. The size of the elementary particles is 4.5 x 2.2 x 10 nm and the formed aggregates have an average diameter of 17 µm. The cohesion of aggregates occurs by way of hydrogen bonds that are stronger than the electrostatic-type interactions. The dissolution of aggregates can only take place at a pH value equal to 2. This means that this dissolution cannot occur in physiological conditions.

$Al(OH)_x(PO4)_y$, used as an adjuvant, has an amorphous structure. The surface is composed of both hydroxyl groups and phosphate groups. The total surface charge at pH = 7.4 is negative. The adjuvant's point of zero charge depends on the Al/P ratio but is generally found in a 4.6-5.6 range. The protein-binding capacity is above 0.6 BSA/mg $Al(OH)_x(PO4)_y$. The primary particles have a diameter of 50 nm and the aggregates formed by these particles have a diameter that can range from 1-10 µm. The diameter of the aggregates depends on the elementary composition.

5.1.2 - Examples of adjuvants used in France

- ***Adjuvants used by Sanofi Pasteur MSD***
  Amorphous aluminium hydroxyphosphate is used as an adjuvant that is a component of the HBVAXDNA® and Gardasil® vaccines. Without antigen adsorption to their surface, the aluminium particles are in the form of agglomerates that are 4-13 µm in size and are composed of primary particles with a diameter of 20-30 nm. It should be noted that the size of the particles after injection and their evolution over time is unknown.

- ***Adjuvants used by Sanofi Pasteur***
  Aluminium hydroxide gels: Superfos gel and Reheis gel. The size distribution of particles is > 100 nm and the "unitary" particles form aggregates of 500 nm to 100 µm.

5.1.3 - Adsorption of aluminium adjuvants

Antigen adsorption in adjuvants is measured, for example, by spectrophotometry using the BCA (bicinchoninic acid assay) method or immunoprecipitation techniques. Antigen adsorption in an adjuvant is dependent on various parameters such as: the nature of the adjuvant, the nature of the adjuvant-antigen interactions (hydrogen, electrostatic and van der Walls), the ionic force of the environment, the pH, the isoelectric point, the surface charge, the antigen's molecular weight, etc. As indicated in the 2012 report of the Académie nationale de médecine [French National Academy of Medicine]: "This is therefore how a vaccine antigen with a large molecular weight and negatively charged at pH 7 is capable of adsorbing itself to the surface of the aluminium gel aggregates, while not penetrating inside the structure. This adsorption phenomenon has direct effects on the vaccine's activity. Understanding the adjuvant's composition and structure allows one to predict whether the antigen-adjuvant interaction will take place on the surface or inside the aggregates or, conversely, whether no adsorption will be observed."

Generally, adsorption is considered to be optimal within a pH range between the antigen's isoelectric point and the aluminium adjuvant's point of zero charge. Within this range, the adjuvant and the antigen will have opposing electrical charges, permitting better electrostatic attraction and thus favoring adsorption.

5.2 - Pharmacokinetics

5.2.1 - Non-vaccine aluminium

- ***Absorption***

  The absorption percentages are similar via the respiratory and digestive routes, on a scale of 0.1% to 1%. This absorption is dependent on the pH value, the identity of the aluminium salt in question and on speciation. Citrates and silicates in particular influence absorption in a contrary manner [1].
  Cutaneous aluminium absorption is generally considered to be low. At the request of the Afssaps, a recent study was conducted on human skin *in vitro* with several aluminium-based cosmetic formulations and according to the OECD (Organization for Economic Co-operation and Development) 428 and SCCP (Scientific Committee on Consumer Products) recommendations. In particular, it was determined that the quantities absorbed at the end of 24 h, corresponding to the systemic absorption of aluminium, were negligible (<0.03% of the applied dose) regardless of the formulation or the skin's condition (normal or peeled). The quantities present in the skin compartments, corresponding to the absorbable aluminium that is likely to be found in systemic circulation from the reservoir that is the skin, were 0.5% (normal skin) and 18% (peeled skin) of the applied dose. The last case is a maximizing scenario [2].

- ***Distribution***

  The aluminium load of the human body, stemming from daily absorption by digestive, pulmonary and cutaneous routes, is primarily distributed in the bone system, the lungs and the liver. One also finds small quantities of aluminium in the central nervous system (CNS), and the hematopoietic and immune systems. Aluminium's transportation protein is transferrin [1].
  In humans, experiments with patients on dialysis have shown that aluminium can accumulate in the skull and in the brain. In the brain, aluminium concentrations increase with age and are higher in gray matter [1].

- ***Mechanisms involved in the transit of aluminium to the brain***

  In plasma, about 91% of aluminium (Al^{3+}) is bound to transferrin, and 7%-8% is found in the form of an aluminium citrate complex [3-4]. At least two mechanisms are involved in aluminium's crossing of the blood-brain barrier (BBB):

  → Transferrin receptor-mediated endocytosis (TfR-ME): this process was demonstrated *in vitro* in primary cultures of rat cells, and *in vivo* in rats using marked aluminium (^{26}Al) [5-6];

  → active transportation (ATP (Adenosine Triphosphate)-dependent and sodium-independent) of aluminium citrate *via* a transporter that is not specifically identified at this time, but which would be one of the transporters of monocarboxylates (MCTs) or one of the transporters of organic anion expressed on the BBB [4, 6-7].

  While TfR-ME is a mechanism that is primarily involved in the transportation of iron to the brain, necessary for proper brain functioning, the same would not be true for aluminium, particularly due to:

  → a difference in transferrin binding: the Fe3+ and Al3+ ions are structurally similar and bind to transferrin. However, transferrin saturated with Al3+ ions or in the presence of an equimolar charge of Fe3+ and Al3+ ions does not interact optimally with its TfR receptor [4];

  → rapid entry of aluminium into the brain following an IV injection of aluminium citrate, which can only be explained by TfR-ME by assuming i) saturation of transferrin by aluminium, ii) comparable influx constant (Kin) values for iron and aluminium by TfR-ME and iii) the initial confinement of aluminium in the brain extracellular liquid [8];

  → the ability of aluminium that is not bound to transferrin to penetrate the brain by a faster mechanism than TfR-ME. The existence of a means of transportation other than TfR-ME is supported by the lack of a significant difference in aluminium brain transit in hypotransferrinemic and control mice, and after pretreatment with antibodies directed against the transferrin receptor (RI7 208) in mice treated with aluminium citrate [9].

  Finally, it has been suggested that the monocarboxylate (MCT) transporter is behind the transit of aluminium across the BBB. Aluminium can form coordination bonds with the carboxylate groups and the citrate hydroxyl group, leaving a terminal hydroxylate group free at physiological pH. Moreover, the transportation speed of a substrate across the BBB via MCT is more than sufficient to explain the speed at which aluminium appears in the brain (dialysate) after the IV injection of aluminium citrate [8].

- ***Interspecies permeability of the BBB***

  The transportation mechanisms detailed above have been described in rodents. Data indicate that they would be relevant to humans, at least from a qualitative point of view:

  → TfR-ME: transferrin is the only aluminium-binding protein in human plasma; approximately 90% of aluminium (Al3+) is bound to transferrin [10]. Moreover, the transferrin receptor was brought to light in the human BBB [4].

  → Active transportation of aluminium citrate: in human serum, the principal species of low-molecular-weight aluminium were citrate, phosphate, tertiary complexes of citrate and aluminium phosphate.

- ***Aluminium's transit to the brain: quantitative aspects***

  A study was conducted in rats to quantify the transit of aluminium from the

circulatory system to the brain, to estimate the time that aluminium remains in the brain and to evaluate whether a treatment repeated with an aluminium chelator (desferrioxamine, DFO) can modulate half-life in the brain [3].

To that end, male rats received 26Al-transferrin via IV (one-hour infusion) and were put down from four hours to 256 days after administration (5-12/sampling time). Starting from the 3rd day after administration, half of the rats received DFO (0.15 mmol/kg, IP route, three times per week). To check the possible influence of the 26Al chemical species on the studied parameters, 14 rats were treated in a similar manner with 26Al citrate and were put down from four hours to four days after administration (4-5/sampling time). The assay of 26Al concentration in the serum samples and the brain tissue was carried out using Accelerator Mass Spectrometry (AMS).

The principal results are as follows:

→ the maximum brain concentration of 26Al (brain Cmax) was 0.005% of the dose/g of the tissue after administration of 26Al-transferrin or 26Al citrate;
→ at the 256th day after administration, the 26Al concentrations were 30% and 10% of this brain Cmax in rats treated with and without DFO, respectively;
→ a significant reduction in 26Al brain concentrations was associated with DFO on the 64th and 128th day after administration;
→ the half-life of 26Al in the brain was 150 days and 50 days in rats treated with and without DFO, respectively.

The study's authors reviewed the prior publications in order to quantify the aluminium transit from the bloodstream to the brain. They conclude that the transit is 0.001%-0.005% per gram of brain tissue under physiological conditions in rats. This value is not influenced by the route of administration or the chemical form when aluminium is administered in a soluble form. From their own efforts, they also conclude that clearance of aluminium in the brain is low in the absence of chelation treatment.

- ***Elimination***

The effectively absorbed aluminium fraction is eliminated via the kidney; when exposure is stopped, the kinetics of the reduction of the urinary concentration are triphasic. The low concentrations of aluminium found in feces after IV administration of radioactive aluminium confirm the existence of an enterohepatic cycle [1].

5.2.2 - Aluminium adjuvants

A team from Purdue University (Indiana, United States) conducted *in vitro* and *in vivo* studies to better characterize the kinetics of aluminium adjuvants.

Dissolution studies were carried out ***in vitro*** with aluminium phosphate (AP) and aluminium hydroxide (AH). [11-12]. Citric acid was used to represent α-hydroxy carboxylic acid at a concentration of 2.7 meq/L. This choice is based on the rational described below. Interstitial fluids contain seven organic acids: three α-hydroxy carboxylic acids (citric, lactic and malic) and acetoacetic, α-ketoglutaric acid, pyruvic and succinic acids. The concentration of each acid is not known, but the total concentration in organic anions varies from 3.4 to 7.0 meq/L for an average concentration of 6.3 meq/L. Only the three α-hydroxy carboxylic acids are capable of chelating aluminium and thus of solubilizing compounds containing aluminium.

Assuming that each organic acid is present interstitially at identical concentrations, the required concentration was 2.7 meq/L (6.3 x 3/7). Citric acid was chosen as a model for α-hydroxy carboxylic acids.

Under the experimental conditions that were used, the results showed that the two adjuvants are solubilized by citrate. Nevertheless, solubilization kinetics are faster for aluminium phosphate than for aluminium hydroxide.

Next, a study was conducted ***in vivo*** in rabbits with these two adjuvants marked by ^{26}Al during their production by substitution of ^{27}Al chloride with ^{26}Al during the precipitation phase [12-13].

A single intramuscular injection of 0.2 mL AP and AH adjuvants marked by ^{26}Al (i.e., 4.5 ng ^{26}Al/0.85 mg Al) was carried out in rabbits (2 females/adjuvant). Blood and urine samples were collected for 28 days, and then the animals were put down. The following tissues were sampled in the necropsy: brain, heart, left kidney, liver, mesenteric lymph node, spleen and bone (femur). However, the bone samples degraded during their preparation, and so did the brain tissue sample from one of the animals in the AP group. ^{26}Al concentrations in the urine, blood and tissue samples were then determined with Accelerator Mass Spectrometry (AMS).

The principal results are as follows:

- The initiation of solubilization of aluminium from the adjuvant is fast, as shown by the detection of ^{26}Al in the blood starting from the time of sampling (1 h post-injection). The systemic absorption speed was higher for AH than for AP during the first 24 hours following administration. Starting with the second day, the systemic absorption speed of aluminium was higher in animals who received AP than in those who received AH, and the aluminium blood concentrations were relatively stable, indicating a relatively constant absorption speed for the two adjuvants up to the end of the study. Over the entire study (28 days), 17% (13-22) and 51% (47-55) of injected aluminium was absorbed from the AH and AP adjuvants, respectively.
- The tissue distribution profile of ^{26}Al was similar, independent of the type of injected adjuvant and was typical of what was reported after administration of ^{26}Al by other routes: kidney > spleen > liver > heart > lymph node > brain. Quantitatively, the ^{26}Al concentrations were on average 2.9 times greater in each type of tissue in rabbits treated with the AP adjuvant, which is consistent with the systemic exposure data.
- The maximum increase of the aluminium plasma concentration (0.85 mg) was 2 ng/mL, or about 7% of the normal aluminium concentration in rabbits (30 ng/mL).
- By extrapolating, one expects that the intramuscular administration of the same dose of adjuvants in humans induces about a 0.04 ng/mL increase in the aluminium plasma concentration, i.e., 0.8% considering a normal value of 5 ng/mL.

Table 3 – Pharmacokinetic parameters after i.m. injection of ^{26}Al-containing aluminium hydroxide and aluminium phosphate adjuvants

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Table 1 Pharmacokinetic parameters after i.m. injection of ^{26}Al-containing aluminium hydroxide and aluminium phosphate adjuvants

Adjuvant	AUC for 0-28 days (mg h g $^{-1}$)	% Absorbed in 28 days	Cumulative aluminium in urine after 28 days (%)
Aluminium hydroxide			
Rabbit 1	2.0 x 10 $^{-4}$	13	5.0
Rabbit 2	3.5 x 10 $^{-4}$	22	6.2
Average	2.7 x 10 $^{-4}$	17	5.6
Aluminium phosphate			
Rabbit 3	2.7 x 10 $^{-4}$	47	10
Rabbit 4	8.7 x 10 $^{-4}$	55	33
Average	8.1 x 10 $^{-4}$	51	22

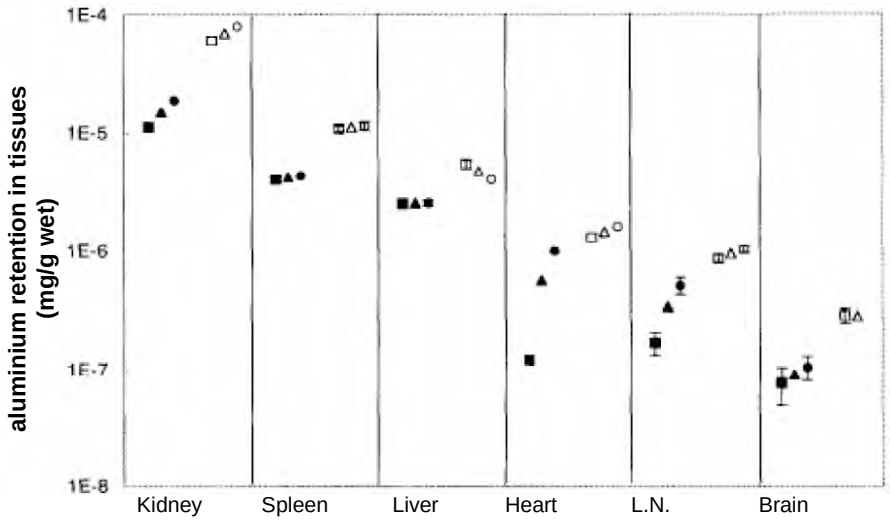

Figure 3 Aluminium tissue concentration 28 days after administration of ^{26}Al-labelled aluminium hydroxide adjuvant: ■, rabbit 1;●, rabbit 2: ▲. mean; or aluminium phosphate adjuvant □. rabbit 3; o rabbit 4: ▵ , mean, L.N., lymph node. Error bars of <5% are not shown

Fig. 5 – Aluminium tissues concentration 28 days after administration of ^{26}Al-labelled aluminium hydroxide adjuvant

Conclusions and notes: it was demonstrated *in vitro* that aluminium can be solubilized from an aluminium adjuvant (AH or AP) by an organic acid at a physiological concentration. Solubilization speed depends on chemical composition. It was shown to be higher for the AP form than the AH form. This was confirmed *in vivo* in rabbits after intramuscular injection of two adjuvants where the evolution of marked aluminium blood concentrations was followed for a period of 28 days. The solubilized aluminium is then absorbed systemically and is distributed to all the studied organs, including the brain which nevertheless showed the lowest tissue concentrations (10^{-8}-10^{-7} mg/g). The bioavailability of the AP form was also higher than that of the AH form, which is consistent with the *in vitro* dissolution studies.

Among this study's weaknesses, the following should be noted:

– the small number of animals (2 females/adjuvant);
– the degradation of bone samples during their preparation, whereas it is known that the skull concentrates significant quantities of systemically distributed aluminium [3,6];
– the degradation of a brain tissue sample in the group treated by AP. The question of the distribution of vaccine aluminium in the brain is an aspect that should also be highlighted given that it is an adjuvant for which aluminium tissue concentrations were higher after 28 days;
– the lack of a determination of the aluminium concentration in the injected muscle; on the basis of the kinetic data obtained (Table 1), one can estimate that it remains at the injection site and, at most, 83% and 49% of the aluminium dose administered 28 days after injection of AH and AP, respectively;
– the use of a single adjuvant, whereas patients are exposed to an antigen-adjuvant pair.

Among this study's strengths, the following should be noted:

– the injection of vaccine adjuvants marked with ^{26}Al and its dosage by AMS. This method allows for a specific dosage of injected aluminium, and thus for the avoidance of:
  - problems involving the contamination of samples by exogenous aluminium;
  - **the question of the mechanism underlying the penetration of aluminium into the brain, after transit that is systemic or *via* the tissue macrophages.** It is in fact established that systemic aluminium can penetrate the brain by at least two mechanisms, as described in the "Distribution" paragraph. Furthermore, the theory is also advanced that aluminium that is phagocyted at the injection site can penetrate the brain via macrophages – the "Trojan horse" hypothesis [14].
– The consistency of the results obtained, the distribution profile of ^{26}Al being similar to that obtained by exposure via other routes of administration.

5.3 - Toxicodynamics

5.3.1 - Summary of aluminium's toxic effects

- ***In animals***

  - ***Central nervous system***

    The central nervous system is one of the most sensitive organs to aluminium.
    The neurotoxicity of aluminium after oral administration primarily manifests itself in neurobehavioral changes, as well as histopathological and biochemical changes, in the absence of encephalopathy or brain tissue injury. The performance changes in treated animals primarily consist of a reduction of activity and motor coordination, sensory deterioration and learning deficiencies. The histopathological changes manifest themselves via cytoplasmic and/or nuclear vacuolization phenomena, neuronal degeneration, in certain regions of the brain such as the cerebral cortex, the hippocampus or the base of the brain. Following oral administration of aluminium, biochemical changes involve, among other things, the cascade of second messengers, the peroxidation of lipids or cholinergic enzymatic activities [1].
    Only unusual routes of exposure (intrarachidial and intracerebral) or parenteral

administration lead to progressive encephalopathy in animals. In the spinal cord, the brain stem and certain parts of the hippocampus, this encephalopathy is associated with the presence of neurofibrillary degeneration. These neurofibrillary degeneration lesions are nevertheless morphologically and biochemically distinct from those that are observed in Alzheimer's disease [1].

- ***Effects on bone***

  Aluminium bone toxicity is recognized in animals. Histopathologically, the observed effects consist of lesions that characterize osteomalacia (rats, dogs and pigs) or an Adynamic bone disease (ABD) (rats). Most studies were conducted by the parenteral route (IV or IP). The data show that kidney failure seems to be a determining but inconstant factor for the impact of aluminium on bone tissue. The mechanism underlying these effects is not known, but it appears that aluminium's bone toxicity is partially linked to the difficulty of incorporating calcium in hydroxyapatite due to the presence of an aluminium deposit [1].

- ***Respiratory effects***

  Via inhalation, respiratory effects (granulomatous lesions) were observed in rats, hamsters and guinea pigs. However, it has not been clearly established whether these effects are linked to a direct effect of aluminium on pulmonary tissue or an indirect effect related to overloading of dust [15].

- ***Haematopoietic effects***

  According to the InVS, animal studies have demonstrated the existence of altered erythropoiesis during long-term, oral exposure; it stems both from direct action on circulating erythrocytes and interference with cellular iron metabolism in erythroid progenitors. Thus, in rats exposed to ≥ 230 mg Al/kg/day for eight months, altered erythropoiesis and erythrocytic damage (reduction of hemoglobin and hematocrit, osmotic fragility and morphological alteration of erythrocytes) were reported [15].

- ***Local effects after intramuscular injection (IM) – vaccine aluminium***

  An injection of GenHevac® (250 µL), a vaccine against hepatitis B adjuvanted with aluminium hydroxide, was carried out in the anterior tibial muscle of four adult Sprague-Dawley rats [16]. The animals were put down 7, 14, 21 and 28 days after injection. Histological cuts of muscle tissue, sampled in proximity and at a distance from the injection site, were prepared and examined with optical and electron microscopes. The results show, on D7 and D15, the presence of a necrotic area at the injection site containing damaged muscle fibers and neutrophils, surrounded by a large number of macrophages and lymphocytes. On D21 and D28, the injury progressed towards a mature lesion consisting of a focal infiltration of PAS+ and macrophages with large cytoplasms of fine granularity, without muscle fiber damage or giant cells. The image is reported to be very similar to macrophagic infiltrate of MMF. In these macrophages, electron microscope examination showed the presence of osmiophilic crystalline inclusions similar to those of MMF. However, the histological examination of muscle tissues at a distance from the injection site did not reveal any anomalies [16].

  Two groups of 12 monkeys received a diphtheria-tetanus vaccine adjuvanted with aluminium hydroxide or aluminium phosphate. After 3, 6 or 12 months, four monkeys from each group were put down. The lesion, focalized and identical to

that observed in humans, was observed for three months with aluminium phosphate and twelve months with aluminium hydroxide [17].

These two studies supported the determination of the provocation of a histological lesion macrophagic myofasciitis following the injection of a vaccine containing an aluminium adjuvant in humans. One also notes that the persistence of the injury, up to three months with aluminium phosphate and 12 months with aluminium hydroxide in monkeys, is consistent with the kinetic data obtained *in vitro* and *in vivo* with aluminium adjuvants.

- ***In humans***

  - ***Central nervous system***

    It has been demonstrated that the accumulation of aluminium in the human body and particularly in gray brain matter can generate encephalopathy-type neurological effects under particular circumstances of exposure that allow the accumulation of large quantities of aluminium or direct contact with the cephalorachidian liquid. This is particularly the case for kidney failure patients on dialysis. In the general population, no publication has reported any cases of encephalopathy linked to aluminium ingestion, including during oral treatment with antacids containing aluminium or under accidental circumstances [1].

    Although it is still difficult to demonstrate the responsibility of aluminium for disruptions of psychomotor-disorder-type neurological functions in the general population and in patients undergoing dialysis, the level of proof found in exposed professions seems, however, to be higher [1]. An association was suggested between exposure to aluminium at work (dust, smoke, McIntyre powder, etc.) and neurological effects related to alterations in psychomotor or cognitive performance during neurobehavioral tests. With the exception of several isolated cases, inhalation exposure has not been associated with manifest symptoms of neurotoxicity [15].

    A decrease in the mental development score (Bayley index) was shown at the age of 18 months in premature children who received prolonged parenteral nutrition (for more than 10 days), with standard solutions at exposures of 45 µg/kg/day of aluminium. A one point drop in score was reported per day of parenteral nutrition. The Bayley score was significantly higher in children receiving parenteral nutrition with aluminium-depleted solutions at exposures of 4-5 µg/kg/day [18].

  - ***Effects on bone***

    An excessive aluminium deposit in the skeleton can lead to the occurrence of a syndrome, commonly called Aluminium-Induced Bone Disease or AIBD, which has two types of histological manifestation in humans:

    → osteomalacia, characterized by lesions presenting large bone tissue scars, few osteoblasts and osteoclasts, suggesting a primary mineralization defect;
    → Adynamic Bone Disease or ABD, for which the width of the bone tissue scars is normal or small, and the number of osteoclasts and osteoblasts is considerably reduced. This reduction is characterized by a primary defect of bone formation, accompanied secondarily by a reduced mineralization [1].

    Premature newborns exposed to aluminium in parenteral nutrition had reduced lumbar and hip bone mass in adolescence [19]. This was a 15-year follow-up of

the children included in the study whose goal was to evaluate the impact on mental development at the age of 18 months of parenteral exposure to aluminium [18]. According to the authors, there are potential risk factors of an occurrence of osteoporosis and hip fracture at a more advanced age. It is nevertheless specified that these results should be confirmed in larger studies.

- ***Respiratory effects***

  In workers exposed to aluminium dust or smoke, damaging effects related to pulmonary fibrosis and function were observed; however, they were not reported consistently between studies and it is possible that co-exposure to other compounds could have contributed to the reported effects [15].

- ***Haematopoietic effects***

  Clinical studies highlight the prevalence of hypochromic microcytic anemia in chronic kidney failure patients with a high aluminium load, whereas chronic kidney failure patients on dialysis usually have normochromic normocytic anemia. The severity of the anemia is correlated with the plasma and erythrocyte levels of aluminium; this anemia is reversible when aluminium exposure is stopped and during aluminium chelater treatments. This type of effect has not been in observed in humans or animals who have normal kidney function [1].

- ***Local effects after IM injection – vaccine aluminium***

  MMF is a histological entity that was initially described in 1998 on examination of the deltoid muscle biopsies carried out in 14 patients. Histologically, the MMF lesion is specifically characterized by centripetal infiltration of the epimysium, the perimysium and the perifascicular endomysium by non-epithelioid macrophages with large basophilic cytoplasms containing periodic-acid-Schiff (PAS)-positive granulations, which are carriers of osmiophilic spiculated crystalline inclusions under an electron microscope. No significant myocytic lesion was observed, nor was any necrotic-type lesion [20-22]. The study of the chemical nature of the crystalline inclusions present in the macrophages has shown that they are made of aluminium salts [16,23].

  Aluminium salts are used as adjuvants in a certain number of vaccines. Given that the location of the lesions corresponds to the site where vaccines are usually injected, the theory was advanced that MMF could be a common reaction to intramuscular injections of vaccines containing aluminium [23-24].

  As reported before, studies conducted in rats and monkeys backed the identification of the association between the aluminium vaccine injection and the occurrence of MMF histological lesions in humans. However, an association between the vaccination and an “MMF clinical syndrome,” which includes myalgia, arthralgia, muscle weakness, asthenia or fever, and symptoms caused by central nervous system damage, principally cognitive function disorders, has not been established. It should be noted that the idea of CNS damage appeared in the R. Gherardi team’s publications [20,25] starting in 2001 and has only been mentioned since 2008 in the review of R. Gherardi’s work by the Afssaps [26].

5.3.2 - Reference toxicological values

The threshold doses relating to systemic effects were determined for chronic parenteral administration [27]:

→ nontoxic dose that does not induce an accumulation of aluminium in tissue: 1 to 2 µg/kg/day;
→ dose with no documented toxicity leading to the accumulation of aluminium in tissue: 15 to 30 µg/kg/day;
→ toxic dose (osteomalacia) leading to the accumulation of aluminium in tissue: 60 µg/kg/day.

It should be noted that the levels considered to be without risk (1-2 µg/kg/day) and toxic (60 µg/kg/day) are consistent with the results from the study showing a decrease in the mental development score (Bailey index) of 18-month-old children exposed to aluminium during their prematurity by way of parenteral nutrition at a level of 45 µg/kg/day. The effect was significant compared to children receiving aluminium-depleted solutions for an induced contribution of 4-5 µg/kg/day [18].

5.4 - Summary of the experimental work carried out by the R. Gherardi team and the ANSM's position

As explained above, MMF is a histological entity initially described in 1998 in patients presenting with myalgia, arthralgia, muscle weakness, asthenia or fever [20-21,28]. If a link between the injection of an aluminium vaccine and the presence of this histological lesion at the injection site is recognized, the team behind the discovery of this MMF injury also establishes a link, which is not recognized, between this MMF histological entity and a systemic clinical syndrome including chronic fatigue, myalgia and arthralgia [25]. Since the R. Gherardi team's initial publication, the agency has paid particular attention to this issue and to the potential health consequences, as seen from the conduct of an epidemiological study, as reported by the Scientific Committee of the Afssaps and the Global Advisory Committee on Vaccine Safety [22,24,29], or the various meetings that were held within the Afssaps, some of which R. Gherardi attended. The work evolved starting in 2008 when, for the first time, R. Gherardi reported the inclusion of cognitive disorders in the MMF clinical syndrome [26]. The agency then focused its monitoring of R. Gherardi's work on this issue until the submission, in 2012 and then in 2013, of two call-for-research-proposal funding requests to the ANSM on the topic "Systemic particle transportation by the phagocyte: safety of vaccine adjuvants."

In January 2008, R. Gherardi presented experimental results and a nonclinical study project to the agency aiming to ascertain the toxic potential of vaccine aluminium, mainly by means of pharmacokinetic investigations seeking to characterize more accurately the tissue distribution of aluminium particles [26]. The principal results presented by R. Gherardi followed by the comments of the preclinical working group of the Afssaps' MA Commission are presented below.

- The intramuscular injection of vaccine (0, 10 or 36 µL) in mice leads to the presence of significantly higher quantities of aluminium in the brain than it does in controls. The average concentration of aluminium reached up to 8 µg/mg of brain tissue (aluminium in an undetermined physicochemical form).
  The preclinical working group noted that the aluminium brain level reported in treated mice (8 µg/mg) corresponds, based on murine brain weight of 400 mg, to an aluminium dose of 3.2 mg. This is higher than the maximum quantity that can be administered. By way of comparison, according to a review published in 2007, the normal concentration of aluminium in the human brain is 2 µg/g of tissue compared to 23 µg/g in individuals having had encephalopathy linked to dialysis

[30]. For information, this would correspond to aluminium quantities of 2.6 and 30 mg, respectively, for an average brain weight of 1.3 kg. Beyond these numbers, there is the question of the homogeneity of aluminium distribution in the brain and whether it is in soluble or particle form.

- In order to study the distribution of vaccine aluminium, fluorescent latex beads of with a diameter of 500 nm were used as a tracer. These beads were admixed beforehand to the vaccine and then this was injected intramuscularly in rodents. R. Gherardi justified the choice of beads as tracers by the fact that the aluminium to be traced comes in particle form and that it follows *a priori*, an identical path to that of the beads, and by the fact that there is no radioactive isotope of sufficient aluminium activity to permit the easy study of its distribution in a more "traditional" manner. The analysis of fluorescence from histological sections performed 4 and 21 days after injection indicates the presence of beads in the drainage lymph nodes starting on the 4th day, as well as in the spleen, the liver and the brain after 21 days. The analysis of the microenvironment close to the beads by an X fluorescence method revealed the presence of aluminium particles.
  The preclinical working group pointed out that the use of latex beads very likely changes the distribution characteristics of aluminium, via the possible adsorption of aluminium to the latex beads in particular.
- In a murine model allowing the tracking of the monocytes-macrophages coming from the bone marrow[2], it was possible to visualize both the migration of monocytes-macrophages from the bone marrow towards the brain (after BBB transit) and the inclusion of latex beads administered via an intramuscular route in the macrophages from the bone marrow. On the basis of these results, Prof. Gherardi advances the hypothesis that particles transit from the point of injection towards the brain via macrophages.
  The preclinical working group was of the opinion that these results did not permit the linking of the two observations that show, on the one hand, macrophages that phagocyte particles *in situ* and, on the other hand, monocytes that migrate into the brain. However, the hypothesis of the migration of local macrophages towards the brain was not excluded and it was specified that it could be further investigated with the help of this model, as proposed by R. Gherardi.

In summary, it was felt in January 2008 that, on the basis of these results, the pursuit of the implemented experimental studies could lead to better characterization of the distribution of vaccine aluminium administered via the intramuscular route. Certain reservations were expressed with regard to:

→ the lack of perspective on the brain concentration of aluminium measured in mice compared to the quantities that can be administered and those that are potentially present in the human brain in individuals who present with and without encephalopathy;
→ the relevance of the use of latex beads as an aluminium tracer;
→ the relevance of the animal model for characterizing, beyond pharmacokinetic parameters, neurological alterations similar to those which characterize the MMF syndrome.

In October 2010, R. Gherardi was again received by the Afssaps to present the results of additional experimental studies carried out since 2008 [14]. It was reported

[2] Irradiated mice transplanted with bone marrow from a transgenic mouse that produces, in a constitutive manner, green fluorescent protein (GFP) mainly within the cells of the bone marrow.

that, in rodents, aluminium-rhodamine (Al-Rho) particles injected via the IM route could be transferred in the form of aggregates into other tissues of the body, particularly the brain. This transit occurred by way of macrophages, which migrate into the tissues by chemotaxis. Additional investigations showed that the phenotype differences induced experimentally in the MCP1 gene, whose product of expression is involved in chemotaxis, changed this transfer. Furthermore, it was reported that the distribution of Al-Rho particles was increased in mice with a more permeable BBB (*mdx* mice). Regarding these experimental results, R. Gherardi refers to a “Trojan horse”-type mechanism based on MCP-1 underlying the brain translocation of particles injected by the IM route *via* macrophages. The underlying question is the possible involvement of translocated particles in the brain, if they accumulate, in the occurrence of aluminium-induced neurotoxicity.

The Afssaps identified biases related to the presented data, as explained below [14]:

- the material used (Al-Rho particles) as a model for aluminium particles is not considered to be representative of the aluminium used as a vaccine adjuvant;
- the demonstration of a signal showing the presence of aluminium particles administered via an IM route is not optimal. Indeed, the detection relies on rhodamine marking and this raises the question whether the aluminium is still bound to the rhodamine nucleus after biodistribution. Furthermore, there is no double-marking in the studies, but a double-revelation whose specificity and quality can be called into question. Morin staining does not have the specificity of modern techniques that are currently available for this type of study;
- establishing a causal link between genetic polymorphism in MCP-1 in connection with cognitive disorders remains risky given the complexity of the study to be put in place and the limited knowledge available at this time about this polymorphism. In the MCP-1 loss of function experiment it is not possible to ascertain whether the loss of function is linked to the primary recruitment of monocytes-macrophages in the injected muscle and/or from secondary translocation towards the brain and other organs. To differentiate these two effects, it would be useful to study the brain’s recruitment of macrophages that are loaded with particles in deficient mice. On the basis of the available data, it is not possible to confirm whether the Trojan-horse mechanism that governs the translocation of particles into the brain is dependent on MCP-1;
- the experiments conducted in animals whose BBB integrity is deficient shows that increased permeability can amplify the phenomenon but not that BBB is involved in the translocation;
- a chemical specificity of the translocation phenomenon has not been demonstrated. Other metals or particles could be subject to the same phenomenon. This is illustrated by the fact that the latex particles used in the previous experiments also undergo this transfer.
- the relationship between the presence, if applicable, of aluminium inclusions in the CNS does not signify the automatic existence of a risk: the relationship with “neurotoxicity” is still a mere hypothesis. Additionally, the search for a dose-effect relationship is essential to permit a risk assessment. Furthermore, R. Gherardi indicated that the concentration of particles that accumulate in the brain is very low, but did not quantify it.

No investigation concerning the potential toxic effects resulting from the presence of particles in the brain of animals was carried out in this study program. They only aimed to study biodistribution, particularly in the brain, of aluminium particles and the implicated mechanisms.

Additionally, this issue concerns the specific case of vaccine aluminium adjuvants

and not aluminium particles alone. In no case was the specific antigen-adjuvant relationship considered in the studies seeking to secure recognition of the MMF clinical syndrome.

Following the creation of the ANSM, R. Gherardi submitted two call-for-research-proposal funding requests in 2012 and 2013 to the ANSM. The details relating to the ANSM's call-for-research-proposal policy can be reviewed at the following address: http://ansm.sante.fr/L-ANSM2/Appels-a-projets-de-recherche/Politique-des-appels-a-projets- de-recherche/%28offset%29/2.

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6 - List of vaccine-schedule vaccines

This list is in Appendix 1.

7 - Recommendations

Overall, the Haut Conseil de la santé publique finds that:

- **most inactivated and subunit vaccines used in the world contain adjuvants that influence their effectiveness;**
- **aluminium is the most frequently used adjuvant;**
- **aluminium salts have been added to vaccine antigens since 1920 without any country or official body calling into question the validity of this addition or the safety of vaccines containing this adjuvant;**
- **the publications concerning series of cases of adult macrophagic myofasciitis come from only one team in the world; the link between vaccination and the presence of granulomas containing aluminium is known but no study in the literature permits confirmation of the causal link between the reported clinical signs and the presence of aluminium-containing granulomas;**
- **the symptomatology described by just this one team primarily concerns adults exposed to an elevated number of vaccinations containing aluminium (5 on average) in the previous ten years. This symptomatology is not reported in infants who nonetheless receive proportionally more aluminium from vaccines, particularly in countries (the United States for example) that have, or that have had, vaccination schemes consisting of a larger number of injections;**
- **the brain toxicity of aluminium at high doses is a known fact and is responsible for clinical symptoms that are distinct from those described as being associated with macrophagic myofasciitis;**
- **recent work in mice, under experimental conditions that is not transposable to humans and to vaccination, provides information that clarifies the mode of transportation of aluminium into various organs, including the brain, without providing any evidence that demonstrates its harmfulness or a link between a potential presence in the brain and the clinical symptoms of macrophagic myofasciitis;**
- **no evidence is provided of genetic factors in humans that could promote the transportation of aluminium into the brain;**
- **adjuvants other than aluminium are or have been used in the past. Up to now, nothing has demonstrated that their effectiveness or their tolerance profile gives them a better risk/benefit balance than that of aluminium;**
- **the development and registration of vaccines incorporating new adjuvants that would replace vaccines containing aluminium (including previously used vaccines) will take several years.**

Thus, the Haut Conseil de la santé publique

- **Deems that the scientific data available today do not allow the safety of vaccines containing aluminium to be called into question with regard to their risk/benefit balance.**
- **Recommends the continuation of vaccinations according to the vaccine schedule in force.**

- **Warns of the consequences, in terms of the reappearance of infectious diseases, that could result from a decrease in vaccine coverage due to aluminium-containing vaccines being called into question without any scientific justification.**
- **Encourages the pursuit of research that aims to evaluate the safety of adjuvants that are available and in development.**

The CTV held session on 4 July 2013: 15 qualified members out of 17 qualified voting members were present, there were 0 conflicts of interest and the text was approved by 15 voters with 0 abstentions and 0 against.

The CSMT held session on 11 July 2013: 8 qualified members out of 15 qualified voting members were present, there were 0 conflicts of interest and the text was approved by 8 voters with 0 abstentions and 0 against.

GLOSSARY

Ab	Antibody
AFM	Association française contre les myopathies [French Myopathy Association]
Afssaps	Agence française de sécurité sanitaire des produits de santé [French Agency for Health Product Safety] (became the ANSM)
Ag	Antigen
ANSM	Agence nationale de sécurité du médicament et des produits de santé [French National Agency for Medicines and Health Product Safety]
ARS	Agence régionale de santé [French Regional Health Agency]
ASIA	Autoimmune/Inflammatory Syndrome Induced by Adjuvants
BBB	Blood-Brain Barrier
CDC	Centers for Disease Control and Prevention
CepiDc	Centre épidémiologique des causes médicales de décès [French Epidemiological Center of Medical Causes of Death]
CNR	Centre national de référence [French National Referral Center]
CNS	Central Nervous System
CPK	Creatine phosphokinase
CRL	Cephalorachidian liquid
CRPV	Centre régional de pharmacovigilance [French Regional Pharmacovigilance Center]
CTV	Comité technique des vaccinations [French Vaccination Technical Committee]
DGS	Direction générale de la santé [French General Directorate of Health]
EMG	Electromyogram
GACVS	Global Advisory Committee on Vaccine Safety
Germmad	Groupe de recherche sur les maladies musculaires acquises et dysimmunitaires [Research Group on Acquired Muscle and Immune Dysfunction Diseases]
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HCSP	Haut Conseil de la santé publique [French High Council for Public Health]
HLA	Human Leukocyte Antigen
IM	Intramuscular
INPES	Institut national de prévention et d'éducation pour la santé [French National Institute for Health Prevention and Education]
Inserm	Institut national de la santé et de la recherche médicale [French National Institute of Health and Medical Research]
InVS	Institut de veille sanitaire [French Institute for Public Health Surveillance]
IP	Intraparenteral
IV	Intravenous
MA	Marketing Authorization
MMF	Macrophagic myofasciitis
MMR	Measles-Mumps-Rubella
MRI	Magnetic Resonance Imaging

MS	Multiple Sclerosis
PCR	Polymerase Chain Reaction
PMI	Protection maternelle et infantile [French Maternal and Infantile Protection System]
PV	Primary Vaccination
SC	Subcutaneous
SG-HCSP	Secrétariat général du Haut Conseil de la santé publique [General Secretariat of the French High Council of Public Health]
SLE	Systemic Lupus Erythematous
SSA	Service de santé des Armées [French Armed Forces Health Department]
WHO	World Health Organization

Appendix – List of vaccine-schedule vaccines

Vaccines without adjuvants that are marketed in France (June 2013)	
Name of vaccine	**Company (MA holder)**
BACTERIAL VACCINES	
***Haemophilus influenzae* type b conjugate vaccine**	
Act-Hib	Sanofi Pasteur
Meningococcal vaccine	
Meningococcal vaccine A+C	Sanofi Pasteur
ACYW135 meningococcal vaccines	
non-conjugate: Mencevax	GSK
Conjugate	
Menveo	Novartis Vaccines and diagnostics
Nimenrix	GSK
Pneumococcal vaccine	
non-conjugate: Pneumo 23	Sanofi Pasteur
Typhoid vaccine	
Typhim V1	Sanofi Pasteur
Typherix	GSK
Live attenuated bacterial vaccine	
Vaccine against tuberculosis : BCG SSI (Bacillus Calmette–Guérin Statens Serum Institute)	Statens Serum Institut
Cholera vaccine (oral route of administration): Dukoral	CRUCELL
VIRAL VACCINES	
KILLED OR INACTIVATED VIRAL VACCINE	
Flu vaccines	
Agrippal	Novartis Vaccines and diagnostics
Fluarix	GSK
Immugrip	Pierre Fabre Médicament
Influvac	Abbott
Vaxigrip	Sanofi Pasteur
Rabies vaccine	
Pasteur rabies vaccine	
Rabipur	Novartis Vaccines and diagnostics
LIVE ATTENUATED VIRAL VACCINES	
Vaccine against yellow fever: Stamaril	Sanofi Pasteur
Measles vaccine: Rouvax	Sanofi Pasteur
Chickenpox vaccine	
Varilrix	GSK
Varivax	Sanofi Pasteur MSD
Zoster vaccine: Zostavax	
Vaccines against rotavirus (*oral route of administration)*	
Rotateq	Sanofi Pasteur MSD
Rotarix	GSK
COMBINATION LIVE ATTENUATED VIRAL VACCINES	
Measles, mumps and rubella vaccine	
M-M-RVAXPRO	Sanofi Pasteur MSD
Priorix	GSK
Measles, mumps, rubella, varicella vaccine	
Priorix tetra	GSK
Proquad	Sanofi Pasteur MSD

Vaccines and adjuvants June 2013 with adjuvant

Adjuvant content in the vaccines marketed in France (June 2013)

Name of vaccine	Company (MA holder)	Aluminium (target value)	Other adjuvants
ASSOCIATED BACTERIAL AND VIRAL VACCINES			
Diphtheria, tetanus, acellular pertussis, poliomyelitis vaccine, against infections of Haemophilus influenzae type b conjugate and hepatitis B			
InfanrixHexa	GSK	Phosphate: 0.3 mg/dose Hydroxide: 0.5 mg/dose (0.5 mL)	
Diphtheria, tetanus, acellular pertussis, poliomyelitis vaccine, against infections of Haemophilus influenzae type b conjugate			
InfanrixQuinta	GSK	Hydroxide: 0.5 mg/dose (0.5 mL)	
Pentavac	Sanofi Pasteur MSD	Hydroxide: 0.3 mg/dose (0.5 mL)	
Diphtheria, tetanus, acellular pertussis, poliomyelitis vaccine			
DTCaPolio			
InfanrixTetra	GSK	Hydroxide 0.5 mg/dose (0.5 mL)	
Acellular tetravac	Sanofi Pasteur MSD	Hydroxide: 0.3 mg/dose (0.5 mL)	
dTcaPolio			
Boostrixtetra	GSK	Hydroxide 0.3 mg/dose + phosphate 0.2 mg/dose (0.5 mL)	
Repevax	Sanofi Pasteur MSD	Phosphate: 0.33 mg/dose (0.5 mL)	
Diphtheria, tetanus, poliomyelitis vaccine			
Revaxis	Sanofi Pasteur MSD	Hydroxide: 0.35 mg/dose (0.5 mL)	
BACTERIAL VACCINES			
Meningococcal vaccine			
Meningococcal C conjugate vaccines			
Meningitec	Pfizer Holding France	Phosphate: 0.125 mg/dose (0.5 mL)	
Menjugatekit	Novar is Vaccines and diagnostics	Hydroxide: 0.3 to 0.4 mg/dose (0.5 mL)	
Neisvac	Baxter	Hydroxide: 0.5 mg/dose (0.5 mL)	
Meningococcal B vaccine			
Bexsero	Novar is Vaccines and diagnostics	Hydroxide: 0.5 mg/dose (0.5 mL)	
Pneumococcal conjugate vaccine			
Prevenar 13	Pfizer Holding France	Phosphate: 0.125 mg/dose (0.5 mL)	
Pasteur tetanus vaccine	Sanofi Pasteur MSD	Hydroxide: 0.6 mg/dose (0.5 mL)	

Vaccines and adjuvants June 2013 with adjuvant

Name of vaccine	Company (MA holder)	Aluminium (target value)	Other adjuvants
VIRAL VACCINES			
KILLED OR INACTIVATED VIRAL VACCINE			
Hepatitis B vaccine			
Engerix 10 µg/0.5 mL	GSK	Hydroxide: 0.25 mg/dose (0.5 mL)	
Engerix 20 µg/1 mL	GSK	Hydroxide: 0.5 mg/dose (1 mL)	
HBVAXPRO 5 µg/0.5 mL	Sanofi Pasteur MSD	Hydroxyphosphate sulfate: 0.25 mg/dose (0.5 mL)	
HBVAXPRO 10 µg/1 mL	Sanofi Pasteur MSD	Hydroxyphosphate sulfate: 0.5 mg/dose (1 mL)	
HBVAXPRO 40 µg/1 mL	Sanofi Pasteur MSD	Hydroxyphosphate sulfate: 0.5 mg/dose (1 mL)	
GenHevac B Pasteur	Sanofi Pasteur	Hydroxide: ≤ 1.25 mg/dose (1 mL)	
Flu vaccines			
Gripguard	Novartis Vaccines and diagnostics	0	MF59
Hepatitis A vaccine			
Avaxim adults	Sanofi Pasteur	Hydroxide: 0.3 mg/dose (0.5 mL)	
Havrix 1440 U/1 mL Adults	GSK	Hydroxide: 0.5 mg/dose (1 mL)	
Havrix 720 U/0.5 mL Infants and [text cut off]	GSK	Hydroxide: 0.25 mg/dose (0.5 mL)	
Vaccine against tick encephalitis			
Ticovac 0.5 mL adults	Baxter	Hydroxide: 0.35 mg/dose (0.5 mL)	
Ticovac 0.25 mL children	Baxter	Hydroxide: 0.17 mg/dose (0.25 mL)	
Encepur	Novartis Vaccines and diagnostics	0.3-0.4 mg/dose (0.5 mL)	
Vaccine against Japanese encephalitis			
Ixiaro		Hydroxide: 0.25 mg/dose (0.5 mL)	
Vaccine against human papillomavirus infections			
Cervarix	GSK	Hydroxide**:** 0.5 mg/dose (0.5 mL)	3-*O* -desacyl-4'-monophosphoryl lipid A (50 µg)
Gardasil	Sanofi Pasteur MSD	Hydroxyphosphate sulfate: 225 µg/dose; 0.5 mL)	
KILLED OR INACTIVATED VIRAL VACCINES+ANATOXINS+POLYSACCHARIDE VACCINES			
***Combined hepatitis A and B* vaccine**			
Twinrix adult	GSK	Hydroxide 0.05 mg/dose + phosphate 0.4 mg/dose (1 mL)	
Twinrix child	GSK	Hydroxide 0.025 mg/dose + phosphate 0.2 mg/dose (0.5 mL)	
Combined typhoid/hepatitis A vaccine			

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TABLES & FIGURES

Dr. Sin Hang Lee

Born in Hong Kong,
Sin Hang Lee, M.D., a practicing pathologist in New Haven, Connecticut, graduated from Wuhan Medical College, China, in 1956 and is qualified to practice medicine in the United States, Canada and the United Kingdom.
He is certified as a medical specialist in pathology by the American Board of Pathology and by the Royal College of Physicians and Surgeons of Canada.
He obtained the F.R.C.P.(C) degree in 1966.
Dr. Lee's postgraduate training and academic experiences took place at Sichuan Medical College, University of Hong Kong, New York Hospital-Cornell Medical Center, Memorial Hospital for Cancer and Allied Diseases, McGill University and Yale University, summarized in the Marquis Who's Who in the World, 7th Edition (1984-1985) and in Who's Who in Frontiers of Science and Technology, 2nd Edition (1985). Dr. Lee has been practicing pathology in New Haven, Connecticut, since 1971 with past and current affiliations listed in the 2003 edition of Marquis Who's Who in America.

Dr. Lee's research interests range from cell biology to cancer. Dr. Lee patented the first FDA-approved histochemical estrogen receptor assay for breast cancers based on his work published in Cancer. This assay identifies human breast cancers that may respond to hormonal manipulation or tamoxifen treatment. Dr. Lee has also patented the most specific FDA-approved method for the serologic diagnosis of Mycoplasma pneumoniae infection.

Dr. Lee’s recent medical research concerns human papillomavirus (HPV) testing by PCR/DNA sequencing, and accurate molecular diagnosis of infections caused by

Chlamydia trachomatis and Neisseria gonorrhoea, and of early Lyme disease caused by Borrelia burgdorferi. Dr. Lee's most recent publications on these projects are:

1. Lee S H, Vigliotti VS, Vigliotti JS, Pappu S. Routine human papillomavirus genotyping by DNA sequencing in community hospital laboratories. Infect Agent Cancer 2007; 2:11.
2. Lee S H, Vigliotti VS, Pappu S. DNA Sequencing Validation of Chlamydia trachomatis and Neisseria gonorrhoeae Nucleic Acid Tests. Am J Clin Pathol. 2008; 129:852-859.
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5. Lee S H, Vigliotti VS, Vigliotti JS, Pappu S. Validation of human papillomavirus genotyping by signature DNA sequence analysis. BMC Clin Pathol. 2009; 9:3.
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Based on his research, Dr. Lee has requested the FDA to revise the classification and the use direction of the current FDA-approved HPV test to reduce the excessive unnecessary harmful colposcopic cervical biopsies on American women

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Detection of human papillomavirus (HPV) L1 gene DNA possibly bound to particulate aluminum adjuvant in the HPV vaccine Gardasil®

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ABSTRACT

Medical practitioners in nine countries submitted samples of Gardasil® (Merck & Co.) to be tested for the presence of human papillomavirus (HPV) DNA because they suspected that residual recombinant HPV DNA left in the vaccine might have been a contributing factor leading to some of the unexplained post vaccination side effects. A total of 16 packages of Gardasil® were received from Australia, Bulgaria, France, India, New Zealand, Poland, Russia, Spain and the United States. A nested polymerase chain reaction (PCR) method using the MY09/MY11 degenerate primers for initial amplification and the GP5/GP6 based nested PCR primers for the second amplification were used to prepare the template for direct automated cycle DNA sequencing of a hypervariable segment of the HPV L1 gene which is used for manufacturing of the HPV L1 capsid protein by a DNA recombinant technology in vaccine production. Detection of HPV DNA and HPV genotyping of all positive samples were finally validated by BLAST (Basic Local Alignment Search Tool) analysis of a 45 60 bases sequence of the computer generated electropherogram. The results showed that all 16 Gardasil® samples, each with a different lot number, contained fragments of HPV 11 DNA, or HPV 18 DNA, or a DNA fragment mixture from both genotypes. The detected HPV DNA was found to be firmly bound to the insoluble, proteinase resistant fraction, presumably of amorphous aluminum hydroxyphosphate sulfate (AAHS) nanoparticles used as adjuvant. The clinical significance of these residual HPV DNA fragments bound to a particulate mineral based adjuvant is uncertain after intramuscular injection, and requires further investigation for vaccination safety.

1. Introduction

The quadrivalent human papillomavirus (HPV) vaccine, Gardasil® (Merck & Co.), has been recommended for prevention of HPV initiated cervical cancer and precancers since 2006 [1]. The active ingredients in the vaccine are genotype specific HPV L1 capsid proteins in the form of virus like particles (VLPs), which are highly effective in eliciting antibody production in the host against future infection by HPV 16, HPV 18, HPV 6 and HPV 11 and contain no viral DNA [2,3]. These VLPs are produced by a DNA recombinant technology in which the genotype specific "viral genes coding for the capsid proteins" [4] are inserted into the plasmid pGAL110 for transformation of the yeast spheroplasts [5]. For the vaccine to be effective, young girls age 9 12 are targeted for vaccination before their sexual activity begins [6].

According to the records kept by the U.S. Centers for Disease Control and Prevention (CDC), an apparently high number of side effects have been reported following HPV vaccinations in certain categories of health disorders [7]. Using the Brighton case definition of anaphylaxis for diagnostic certainty, the estimated rate of anaphylaxis in young women after HPV vaccination was found to be 5 to 20 times higher than those identified in comparable school based vaccination programs [8]. Rheumatoid arthritis, including juvenile rheumatoid arthritis, was recorded 3 times more frequently in the Gardasil® vaccinated subjects than in the control group receiving amorphous aluminum hydroxyphosphate sulfate (AAHS) adjuvant during clinical trials [9]. A number of cases of possibly immune based inflammatory neurodegenerative disorders involving the central nervous system, known as acute disseminated encephalomyelitis, following Gardasil® injections have been reported in world literature [10 16]. Physicians from several countries submitted samples of this quadrivalent HPV vaccine currently being used in the market to the author's laboratory contracted by a nonprofit organization (SANE VAX Inc.) to be tested for the presence of HPV DNA in the vaccine samples. These health care providers and some of their patients suspected that residual recombinant HPV DNA left in Gardasil® might have contributed to some of the unexplained post vaccination side effects.

To clarify the vaccine specification, the U.S. Food and Drug Administration has recently announced that Gardasil® indeed does contain recombinant HPV L1 specific DNA fragments [17]. However, the physical conditions of these HPV DNA fragments in the final vaccine products have not been characterized. It is not clear if they are in the form of free HPV DNA molecules in the aqueous phase of a suspension, encapsulated inside the VLPs [18,19], reversibly bound to the insoluble AAHS adjuvant as the VLPs, or irreversibly bound to the mineral aluminum (Al^{3+}) [20]. Free foreign DNA molecules are

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known to be degraded and eliminated quickly by the mammalian hosts [21]. The DNA fragments encapsulated in the VLPs [22,23] or bound to the particulate aluminum adjuvant [24] may be delivered into the antigen presenting cells or macrophages after injection. Their physical condition in the vaccine may determine the fate of these foreign DNA fragments in a vaccinated person and their variable physiopathological effects on the host. Different inorganic aluminum compounds with their specific physicochemical characteristics have been a subject of intense research [24 27] because they can boost the host's immunity response to both protein based [27] and DNA based [24,28] vaccination.

This article reports the results of HPV DNA testing performed on 16 samples of Gardasil® each bearing a different batch lot number re ceived from 9 countries and shows that the residual HPV L1 gene DNA fragments are probably firmly bound to AAHS nanoparticles.

2. Experimental

Since Gardasil® is a prescription drug, all samples tested were pur chased by licensed medical practitioners in the country of origin from their respective local drug suppliers. Gardasil® is marketed in 0.5 mL suspensions for injection in a single dose vial and in a manufacturer sealed prefilled syringe. A total of 16 Gardasil® samples with intact orig inal packages, including 3 unopened vials and 13 unopened prefilled syringes were received from physicians in Australia, Bulgaria, France, India, New Zealand, Poland, Russia, Spain and the United States, each bearing one of the following lot numbers: #1437Z, #1511Z, # 0553AA, #NL35360, #NP23400, #NN33070, #NL01490, #NM25110, #NL39620, #NK16180, #NK00140, #NM08120, #NL13560, #NL49190, #NN28160, or #NM29390. The 3 samples sent from the medical doctors located in the U.S. were delivered to the laboratory with cold packs in thermally in sulated containers. The other 13 samples from various countries outside the U.S. were transported in non insulated containers exposed to ambi ent temperatures.

A commonly targeted region of the HPV L1 gene DNA was first am plified by a primary PCR using the MY09/MY11 degenerate primer pair, followed by a nested PCR using a pair of GP5/GP6, a pair of GP6/MY11, or a pair of GP5/MY09 general consensus primers. Three primer pairs were chosen to perform the second PCR to amplify multiple nests of the 450 bp MY09/MY11 PCR products in an attempt to cover possible sequence variants of the genotype specific L1 genes that are used for manufacturing of the quadrivalent HPV vaccines by the DNA recombi nant technology [29], but may not be identical to those which the GP5/GP6 general primers were designed for. The PCR products were vi sually identified by standard agarose gel electrophoresis. The relative positions of these primer binding sites in the open reading frame of the HPV L1 gene, the sequences of the primers used, and the expected size of the amplicon terminated by each primer pair are summarized in Fig. 1. The presumptive nested PCR amplicons of HPV DNA were subjected to automated direct DNA sequencing, using a GP6 or a GP5 ol igonucleotide as the sequencing primer. A segment of 45 60 bases of the hypervariable region of the L1 gene sequence was excised from the computer generated base calling electropherogram for BLAST anal yses for confirmation of the HPV DNA detected and to validate its genotype. The technical detail of this nested PCR/DNA sequencing methodology has been previously reported [30 35].

Experiments were first designed to determine if free HPV DNA was detectable in the solution of the Gardasil® vaccine. To this end, an aliquot of 100 μL of the vaccine suspension was centrifuged at ~16,000×*g* for 10 min in a 1.5 mL microcentrifuge tube at room tem perature. The entire supernatant was transferred to another 1.5 mL microcentrifuge tube containing 500 μL of 95% ethanol, 12 μL of water, and 68 μL of 3 M sodium acetate. After the pellet was washed 3 times with 1 mL of 70% ethanol each and the final ethanol suspen sion was centrifuged at ~16,000×*g* for 5 min, the pellet was air dried. The dried pellet was re suspended in100 μL of 0.1 mg/mL proteinase K (Sigma Chemical Co., St. Louis, MO) in a buffer consisting of 50 mM Tris HCl, 1 mM EDTA, 0.5% Tween 20, pH 8.1. The mixture was digested at 45 55 °C overnight. After inactivation of the proteinase K solution in a metal block heated at 95 °C for 10 min, 1 μL of the unspun digestate was used for each primary PCR followed by nested (or hemi nested) PCR with various nested PCR primer pairs described above.

To test for HPV DNA in the insoluble part of the Gardasil® vaccine, the pellet of the centrifuged 100 μL vaccine suspension described above was washed twice with 1 mL of 70% ethanol each and the final ethanol suspension was centrifuged at ~16,000×*g* for 5 min. The washed pellet was air dried. The dried pellet was re suspended in100 μL of proteinase K solution. The suspension was digested at 45 55 °C overnight and centrifuged at ~16,000×*g* for 5 min the next day. The supernatant of the digestate was transferred to a 1.5 mL centrifuge tube and was heated at 95 °C for 10 min to inacti vate the proteinase K. One microliter of the unspun digestate super natant was used for each primary PCR followed by nested PCR.

The pellet of the proteinase K digestate of the insoluble part of Gardasil® was exhaustively washed with the buffer solution (50 mM Tris HCl, 1 mM EDTA, 0.5% Tween 20, pH 8.1) 4 times, 1 mL each time. The final washed pellet, presumably consisting of protein free AAHS particles, was re suspended in 100 μL of buffer. After heating to 95 °C for 10 min, 1 μL of the washed and heated insoluble particle sus pension was used for each primary PCR followed by nested PCR.

Short target sequence HPV genotyping was performed by direct au tomated cycle DNA sequencing [30 35]. Briefly, a trace of the positive nested PCR product was transferred directly with a micro glass rod

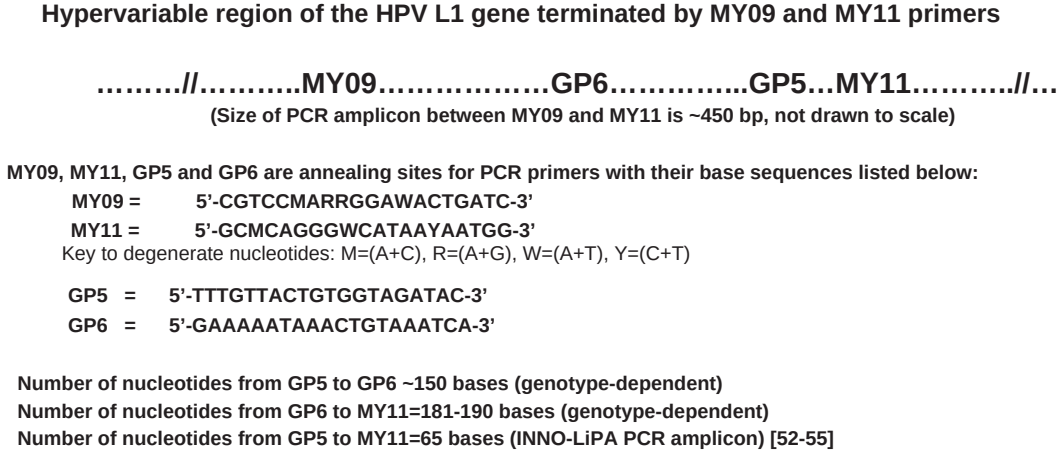


Fig. 1. Title: position map of four PCR primers for the detection of HPV L1 gene DNA. Description: relative locations of MY09, GP6, GP5 and MY11 primer sites.

from the positive nested PCR tube into a 20 μL volume of a cycle sequencing reaction mixture consisting of 14.5 μL water, 3.5 μL of 5 X buffer, 1 μL of BigDye Terminator 1.1 (Applied Biosystems) and 1 μL of 10 μM GP6, or GP5 sequencing primer. After thermal cycling according to the manufacturer's recommendation, the reaction mixture was loaded in an automated ABI 3130 four capillary Genetic Analyzer for sequence analysis. Alignment analysis of a 45 60 bases sequence in the hypervariable region of the L1 gene excised from the computer generated base calling electropherogram was performed against various standard HPV genotype sequences stored in the GenBank, using the on line BLAST system to validate the specific HPV genotyping.

Extraordinary precautionary steps were taken to ensure that the detection of any HPV target DNA was not due to inadvertent amplification of ambient HPV DNA sequences. A molecular laboratory was dedicated exclusively to this vaccine testing project from June 1 to October 31, 2011. During this period the entire project was completed and no other nucleic acid work was performed in the same facility. Transferring of primary PCR products to the nested PCR mixture and nested PCR products to the Sanger reaction mixture was accomplished with micro glass rods to avoid micropipetting aerosol and all procedures were carried out by highly trained, experienced molecular technologists according to a set of guidelines for the nested PCR technology applied in clinical diagnostic laboratories [35]. Negative water and primer controls were included in each PCR run of no more than 4 samples in one run. All PCR primers, including the MY09, MY11, GP5 and GP6 oligonucleotides, were tested, as previously described [30], against standard plasmid DNA of HPV type 16, 18, 11 or 6B purchased from American Type Culture Collection to ensure that 1 10 copies of plasmid DNA from each genotype could be detected by the nested PCR protocol designed for this project.

3. Results

Agarose gel electrophoresis of the GP6/MY11 or GP5/GP6 nested PCR products of all 16 Gardasil® samples tested revealed bands of expected size for HPV DNA when the proteinase K resistant insoluble part of the vaccine, presumably containing HPV DNA fragments bound to AAHS, was used as the template to start the primary PCR. However, primary PCR with the degenerate MY09/MY11 primer pair did not generate a visible PCR product band on any of the samples tested. When the GP5/MY09 primer pair instead of the GP6/MY11 primers was used, no nested PCR products were observed under identical experimental conditions, even when the entire pellet was used for starting a primary PCR. Since there were no primary PCR products observed, it remains questionable if the accumulation of the target DNA copies was numerically exponential or linear in the MY09/MY11 primary PCR.

No PCR products, primary or nested, were obtained when the supernatant of the Gardasil® vaccine or the supernatant of the proteinase K digestate of the insoluble particles of the Gardasil® vaccine was used as the starting material to initiate the primary PCR.

After the above results were obtained, a vial of recombinant hepatitis B vaccine, Recombivax HB® which also uses AAHS as the adjuvant for its formulation [36], was tested in parallel with 4 Gardasil® lot samples to determine if the HPV DNA fragments detected in the Gardasil® vaccine lots might have been a contaminant bound to the AAHS adjuvant ingredients in general use for other vaccine formulations by the manufacturer (Merck & Co.). The results of this parallel comparative nested PCR experiment showed no evidence of HPV DNA in the AAHS particles in a vial of Recombivax HB® purchased on the U.S. market (Fig. 2). The latter finding supported the interpretation that the HPV DNA fragments bound to the AAHS particles were associated with the Gardasil® manufacturing process, not a contaminant of the adjuvant used in the vaccine formulation.

To determine if the HPV L1 gene DNA detected in the AAHS particulate fraction might be in free solution after re suspension of the insoluble particles for PCR amplifications, serial double dilutions of the 4 proteinase K digested Gardasil® particle suspensions used for the above experiment (Fig. 2) were made in buffer, and 1μL from each dilution was used as the starting material for primary and then nested PCR. The results showed that the distribution of the HPV L1 gene DNA fragments in these samples was not homogeneous. The concentration of the HPV DNA templates amplifiable by the nested PCR from the dilution ladder did not decrease accordingly while the dilution factors increased progressively toward the endpoint (Fig. 3), as would be expected if free HPV DNA in true solution was titrated by serial dilutions [30]. This finding supported the interpretation that the HPV L1 gene DNA fragments existed in aggregation, which would prevent the success of using serial dilution methods to obtain single HPV DNA molecule samples to perform nested PCR for the preparation of sequencing template on some Gardasil® lots containing HPV DNA molecules of more than one genotype (Fig. 6).

All positive nested PCR amplicons were proven to contain a hypervariable sequence of the L1 gene open reading frame of an HPV 11 DNA synthetic construct (Fig. 4), an African variant of HPV 18 DNA (Fig. 5), or a mixture of these two (Fig. 6). No HPV 6 or HPV 16 DNA residues were detected in this study.

The HPV genotypes detected in the 16 lot samples of Gardasil® are summarized in Table 1. Part of the results presented in this article was previously reported to the FDA by SANE VAX, Inc.

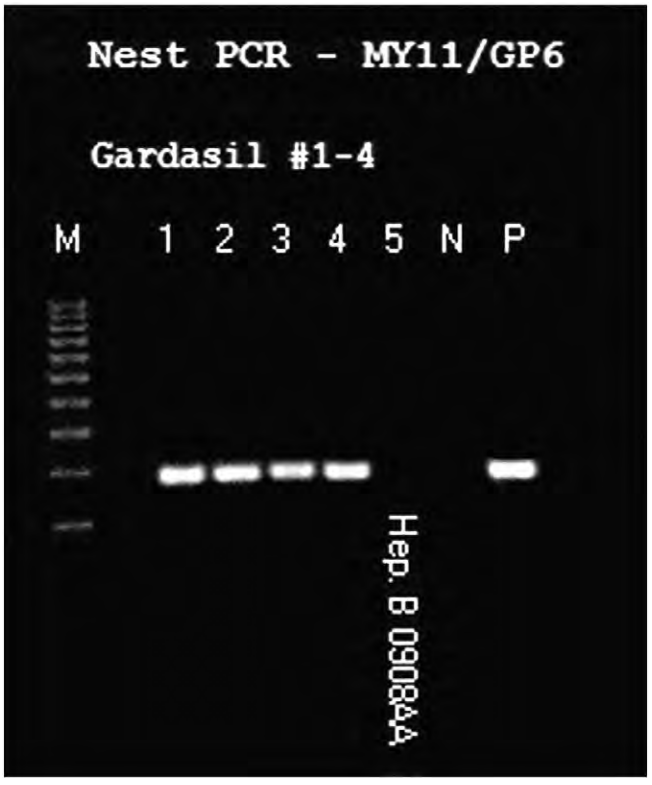


Fig. 2. Title: nested PCR with AAHS recovered from HPV vaccine and hepatitis B vaccine. Description: HPV L1 gene DNA fragments detected in AAHS particles of Gardasil®, but not in AAHS particles of Recombivax HB®. Gel electrophoresis of GP6/MY11 nested PCR products on 4 of the 16 Gardasil® vaccine samples and 1 Recombivax HB® sample (Lot # 0908AA). The MY09/MY11 primary PCR was initiated with 1 μL suspension of the insoluble and proteinase K-resistant fraction derived from each vaccine sample. Visualization of a ~190 bp nested PCR amplicon in lanes #1–4, but not in lane #5, indicates the presence of HPV L1 gene DNA residues possibly bound to the AAHS adjuvant in the HPV vaccine, but not in the AAHS adjuvant of the hepatitis B vaccine. All ~190 bp nested PCR amplicons were confirmed by DNA sequencing to be HPV-11 or HPV-18 DNA or a mixture of both. M = molecular ruler, 100–1000 bp. Lanes 1–4 = Gardasil® GP6/MY11 nested PCR products. Lane 5 = no GP6/MY11 nested PCR products in Recombivax HB®. N = negative water control. P = HPV-16 DNA positive control.

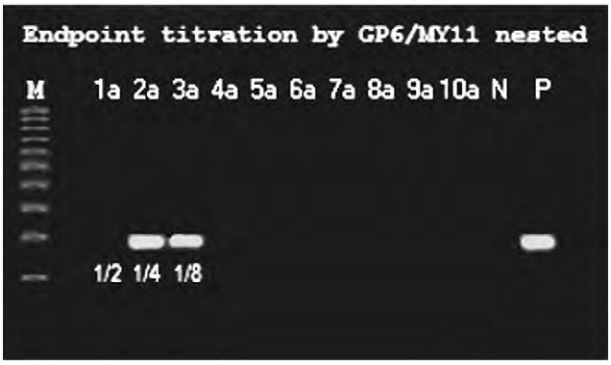


Fig. 3. Title: HPV DNA nested PCR on serially diluted Gardasil® AAHS suspensions. Description: serial dilution experiment suggests HPV DNA bound to insoluble AAHS aggregates. Gel electrophoresis of the nested PCR products after 10 serial double dilutions of 1 of the 4 Gardasil® particulate suspensions (Fig. 2) were used to initiate the respective primary PCRs. Dilution factors covered from ½ in Lane 1a to 1/1024 in Lane 10a. The results showed no PCR product band with sample of a low dilution factor (Lane 1a) followed by heavy product bands of PCR started with samples of higher dilution factors (Lanes 2a and 3a). No gradual reduction in PCR product band density in the dilution ladder was observed, suggesting that the HPV DNA was bound to AAHS particles in solid aggregates. M = molecular ruler, 100–1000 bp. Lanes 1a–10a = GP6/MY11 nested PCR products after the AAHS particle suspension was serially double-diluted from ½ to 1/1024 in buffer. N = negative water control. P = HPV-16 DNA positive control.

4. Discussion

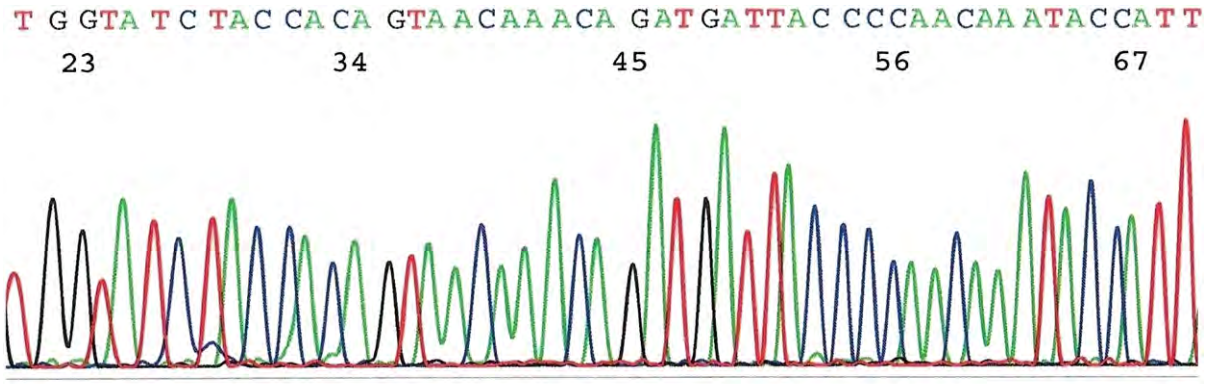
Since the quadrivalent HPV vaccine, Gardasil®, is produced by a DNA recombinant technology in which viral genes coding for the major L1 capsid proteins (4) are inserted into plasmid pGAL110 for transformation of the yeast spheroplasts [5] to manufacture the desired genotype specific VLPs, any residual HPV DNA detected in the vaccine represents a fragment or fragments of the genetically modified viral DNA for vaccine manufacturing, and not a viable or self replicating virus.

The presence of HPV DNA in Gardasil® is a surprise to most medical practitioners because this protein based vaccine has been purified to remove all contaminating components, including viral and plasmid DNA, by a highly effective patented process [37]. According to the published specification of Gardasil®, the active ingredients of the vaccine are "highly purified virus like particles (VLPs) of the recombinant major capsid (L1) protein of HPV types 6, 11, 16, and 18. As the VLPs do not contain viral DNA, they cannot infect the cells or reproduce [2,3]."

This study shows that the HPV L1 gene DNA fragments are bound to the AAHS nanoparticles, not in the aqueous phase or within the VLPs in the Gardasil® vaccine because they are not detectable in the supernatant of the vaccine, or in the supernatant of the proteinase K digestate of the vaccine precipitates. According to the Gardasil® formulation, the only water insoluble, proteinase K resistant excipient in the vaccine is the AAHS precipitates which the manufacturer chooses and specifically prepares as a mineral based adjuvant. It is inconceivable that an unidentified proteinase resistant particulate foreign substance could have contaminated all of the Gardasil® vaccine lots tested. Interpretation of the study findings is further explored as follows.

Based on information available in the public domain, in addition to the HPV 6, 11, 16, and 18L1 protein VLPs, Gardasil® contains AAHS adjuvant, sodium chloride, L histidine, polysorbate 80 (PS 80), sodium borate, and water for injection as excipients [2,3] with a buffer system which provides for a range from pH 6.0 to 6.5 [38]. Except for the VLPs and AAHS adjuvant, all other excipients listed in the formulation are common laboratory chemicals well known for their highly water soluble properties [39]. The insoluble AAHS adjuvant and VLPs are expected to be in the pellet of the vaccine after centrifugation while all other excipients remain in the supernatant.

If the residual HPV L1 gene DNA fragments existed freely in the aqueous solution as the water soluble excipients do in the vaccine, they would be rapidly degraded by the nucleases in the serum after intramuscular injection and eliminated from the body of the host (21). However, intramuscular injection of 100 μg of free HPV 16L1 plasmid DNA in BALB/C mice without adjuvant invariably induces a strong CD8 T cell response [27], indicating that under certain conditions free non replicating HPV L1 gene DNA can activate the immune system.



>gb|U55993.1|SCU55993 Synthetic HPV11 major capsid protein L1 gene, complete cds
Length=1506

Score = 97.6 bits (49), Expect = 5e-18
Identities = 49/49 (100%), Gaps = 0/49 (0%)
Strand=Plus/Minus

```
Query  1     TGGTATCTACCACAGTAACAAACAGATGATTACCCCAACAAATACCATT  49
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1000  TGGTATCTACCACAGTAACAAACAGATGATTACCCCAACAAATACCATT  952
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Fig. 4. Title: HPV-11 L1 gene DNA short target sequence. Description: synthetic construct of HPV-11 DNA detected in Gardasil® AAHS adjuvant. HPV-11 L1 gene DNA detected in the insoluble part of Gardasil® after proteinase K digestion and exhaustive washings in detergent buffer pH 8.1. This is the electropherogram of a short target DNA sequencing, using a 181 bp GP6/MY11 nested PCR amplicon as the template. The sequencing primer was GP6. BLAST alignment of 49 bases of the DNA sequence confirmed that the HPV DNA detected was part of the synthetic construct (GenBank Locus SCU55993) designed for production of HPV-11 VLPs used in Gardasil®.

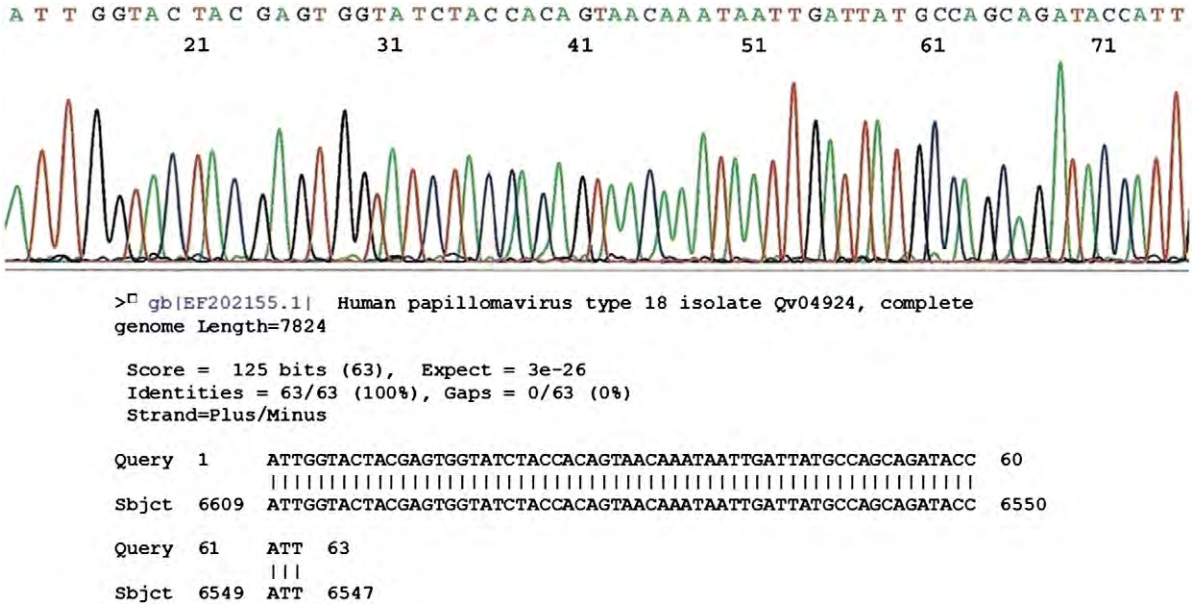

Fig. 5. Title: HPV-18 DNA short target sequence. Description: HPV-18 DNA fragment detected in Gardasil® AAHS adjuvant. HPV-18L1 gene DNA detected in the insoluble part of Gardasil® after proteinase K digestion and exhaustive washings. This is the electropherogram of a short target DNA sequencing, using a 187 bp GP6/MY11 nested PCR amplicon as the template. The sequencing primer was GP6. BLAST alignment of 63 bases of the DNA sequence confirmed that the HPV DNA detected was a hypervariable segment of the HPV-18L1 gene (GenBank Locus EF202155).

HPV VLPs are irregularly shaped 30 50 nm structures composed of self assembled HPV major capsid L1 protein pentamers [40]. VLPs pro duced by various viral genes, including the HPV VLPs, have been shown to be able to encapsulate non viral DNA for transfer of the latter into tar get cells [19,23,41]. If the L1 gene DNA fragments in Gardasil® were en capsulated inside the VLPs, the HPV DNA fragments could be delivered into the antigen presenting cells (APCs) or macrophages, which may trigger a series of immunological reactions. The VLPs carrying encapsu lated DNA can be digested by proteinase K in vitro and release the pack aged DNA inside into solution [42].

AAHS is Merck's proprietary mineral based adjuvant which con sists of amorphous precipitates prepared by mixing solutions of NaH_2PO_4, $KAl(SO_4)_2$ and ammonium hydroxide under special con trolled conditions (25 27). The mechanism for its extraordinarily high VLP binding capacity has been investigated (27), but is still not fully understood. It has been attributed to a nonspecific binding due to the unique amorphous mesh ultrastructure of the AAHS precipi tates and an electrostatic attraction between the AAHS nanoparticles and the VLPs (27). However, the isoelectric points of the HPV L1 cap sid proteins are pH 7.95, 8.35 and 8.55 for HPV 16, HPV 18 and HPV 6, respectively [43], and are positively charged in the Gardasil® vaccine at pH 6.0 6.5. The point of zero charge (PZC) for AAHS is 7, compared to a PZC of aluminum phosphate at pH 5 and a PZC of alu minum hydroxide at pH 10. Yet, the HPV VLP binding capacity for AAHS is twice as high as that for aluminum phosphate or for alumi num hydroxide (27), indicating that electrostatic attraction plays lit tle role, if any, in the binding between HPV VLPS and AAHS.

The mechanism of binding between AAHS and DNA fragments in Gardasil® is different from that between AAHS and VLPs. DNA molecules are very small, but linear and long, have an isoelectric point at about pH 5.0 and carry a negative charge at pH 6.0 6.5. As a result, DNA molecules in the Gardasil® vaccine can bind to the pos itively charged AAHS particles electrostatically. However, the HPV L1 gene DNA fragments in the vaccine are detected in the proteinase K digested precipitates, presumably AAHS nanoparticles which have been exhaustively washed in a nonionic detergent buffer, pH 8.1.

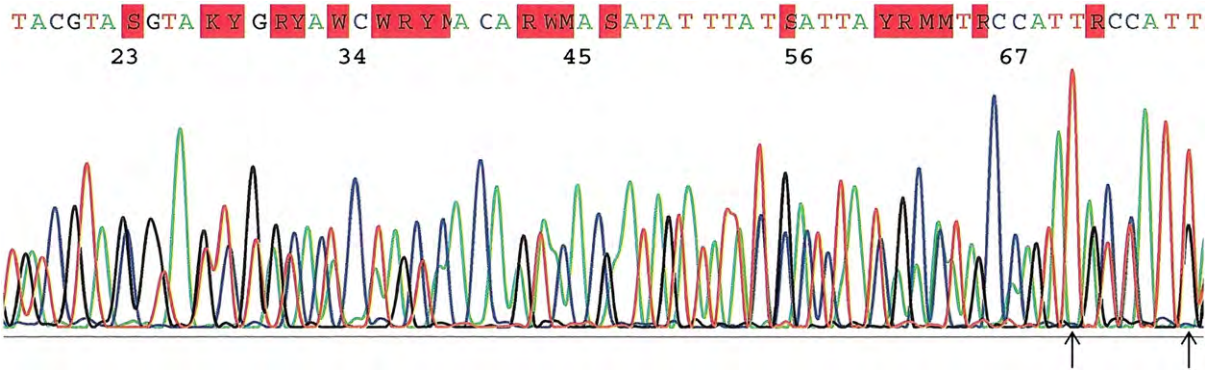

Fig. 6. Title: mixed HPV-11 and HPV-18 short target sequences. Description: mixed HPV-11 and HPV-18L1 gene DNA sequences detected in the insoluble part of a Gardasil® sample after proteinase K digestion and exhaustive washings. This is the electropherogram of a short target DNA sequencing of one of the GP6/MY11 nested PCR products, illustrating two superimposed DNA sequences. Since the GP6/MY11 PCR amplicon for HPV-11 is 181 bp and the GP6/MY11 PCR amplicon for HPV-18 is 187 bp in size [35], some conserved se quences shared by these two genotypes in this composite electropherogram may be recognized in two different positions 6-nucleotides apart. For easy identification, the first 5′ T (counting from the right of the color tracing) in the characteristic CCATT--5′ ending of the MY11 primer site for these two sequences is pointed with a black arrow. These two Ts are separated exactly six bases apart. Since numerous HPV DNA fragments of both genotypes were firmly bound to any individual AAHS particles, it was not possible to ob tain a single DNA fragment by making serial dilutions of the AAHS particles for PCR amplification to prepare a pure DNA template for Sanger sequencing reaction.

Table 1
Gardasil® lot numbers, countries of origin and HPV L1 gene DNA found.

Lot #	Country/source	Genotype
1437Z vial	USA, Connecticut	HPV-11
		HPV-18
1511Z prefilled syringe	USA, New York	HPV-18
0553AA vial	USA, New Jersey	HPV-11
		HPV-18
NL35360 prefilled syringe	France	HPV-11
		HPV-18
NP23400 prefilled syringe	Spain, Valencia	HPV-11
		HPV-18
NN33070 prefilled syringe	Spain, Valencia	HPV-11
		HPV-18
NM25110 prefilled syringe	Australia, Sydney	HPV-11
		HPV-18
NL01490 prefilled syringe	New Zealand, Tauranga	HPV-18
NK16180 prefilled syringe	New Zealand, Northland	HPV-18
NK00140 prefilled syringe	New Zealand, Tauranga	HPV-11
		HPV-18
NM08120 prefilled syringe	New Zealand, Christchurch	HPV-11
		HPV-18
NL13560 prefilled syringe	New Zealand, Wellington	HPV-11
		HPV-18
NL39620 prefilled syringe	Poland	HPV-11
NN28160 vial	Russia	HPV-11
		HPV-18
NL49190 prefilled syringe	Bulgaria	HPV-11
		HPV-18
NM29390 prefilled syringe	India	HPV-18

The latter pH is way above the PZC of the AAHS nanoparticles and the isoelectric point of DNA. Any DNA molecules initially bound to the in soluble AAHS electrostatically would have been washed off because both the DNA molecules and the AAHS particles carry a negative charge at pH 8.1, thus electrostatically repulsive.

The more likely binding mechanism between the HPV L1 gene DNA and the AAHS nanoparticles in Gardasil® is of a chemical na ture through ligand exchange of phosphate for hydroxyl, indepen dent of the electrostatic forces [20,44]. When aluminum (Al^{3+}) and DNA interact, the binding site for Al^{3+} on the DNA chains is the phosphate groups on the DNA backbones, not the bases of the DNA molecule [20]. Aluminum DNA complexes differ from other metal DNA complexes. Apparently more than one form of DNA can exist at any time in the presence of aluminum. DNA aluminum complexes formed at different pH are known to have variable DNA melting profiles that only a portion of the DNA is "stabilized" or "denatured" [45], which may explain the success of amplification of the HPV 11 and HPV 18L1 gene DNA fragments when the MY11, GP6 and GP5 PCR primers are used and the failure of amplifi cation with an MY09 PCR primer in the present study. Existent enzyme based biochemical methods cannot quantify the DNA bound to a particulate aluminum adjuvant. Quantitation of HPV by qPCR assay involves phenol/chloroform extraction of the HPV DNA from proteinase K digestate [46].Such assays cannot be used to quantify the mineral bound DNA which requires a nested PCR with a highly processive DNA polymerase for detection.

This report provides the first evidence that a chemical binding may have occurred between naked DNA fragments and an aluminum based adjuvant to form a highly stable complex in a vac cine. However, the evidence is still indirect. Such a new chemical complex may need direct physical analyses of the molecular structure for final validation. A stable HPV DNA AAHS complex, chemical in na ture and protected in the cytoplasm of the macrophages, may explain why HPV L1 gene DNA fragments can be detected in the post mortem blood and spleen tissue obtained at autopsy after a teenage girl suf fered a sudden unexpected death 6 months after receiving the last dose of Gardasil® vaccination (S.H. Lee, manuscript in preparation based on a report submitted to the Coroner of Wellington, New Zealand for an inquest on August 8 9, 2012). Macrophages laden with particulate aluminum based adjuvant are known to travel from the site of intramuscular vaccine injection through the blood to the spleen and to other organs [47 49].

One may speculate on the possible answers to the question as to why only residual fragments of HPV 11 and HPV 18 DNA, but not those of HPV 6 or HPV 16 DNA, were detected in a quadrivalent HPV vaccine in the current study. First, it is entirely possible that the nested PCR method using the consensus general primers is not suitable for detecting a small amount of residual HPV 6 and HPV 16 DNA residues bound to the AAHS particles when the HPV 11 and HPV 18 DNA residues are present in overwhelming proportions. An other possibility is that the vaccine manufacturer may have a more ef fective procedure in cleaning up the HPV 6 and HPV 16 DNA residues from the vaccine products than the procedure used to remove the residues of HPV 11 and HPV 18 DNA fragments. (Author's note after submission of this manuscript HPV 16L1 gene DNA fragments have been detected in at least some of the Gardasil® vaccine lots using various sets of genotype specific PCR primers).

All existent commercial test kits and published protocols for HPV DNA assays are designed to test for an analyte in solution. Diligent search of the literature has failed to find a publication dealing with detection of HPV DNA bound to aluminum based particles. Both the developer [50,51] and the manufacturer [52,53] of the Gardasil® vac cine seem to heavily rely on using the INNO LiPA kit [54,55] for HPV DNA testing. If the latter procedure was used as the tool to test for HPV DNA residues during vaccine manufacturing, the unrecognized HPV 11 and HPV 18 DNA residues might have been a result of techni cal failure in detection. In a recently published WHO sponsored sur vey, 9 of 12 laboratories using the INNO LiPA kit were found to be not proficient of detecting the HPV types tested for [56].

As shown in Table 2, the INNO LiPA probe for detection of the HPV 11 DNA sequence immediately downstream of the MY11 primer region is designed for naturally occurring HPV 11 isolates. In contrast to all known HPV 11 variants isolated from patients, the genetically engineered HPV 11L1 protein coding synthetic construct for Gardasil® vaccine production has 4 base mismatches against the sequence of the probe in this 22 base interprimer region targeted for hybridization which may fail to take place because the probe is not matched with the target sequence. To allow detection of at least 43 different HPV genotypes in a 22 base sequence region, mismatched hybridization probes must be washed off from the target sequence [54].

Table 2
Mismatched HPV-11 and HPV-18 DNA sequences between the target regions of the HPV DNA in Gardasil® and the INNO-LiPA probes.

Gardasil® target/probe	DNA sequence (22 bases)
Gardasil® HPV-11 L1 DNA[a]	CAGATGATTACCCCAACAAATA-5′
INNO-LiPA HPV-11 probe	CAAGTGGTTTCCCCAGCAAATA-5′
Gardasil® HPV-18 L1 DNA[b]	TAATTGATTATGCCAGCAGATA-5′
INNO-LiPA HPV-18 probe[c]	TAATTGATTATGCCAGCAAACA-5′

[a] The 22-base interprimer segment of Gardasil® HPV-11 L1 gene DNA to the right can be amplified by the INNO-LiPA PCR primers designed for all common anogenital HPV DNA variants in this L1 region. However, the viral gene encoding the HPV-11 major capsid L1 protein for production of the Gardasil® HPV-11 VLPs is a synthetic construct which has a unique DNA sequence in this region with the interprimer location at 978 to 957 in the gene map depicted in GenBank Locus SCU55993. The 4 mismatched bases between the INNO-LiPA HPV-11 probe [54] and the Gardasil® HPV-11 in this region are underlined.

[b] The segment of the Gardasil® HPV-18 L1 gene DNA amplified by the INNO-LiPA PCR primers corresponds to a 22-base interprimer sequence from location 6573 to 6552 of the complete genome map of HPV-18, African type, as depicted in GenBank Locus EF202155.

[c] The INNO-LiPA HPV-18 probe [54] is designed to target a 22-base interprimer sequence shared by all HPV-18 European variants, e.g. GenBank Locus EF202148, and Asian-American variants, e.g. GenBank Locus EF202145. The two mismatched bases between the INNO-LiPA HPV-18 probe and the Gardasil® HPV-18 in this interprimer region are underlined.

There are numerous DNA sequence variations to the L1 gene with in the genotype of HPV 18 which ultimately determines the amino acid composition of the major capsid protein of a virion. Phylogenetic analysis has shown that all HPV 18 isolates can be classified into 3 subtypes based on alignments of the DNA sequences of the variants, i.e. the European, the Asian American and the African subtypes [57]. In Europe, it has been reported that all of the HPV 18 isolates from patients are found to be of the European or Asian American variants [58]. In the U.S., 91% of the HPV 18 isolates from white women are reported to be of the European and Asian American variants, and 64% of the HPV 18 isolates from African American women belong to the African variants [59]. Since the prevalence of the African variants of HPV 18 among European patients is negligible [58], the Dutch researchers who originally developed the HPV INNO LiPA kit [54] naturally selected an HPV 18 probe targeting a homologous sequence shared by all European and Asian American HPV 18 variants for the testing. However, the HPV 18L1 protein coding gene chosen by the manufacturer for Gardasil® production is closely related to an African subtype [57,60]. Failure to detect a target sequence of an African variant HPV 18 DNA in the vaccine Gardasil® with a hybridization probe specifically designed for the European and Asian American variant DNA may simply reflect the diversity of the L1 protein amino acid sequences within the genotype of HPV 18 (Table 2).

To reach the conclusion of finding unexpected HPV DNA residues in a vaccine product is a serious consideration. In addition to the extraordinary precautionary measures undertaken to avoid potential amplification of ambient HPV DNA sequences in conducting this research project as described in the Experimental section, the DNA sequence illustrated in Fig. 4 assures that the DNA template of this sequence has its origin in the Gardasil® vaccine because it represents a signature sequence of the HPV 11L1 gene synthetic construct specifically designed by the manufacturer for vaccine production. This HPV 11 DNA template does not exist in the environment and cannot come from any patient samples. In the author's laboratory serving a women population under the care of private gynecologists, HPV 18 is detected in about 6% of the routine HPV isolates [33], and about 80% of the HPV 18 isolates from the clinical samples belong to the European/Asian American subtype (author's unpublished data). All of the HPV DNA fragments detected in the vaccine samples other than those of the HPV 11 synthetic construct are of the African subtype of HPV 18. It is highly unlikely to contaminate the vaccine samples only with one HPV subtype so rarely encountered in the environment.

A major limitation of this study is that only 16 randomly selected Gardasil® samples have been tested. The findings presented in this report cannot prove that the Gardasil® vaccines being marketed other than these 16 samples also contain HPV 11 or HPV 18 DNA fragments; nor can the findings rule out the possibility that the Gardasil® vaccines on the world market may contain HPV 6 or HPV 16L1 gene DNA residues in addition to HPV 11 and HPV 18L1 gene DNA residues, or DNA residues from the plasmid pGAL110 and the yeast cells used in the production of the HPV VLPs.

The Gardasil® formulation with AAHS adjuvant has significantly increased the peak neutralizing antibody titers in vaccinated mammals based on poorly understood mechanisms [61]. The findings presented in this paper suggest that the residual HPV L1 gene DNA bound to a particulate aluminum mineral based adjuvant in Gardasil® may activate the antigen presenting cells or macrophages in an innate immunity reaction after endocytosis, which may have played a significant role in augmenting its immunogenicity. Co delivery of a DNA vaccine and a protein vaccine with aluminum phosphate salts is known to stimulate a potent and multivalent immune response [62]. However, development of preventive human DNA vaccines is still at an experimental stage [63,64]. The potential risks of a DNA vaccine may include DNA integration into the host genome, induction of anti DNA antibodies [65] and activation of the macrophages with release of cytokines, including tumor necrosis factor [27,66].

For Gardasil® vaccine production, the HPV DNA encoding the L1 capsid proteins is inserted into the plasmid pGAL110 to transform yeast cells for production of VLPs [5,67,68]. Possible expression systems also include mammalian cells [69]. Retention of residual recombinant DNA in protein based vaccines has been a concern in the industry since induction of cancer is a single cell phenomenon, and a single functional unit of foreign DNA integrated into the host cell genome might serve to induce cell transformation as a single event or part of a series of multifactorial events [70]. Chromosomal integration of foreign DNA may occur through poorly understood mechanisms [71,72] with uncertain consequences [73]. The short term and long term impact of the residual fragments of HPV L1 gene DNA or plasmid DNA if chemically bound to the mineral aluminum of AAHS nanoparticles is largely unknown and warrants further investigation.

5. Conclusion

Residual HPV L1 gene DNA fragments are present in the protein based quadrivalent HPV vaccine. These DNA fragments are found to be firmly bound to the insoluble AAHS adjuvant particles. The clinical significance of these residual HPV DNA fragments bound to AAHS is not clear after intramuscular injection, and needs further investigation for vaccination safety.

Conflict of interest

This study was commissioned and sponsored by SANEVAX, Inc. for a future payment not to exceed one U.S. dollar.

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Detection of human papillomavirus L1 gene DNA fragments in postmortem blood and spleen after Gardasil® vaccination—A case report

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ABSTRACT

A same-nested PCR was used to re-amplify the amplicon of a hypervariable region of the HPV-16 L1 gene DNA in the postmortem blood and splenic tissue obtained at autopsy of a formerly healthy teenage girl who suffered a sudden unexpected death in sleep 6 months after 3 intramuscular injections of a quadrivalent HPV vaccine, Gardasil®. A full autopsy analysis revealed no cause of death. The HPV-16 gene DNA detected in the postmortem materials was similar to the HPV-16 gene DNA fragments in Gardasil® in that both were in non-B-conformation, requiring non-degenerate GP6 and MY11 primers to re-amplify the PCR amplicon for detection and to generate a template useful for direct DNA sequencing. A sequence excised from the base-calling DNA sequencing electropherogram was analyzed by Basic Local Alignment Search Tool (BLAST) alignment and a 45 - 60 base sequence fully matched with a standard hypervariable region of the HPV-16 L1 gene retrieved from the National Center for Biotechnology Information database validated the correct genotyping for HPV-16 L1 gene DNA. These naked non-proliferating HPV-16 L1 gene DNA fragments appeared to be in the macrophages of the postmortem blood and spleen, and were protected from degradation by binding firmly to the particulate aluminum adjuvant used in vaccine formulation. The significance of these HPV DNA fragments of a vaccine origin found in postmortem materials is not clear and warrants further investigation.

Keywords: Gardasil®; Postmortem; HPV; DNA; Aluminum; Vaccine

1. INTRODUCTION

Virus-like particles (VLPs) of human papillomavirus (HPV) are irregularly shaped 30 - 50 nm structures composed of self-assembled HPV major capsid L1 protein pentamers manufactured by a DNA recombinant technology [1,2]. Genotype-specific VLPs for HPV-16, -18, -11 and -6 are used as the active ingredient of a quadrivalent HPV vaccine Gardasil® which has been shown to reduce the incidence of cervical intraepithelial neoplasia grade 2 and grade 3 in vaccinated women [3], and is approved as a vaccine against cancer of the uterine cervix [4]. HPV VLPs adsorbed to particulate amorphous aluminum hydroxyphosphate sulfate (AAHS) adjuvant are exceptionally effective in eliciting production of neutralizing antibodies against HPV-16 in clinical trials [5-7]. The recent revelation that Gardasil® does contain recombinant HPV L1 gene DNA fragments [8,9] which appear to be firmly bound to AAHS nanoparticles [9] may offer a plausible explanation for the high immunogenicity of Gardasil® because co-delivery of both a DNA component and a protein component of a vaccine with aluminum phosphate salts may have the advantage of stimulating a potent and multivalent immune response [10].

On the other hand, the estimated rate of anaphylaxis in the young women receiving Gardasil® vaccination is also high and has been reported to be 5 to 20 times those identified in comparable school-based vaccination programs, using the Brighton case definition of anaphylaxis for diagnostic certainty [11]. A number of cases of possibly immune-based inflammatory neurodegenerative disorders involving the central nervous system, known as acute disseminated encephalomyelitis, following Gardasil® injections have been reported in world literature [12-18]. Among 12,424 reported adverse events following Gardasil® vaccination from June 1, 2006 through December 31, 2008, there were 32 deaths with a mean age of 18 years old, who died 2 to 405 days after the last Gardasil® injection [19]. Medical records and autopsy reports on 20 of the 32 deaths were available for review and confirmed there were 4 unexplained deaths and 6 cardiac-related deaths [19]. No investigative work was

attempted to confirm or to exclude any link of a death to Gardasil® vaccination although there was disproportional reporting of syncope among the Gardasil® recipients [19].

The parents of a formerly healthy New Zealand young woman who suffered a sudden unexpected death in sleep 6 months after Gardasil® vaccination requested testing for the presence of HPV L1 gene DNA in the post-mortem samples of their deceased daughter collected at the time of autopsy. Some of the consultants to the parents suggested that if residual HPV L1 gene DNA which is known to be present in the Gardasil® vaccine [8,9] were present in the postmortem samples, there might be a potential link between the residual HPV DNA and the unexplained death of their daughter. This paper reports the experience in developing a method for the detection and validation of minute quantities of HPV-16 L1 gene DNA in the postmortem blood and spleen obtained at autopsy. The data reported in this paper were extracted from a full report which was submitted to the Wellington coronial court at a public inquest held on August 8-9, 2012.

The parents of the deceased have granted their permission to the author to publish the data contained in this report.

2. MATERIALS AND METHODS

2.1. Postmortem Samples

An 18-year old healthy woman living with her parents was found dead in bed. The only relevant medications that she received before death were three injections of Depo Provera over a period of four years and three doses of intramuscular injections of the HPV vaccine, Gardasil®, in the last year of her life with the last dose of Gardasil® vaccination given six months prior to her demise. There was no history of alcohol or drug abuse. According to the documents presented at the inquest, the patient experienced temperament changes shortly after the first dose of Gardasil® injection, started to have dizziness spells, pins-and-needles feelings in her hands, memory lapses and abdominal pains after the second injection, and developed intermittent weak arm, frequent tiredness requiring daytime naps, increased pins-and-needles feelings in hands causing things to drop from hands, appetite increase with no weight gain, night sweats, loss of ability to use common objects, intermittent chest pain and sudden unexpected "racing heart". A full autopsy analysis revealed no anatomical, histological, toxicological, genetic or microbiological findings that might be linked to a potential cause of death.

At the parents' request and by order of the coronial court, DNA samples of the postmortem blood and splenic tissue of the deceased were prepared by Dr. Donald Love at Auckland Hospital Molecular Genetics Laboratory to be tested for the presence of HPV L1 gene DNA. According to Dr. Love, the commercial Gentra® Puregene® Blood Kit (Qiagen) was used to extract the DNA from the nucleated cell fraction of the unfixed blood and splenic tissue which were obtained at the time of autopsy and had been stored at –80˚C. The purified DNA was finally dissolved in TE buffer at the concentration of 0.5 µg of DNA per µL, speedvac dried in plastic tubes, and sent to the author's laboratory for analyses. Based on the second edition of Gentra® Puregene® Handbook (September 2007), the DNA yield with this method of preparation is about 6 pg DNA per human diploid cell. Therefore, 0.5 µg of DNA was equivalent to the amount of DNA extracted from ~80,000 nucleated cells (500 ng/6pg). A few split samples of the dried DNA are stored at Auckland Hospital for possible future investigation by independent laboratories at the order of the coronial court.

2.2. Low Temperature PCR

The traditional heat-resistant *Taq* DNA polymerase could not generate a useful nested PCR amplicon from a minute quantity of target HPV DNA in the postmortem materials to be used as a template for direct DNA sequencing. As a result, a LoTemp® PCR with a highly processive HiFi® DNA polymerase system programmed at thermocycling steps not to exceed 85˚C was selected for this study. The general method used to detect HPV L1 gene DNA by heminested (nested) LoTemp® PCR amplification with the GP/MY degenerate consensus primers and validation with direct automated DNA sequencing for genotyping has been described in detail elsewhere for clinical samples [20-25] and for detecting residual HPV DNA fragments in the Gardasil® vaccine [9]. Each primary PCR consisted of 1 µL of reconstituted DNA solution in molecular grade water containing about 0.5 µg human DNA, 2 µL of water, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, and 20 µL of ready-to-use LoTemp® PCR master mix with HiFi® DNA polymerase (www hifidna.com) in a total volume of 25 µL. The thermocycling steps for the LoTemp® PCR system were programmed for an initial heating at 85˚C for 10 min, followed by 30 cycles, each set at 85˚C for 30 sec, 40˚C (low stringency PCR) or 50˚C (high stringency PCR) for 30 sec, and 65˚C for 1 min. The final extension was 65˚C for 10 min (30-cycle Lo-temp program). One µL of each reconstituted sample was placed in a separate PCR tube with a β-globin primer pair for human genomic DNA amplification to assure specimen adequacy.

2.3. HPV-16 L1 Gene DNA Detected by Same-Nested PCR

Transferring of PCR products was accomplished by a

micro-glass rod to eliminate the need for micropipetteting to avoid aerosol contamination [25]. The nested PCR mixture consisted of 3 μL of water, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, and 20 μL of ready-to-use LoTemp® PCR mix with HiFi® DNA polymerase in a total volume of 25 μL. The thermo-cycling steps for the nested PCR were identical to those for primary PCR.

A "same-nested" PCR was introduced for re-amplification of a target region of an HPV L1 gene in the postmortem samples in this case. To perform a same-nested PCR, the primary PCR and the subsequent same-nested PCR(s) were conducted with an identical pair of PCR primers, or the subsequent same-nested PCR was conducted with a pair of the same primers having a few new bases added to the 3'end for one or for both of the primers which had been used in the prior PCR. As a result, all same-nested PCR products were terminated by the first pair of PCR primers used to initiate the primary PCR. The same-nested PCR protocol was found to be necessary to amplify the HPV-16 L1 gene DNA fragments in the postmortem materials in this case and the HPV-16 L1 gene DNA fragments in the Gardasil® vaccine [26].

After completion of the primary and the nested PCR, a 5 μL aliquot of the PCR products was pipetted out from each tube and mixed with 2 μL loading fluid for electrophoresis in a 2% agarose gel containing ethidium bromide. The gel was examined under UV light for various PCR product bands in the agarose gel.

2.4. Direct DNA Sequencing of Nested PCR Amplicon

For DNA sequencing, a trace of the positive nested PCR product was transferred directly with a micro-glass rod from the positive nested PCR tube into a 20 μL volume of a cycle sequencing reaction mixture consisting of 14.5 μL water, 3.5 μL of 5× buffer, 1 μL of BigDye Terminator 1.1 (Applied Biosystems) and 1 μL of 10 μM sequencing primer. After thermal cycling according to the manufacturer's recommendation, the reaction mixture was loaded in an automated ABI 3130 four-capillary Genetic Analyzer for sequence analysis. Alignment analysis of a 45 - 60 base sequence in the hypervariable region of the L1 gene excised from the computer-generated base-calling electropherogram was performed against various standard HPV genotype sequences stored in the GenBank, using the on-line BLAST (Basic Local Alignment Search Tool) system to validate the specific HPV genotyping.

2.5. Oligonucleotides Used as Primers

The PCR and DNA sequencing primers used and referred to in this report with their DNA sequences are listed as follows.

Degenerate HPV L1 gene primers:
MY09 = 5'-CGTCCMARRGGAWACTGATC-3'
MY11 = 5'-GCMCAGGGWCATAAYAATGG-3'
Key to degenerate nucleotides: M = (A + C), R = (A + G), W = (A + T), Y = (C + T)

Non-degenerate HPV L1 gene primers:
GP5 = 5'-TTTGTTACTGTGGTAGATAC-3'
GP6 = 5'-GAAAAATAAACTGTAAATCA-3'
GP6+ = 5'-GAAAAATAAACTGTAAATCATATTC-3'
MY11 (1) = 5'-GCACAGGGACATAACAATGG-3'
MY11 (2) = 5'-GCACAGGGACATAATAATGG-3'
MY11 (3) = 5'-GCACAGGGTCATAACAATGG-3'
MY11 (4) = 5'-GCACAGGGTCATAATAATGG-3'
MY11 (5) = 5'-GCCCAGGGACATAACAATGG-3'
MY11 (6) = 5'-GCCCAGGGACATAATAATGG-3'
MY11 (7) = 5'-GCCCAGGGTCATAACAATGG-3'
MY11 (8) = 5'-GCCCAGGGTCATAATAATGG-3'
HPV16MY11+ = 5'-GCACAGGGCCACAATAATGGCAT-3'

The choice of an appropriate combination of PCR primers at different stages was crucial to generate a relatively pure template useful for direct DNA sequencing. Lengthening a PCR primer increased the specificity of target DNA amplification at the expense of sensitivity in this case. The purified full-length HPV-16 plasmid DNA purchased from American Type Culture Collection, diluted to a concentration of 1 copy of HPV-16 L1 gene DNA per μL in TE buffer, was used as the positive control. At the latter theoretical concentration, about 50% of the primary PCRs and 100% of the same-nested PCRs would generate a PCR amplicon of ~190 bp in size at agarose gel electrophoresis when 1 μL of the HPV-16 positive control was used as the template and the degenerate consensus 20-base GP6/MY11 primer pair as the same-nested PCR primers.

3. RESULTS

3.1. Genomic DNA Interfered with Re-Amplification of Target HPV DNA

To detect minute quantities of HPV L1 gene DNA fragments in the whole blood DNA, 1 μL of undiluted reconstituted sample containing 0.5 μg of human genomic DNA was used to start each primary PCR. The general protocol of MY09/MY11 degenerate primer amplification followed by degenerate consensus GP6/MY11 heminested PCR amplification, which has proved highly successful in detecting HPV DNA and in preparing templates for direct DNA sequencing for testing HPV DNA in clinical specimens [25] and in Gardasil® samples [9, 26], generated numerous overlapping PCR product bands at agarose gel electrophoresis when the same protocol was used for testing the postmortem materials. None of

such PCR products could be used for direct DNA sequencing. When the 8 individual non-degenerate MY11 primers, *i.e*. MY11 (1), MY11 (2), MY11 (3), MY11 (4), MY11 (5), MY11 (6), MY11 (7), or MY11 (8), were paired with a GP6 primer to perform 8 individual parallel same-nested PCRs, numerous PCR products of various bp sizes were generated by the same-nested PCR as shown in a gel electrophoresis (**Figure 1**). Only one of these bands, ~190 bp in size, suggestive of a possible HPV DNA amplicon, although light in intensity, was obtained by PCR with the MY11 (1)/GP6 primer pair (**Figure 1**, lane 1). DNA sequencing of this same-nested PCR product, using GP6 nucleotide as the sequencing primer, showed an electropherogram of mixed DNA fragments, possibly including an HPV-16 L1 gene DNA sequence by visual analysis (**Figure 2**). Heminested PCR using a pair of MY09/GP5 primers did not generate an amplicon compatible with an HPV DNA PCR product at gel electrophoresis.

3.2. Second Same-Nested PCR with Elongated Primer for HPV-16 L1 DNA Amplification

To generate an amplicon suitable for DNA sequencing validation, a second same-nested PCR was performed, using a pair of HPV16MY11+/GP6 primers for re-amplification (**Figure 3**). A two-directional sequencing carried out on this new same-nested PCR amplicon, using the GP6 and the HPV16MY11+ oligonucleotide as the sequencing primer, respectively, showed a typical segment of HPV-16 L1 gene DNA (**Figures 4** and **5**).

3.3. Co-Amplification of Human Genomic DNA by HPV DNA Primers

In order to characterize the non-target PCR products resulting from a GP6/MY11 primer nested PCR amplification, the amplicon illustrated in lane 2 of **Figure 1** was sequenced from its both ends, using the MY11 (2) and GP6 oligonucleotide as the sequencing primer, respectively. The base-calling electropherograms of DNA sequencing confirmed that the amplicon observed in lane 2, **Figure 1** represented a 318-bp human genomic DNA sequence terminated by a GP6 primer and an MY11 primer (**Figures 6** and **7**). The 20-base GP6 and MY11 HPV DNA primers were apparently able to anneal to multiple sites of the human genome with various degrees of complementary base matches and were capable of initiating non-target DNA PCR amplifications in a same-nested PCR setting.

3.4. Selection of Non-Degenerate MY11/GP6 Primers to Prepare Template for DNA Sequencing

Based on the above experiments, a same-nested PCR

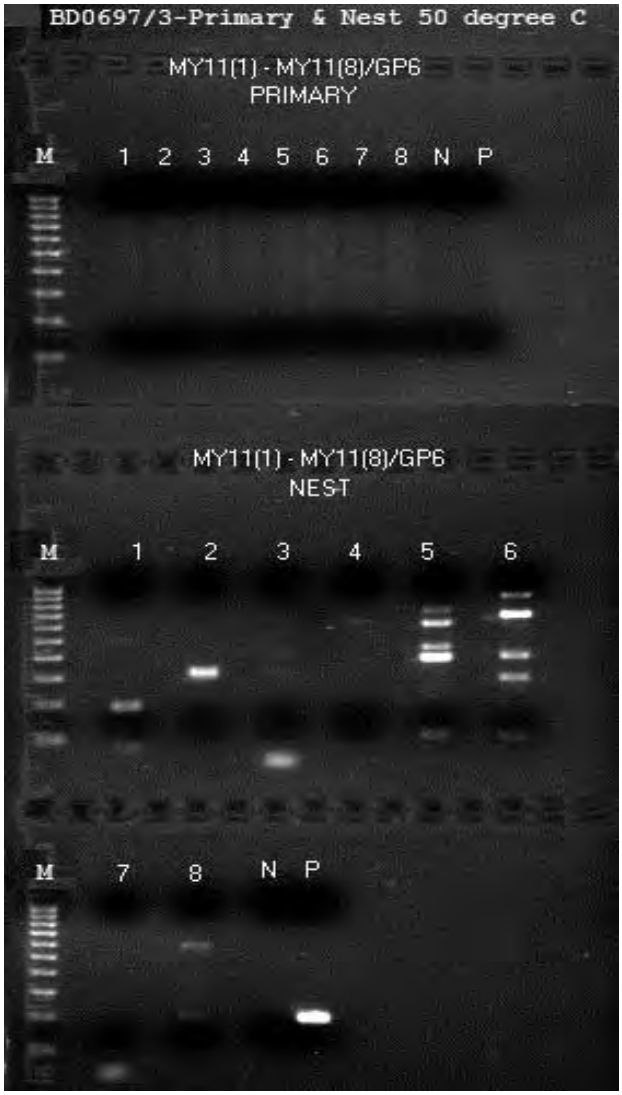

Figure 1. Same-nested PCR amplification with individual non-degenerate MY11 primers pairing with the GP6 primer for detection of HPV L1 gene DNA in a postmortem blood sample. This is a gel electrophoresis showing various same-nested PCR products with different primer pairs. Both the primary PCR and the same-nested PCR were performed in a 25 µL volume containing 20 µL of LoTemp® PCR master mix with HiFi® DNA polymerase, 1 µL of 10 µM GP6 primer and 1 µL of 10 µM of 1 of the 8 non-degenerate MY11 primers labeled MY11 (1) to MY11 (8) with their individual sequences described in the Materials and Methods section. For thermocycling, after an initial heating for 10 min at 85˚C, a 30-cycle amplification with 85˚C for 30 sec, 50˚C for 30 sec, and 65˚C for 1 min was programmed, with a final extension at 65˚C for 10 min for both primary and nested PCRs. The results show that only the MY11 (1)/GP6 primer pair generated a possible HPV DNA amplicon of ~190 bp in lane 1. Numerous nonspecific PCR products were generated when GP6 paired with other non-degenerate MY11 primers. Note: BD0697/3 = Batch No. assigned by Auckland Hospital. Molecular ruler 100 - 1000 bp on the left; N = negative water control; P = HPV-16 plasmid DNA amplified by a pair of degenerate consensus GP6/MY11 primer pair.

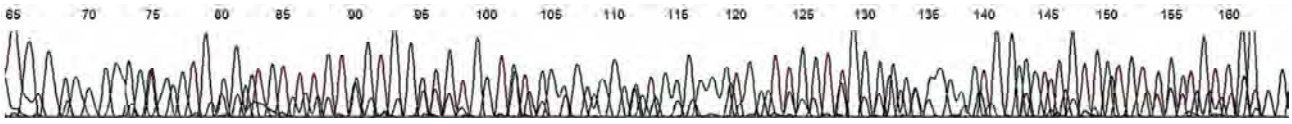

Figure 2. DNA sequencing electropherogram on a possible HPV DNA amplicon. Using GP6 as the sequencing primer, the sequencing electropherogram of the ~190 bp MY11 (1)/GP6 same-nested PCR amplicon shown in lane 1, **Figure 1**, suggested an HPV-16 L1 gene DNA fragment. However, the coexistent interfering nonspecific PCR products prevented a base-calling for validation.

Figure 3. Second nested PCR to generate an amplicon for direct DNA sequencing. The 1st same-nested PCR product shown in lane 1, **Figure 1**, was further amplified by a pair of HPV16MY11+ and GP6 primers in a 2nd nested PCR. This gel electrophoresis shows a clean band of HPV amplicon in lane 1. Note: Lane 1 = 2nd nested PCR amplicon using the 1st nested PCR product described in lane 1, **Figure 1** as the template; N = negative water control; P = PCR product from the P control in 1st nested PCR was used as the template.

with a pair of 20-base non-degenerate MY11 (1)/GP6 primers was chosen for the initial detection of HPV-16 L1 gene DNA fragments in all batches of DNA extraction on this case. It turned out that only about 1/3 of the same-nested PCRs with this protocol showed a positive amplicon of HPV-16 L1 gene DNA, eventually validated by DNA sequencing. The non-target human genomic DNA fragments which were co-amplified in the same-nested PCR varied considerably. For example, in a splenic DNA extract, when four 1 µL aliquots of the reconstituted DNA sample were used for a parallel same-nested PCR experiment, all using the non-degenerate MY11 (1)/GP6 primer pair for amplification, and two of the PCRs were cycled under a low stringency condition with a 40˚C annealing temperature while two under a high stringency condition with a 50˚C annealing temperature, only one of the PCRs generated a ~190 bp HPV DNA amplicon. Even in the latter nested PCR, there was a heavy ~500 bp product which was the result of co-amplification of non-target DNA in a heminested PCR setting (**Figure 8**, lane 4). A 3rd nested PCR using a non-degenerate elongated HPV16MY11+/GP6+ primer pair was needed to selectively amplify the target HPV DNA (**Figure 9**) in preparation of an amplicon suitable to be used for DNA sequencing (**Figure 10**) to confirm that HPV-16 L1 gene DNA was also present in the splenic tissue obtained at autopsy.

4. DISCUSSION

A same-nested PCR in which one identical pair of non-degenerate primers selected from the well-characterized degenerate consensus 20-base GP6/MY11 primer group, referred to as the MY11 (1)/GP6 primers in this report, was needed to detect the HPV-16 L1 gene DNA fragments present in the postmortem blood and the splenic tissue obtained at autopsy of a teenage girl who suffered a sudden unexpected death in sleep 6 months after Gardasil® vaccination. Since the human genomic samples contain numerous DNA fragments which are substantially complementary to the base sequences of the HPV PCR primers, co-amplification of non-target DNAs of the human genome invariably occurs in the same-nested PCR settings when PCR amplicons are re-amplified with the same primer(s). These human genomic DNA segments may act as primer-binding PCR inhibitors even when the partially matched primer-binding does not generate PCR amplicon bands visible at agarose gel electrophoresis. The same-nested PCR procedure has the effects of reducing the concentration of inhibitors carried over from the original sample by simple dilution so that the chance of obtaining a target DNA amplicon is significantly increased [25]. However, additional same-nested PCR with an elongated 23-base HPV16MY11+ primer and an elongated 25-base HPV16GP6+ primer may be needed to selectively generate a specific 184-bp HPV-16 PCR amplicon to be used as the template for direct DNA sequencing.

The elongated HPV16MY11+/HPV16GP6+ primers cannot be used to generate a PCR amplicon directly from the HPV-16 DNA template in the postmortem materials in this case. In comparison, the 184-bp L1 gene DNA

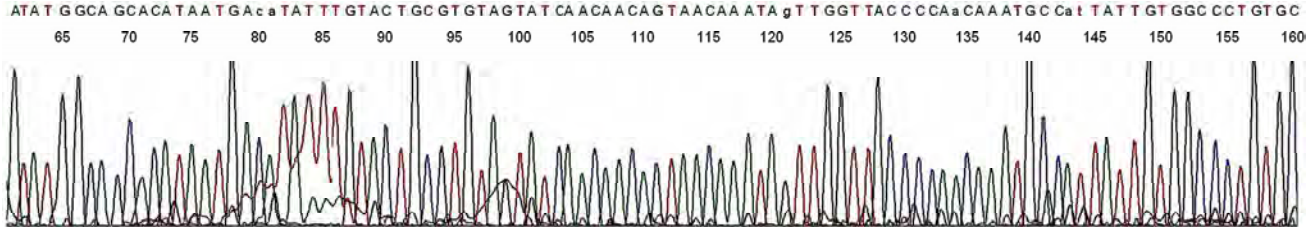

Figure 4. Base-calling electropherogram of DNA sequencing of an HPV PCR amplicon using GP6 as the sequencing primer. The 2nd nested PCR products described in lane 1, **Figure 3**, was used as the template for sequencing. The last 23 bases are the HPV16MY11+ primer-binding site.

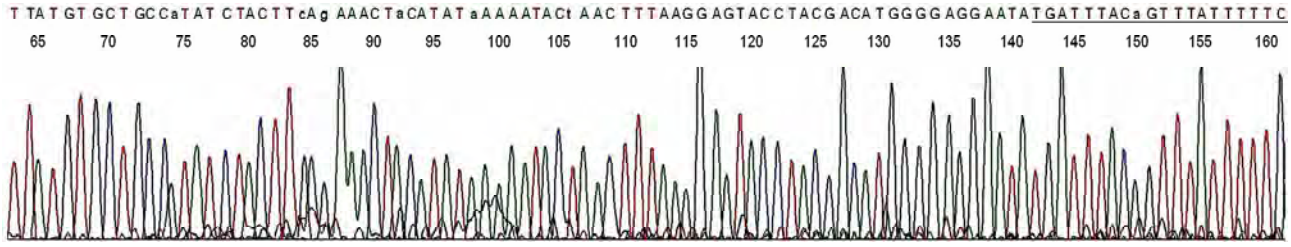

Figure 5. Base-calling electropherogram of DNA sequencing of an HPV PCR amplicon using HPV16MY11+ as the sequencing primer. The 2nd nested PCR products described in lane 1, **Figure 3**, was used as the template for sequencing. The last 20 bases are the GP6 primer-binding site (underlined). A composite two-directional 184-base sequence derived from the base-calling electropherograms depicted in **Figures 4** and **5** is 100% matched with that of the standard HPV-16 L1 gene DNA as follows: GCACAGGGCCACAATAATGGCATTTGTTGGGGTAACCAACTATTTGTTACTGTTGTTGATACTACACGCAGTACAAATAT GTCATTATGTGCTGCCATATCTACTTCAGAAACTACATATAAAAATACTAACTTTAAGGAGTACCTACGACATGGGGAGGA ATATGATTTACAGTTTATTTTTC-3' (HPV-16 genome Locus ID AF125673, location 6582-6765, direction 5'-3', retrieved from the National Center for Biotechnology Information database).

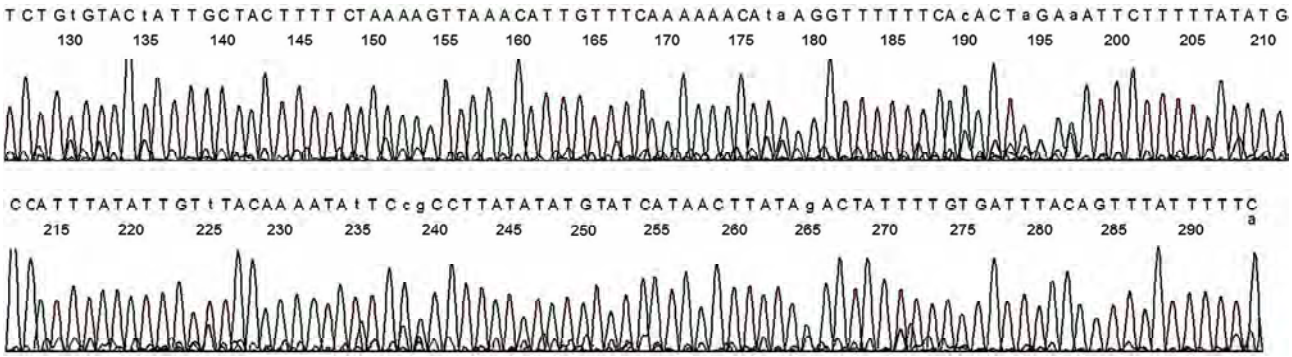

Figure 6. Base-calling electropherogram of DNA sequencing of a non-target PCR amplicon, using MY11 (2) as the sequencing primer. The nested PCR amplicon shown in lane 2, **Figure 1**, was used as the template for sequencing. The 20 bases of GP6 primer-binding site are in the end as for the HPV-16 L1 gene DNA sequence shown in **Figure 5**.

template in the HPV-16 plasmid DNA control and in the HPV-16 DNA isolated from clinical cervicovaginal cytology samples is always successfully amplified by the 20-base degenerate consensus GP6/MY11 primer pair and by the elongated HPV16MY11+ /HPV16GP6+ primer pair under identical same-nested PCR conditions. Unlike the L1 gene in the HPV-16 plasmid DNA and in the HPV-16 isolates from clinical cervicovaginal samples, the HPV-16 L1 gene DNA fragments found in the postmortem blood and splenic samples cannot be amplified under low stringency PCR condition and lacks a useful MY09 primer-binding site for PCR amplification. These variances in PCR amplification characteristics indicate that there are topological conformational changes in the HPV-16 L1 gene DNA fragments in the postmortem samples. Similar topological non-B conformational changes in HPV L1 gene DNA fragments bound to the AAHS particles in the Gardasil® vaccine have been demonstrated by a low temperature (LoTemp®) PCR catalyzed by a highly processive DNA polymerase with proof-reading function [26]. Aluminum, unlike other metals, is known to stabilize and destabilize portions of a dsDNA molecule at different pHs, cause intrastrand cross-links, and create a "non-cooperative melting profile" for the bound

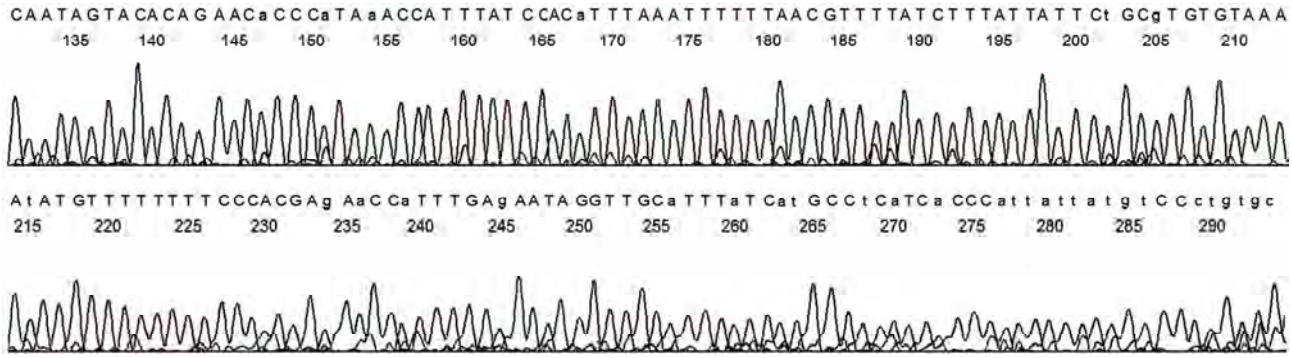

Figure 7. Base-calling electropherogram of DNA sequencing of a non-target PCR amplicon, using GP6 as the sequencing primer. The nested PCR amplicon shown in lane 2, **Figure 1**, was used as the template for sequencing. The 20 bases of MY11 (2) primer-binding site are in the end of the electropherogram as for the HPV-16 L1 gene DNA shown in **Figure 4**. A composite two-directional inter-primer sequence derived from the base-calling electropherograms depicted in **Figures 6** and **7** shows a human genomic DNA sequence flanked by a GP6 primer-binding site (underlined small letters) and by an MY11 primer-binding site (underlined capitalized letters) as follows:

gcactgggacataataatggGTGATGAGGCATGATAAATGCAACCTATTCTCAAATGGTTCTGGTGGGAAAAAAAACATATTTTACA
CACGCAGAATAATAAAGATAAAACGTTAAAAAATTTAAATGTGGATAAATGGTTTATGGGTGTTCTGTGTACTATTGCTAC
TTTTCTAAAAGTTAAACATTGTTTCAAAAAACATAAGGTTTTTTCACACTAGAAATTCTTTTTATATGCCATTTATATTGTT
TACAAAATATTCCGCCTTATATATGTATCATAACTTATAGACTATTTTG**TGATTTACAGTTTATTTTTC.**

DNA molecule [27].

Gardasil® is a quadrivalent vaccine which is known to contain residual recombinant HPV L1 gene DNA fragments [8]. As vaccine excipient, HPV L1 gene DNA fragments of all four genotypes, namely HPV-16, -18, -11 and -6, are expected to be present in any vaccine lot. However, previous studies have shown that only the L1 gene DNA of HPV-11 or HPV-18, or a combination of both was successfully amplified by a pair of degenerate consensus GP6/MY11 PCR primers [9], and that specially modified non-degenerate GP6/MY11 primers were needed to amplify the HPV-16 L1 gene DNA fragments bound to the insoluble fraction of the vaccine [26], indicating conformational changes taking place when naked HPV DNA is bound to the AAHS adjuvant during vaccine formulation. The topological conformational changes in the bound DNA may be genotype-related. The current study shows that only HPV-16 L1 gene DNA was detected 6 months after last vaccination, further suggesting that the non-B-conformation has protected the HPV-16 L1 gene DNA fragments from being degraded by various nucleases in the human body. Unprotected foreign DNA fragments in B conformation introduced into peripheral blood of a mammalian host are known to be degraded and eliminated within 48 hours [28].

HPV-16 is a virus which only infects human mucosal epithelial cells. HPV-16 DNA may be detected in the plasma of patients with invasive squamous cervical cancer harboring the same genotype of virus, but not in the control subjects without cervical cancer [29]. HPV-16 DNA has been reported to be present in peripheral blood mononuclear cells from human immunodeficiency virus (HIV)-infected pediatric patients and even from healthy blood donors [30]. However, unlike the HPV-16 L1 gene DNA fragments found in the Gardasil® vaccine and in the postmortem materials in this autopsy case, the HPV-16 L1 gene DNA in those reported clinical samples is always in B conformation which is readily amplified by a pair of degenerate or consensus PCR primers from both ends defined by the MY09 and MY11 binding sites [30].

The HPV-16 L1 gene DNA fragments detected in the postmortem blood and splenic tissue in this case are presumably in minute quantities and in the nucleated cells, probably macrophages. Naked viral and bacterial DNA fragments firmly bound to insoluble aluminum salts can be carried into tissue macrophages through phagocytosis to initiate a series of DNA-related immune reactions [31-34]. Intramuscular injection of free HPV-16 L1 plasmid DNA in BALB/C mice without adjuvant has been known to induce a strong CD8 T cell response [35], indicating that under certain conditions non-replicating HPV L1 gene DNA can activate the immune system. However, to be detectable 6 months after intramuscular injection, the naked foreign DNA in the host must be in a stabilized physical condition, either by remaining bound to the AAHS nanoparticles or by integration into the human genome through hitherto poorly understood mechanisms [36-40].

The presence of HPV-16 L1 gene DNA fragments of a vaccine origin indicates possible co-existence of other companion microbial DNA, such as DNA fragments of the plasmid pGAL110 and yeast cells which are used in the vaccine production by the manufacturer [2]. A potential consequence of these viral and microbial DNA fragments with their unmethylated CpG motifs in macro-

Figure 8. Detection of HPV L1 gene DNA in a postmortem splenic sample by a second same-nested PCR amplification with a non-degenerate HPV16MY11+/GP6 primer pair. Description: Four parallel same-nested PCRs with the MY11 (1)/GP6 primer pair, each started with 0.5 μg of human genomic DNA extracted from spleen, were performed, two under low stringency condition with 40˚C annealing temperature and two under high stringency condition with 50˚C annealing temperature. No HPV amplicon was obtained in the 1st same-nested PCRs. Then the second same-nested PCRs each with an HPV16MY-11+/GP6 primer pair were performed. As depicted in this gel electrophoresis, an intense HPV DNA amplicon band of ~190 bp in size was generated and shown in lane 4 in one of the two second nested PCRs under high stringency condition only. However, due to co-amplification of human genomic DNA which generated a large amount of high molecular weight PCR products, it was impossible to use this material as the template for DNA sequencing.

Figure 9. Third same-nested PCR amplification with two elongated primers to prepare HPV-16 DNA template from splenic DNA for sequencing. The second same-nested PCR products of the splenic DNA depicted in lane 4, **Figure 8**, were selectively amplified by a pair of HPV16MY-11+ and GP6+ primers to obtain a target HPV-16 amplicon for DNA sequencing. Note: M = molecular ruler; 4 = re-amplification of the lane 4 PCR products depicted in **Figure 8** with two elongated primers; N = negative water control; P = HPV-16 plasmid DNA control.

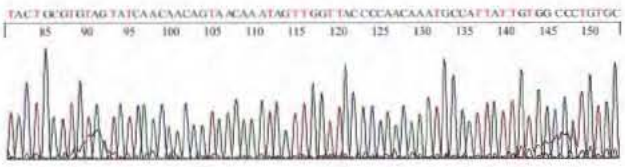

Figure 10. Base-calling electropherogram of DNA sequencing on HPV PCR amplicon from postmortem splenic DNA. This is a typical HPV-16 L1 gene DNA sequence using GP6+ as the sequencing primer and the #4 PCR amplicon described in **Figure 9** as the template.

phages [41-46] is to cause release of various cytokines, including tumor necrosis factor (TNF), a recognized myocardial depressant [47-51]. TNF-induced hypotensive shock is a documented observation among animals [52,53] and humans [54,55]. To answer the question whether the quantity of these persistent viral or microbial DNA fragments can stimulate the macrophages to release enough TNF to generate a significant pathophysiological impact following Gardasil® vaccination needs expanded research.

5. CONCLUSION

Detection of HPV-16 L1 gene DNA fragments in non-B-conformation in postmortem blood and spleen from a person who died suddenly and unexpectedly 6 months after quadrivalent HPV vaccination has not been previously reported and warrants further investigation.

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Landon James Watt

From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Tuesday, 18 February 2014 3:29 p.m.
To: Helen Petousis-Harris
Subject: RE: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

Dear Helen,

It was so nice talking to you and thank you so much for agreeing on joining our meeting.
It is indeed very helpful.

Just quickly, **I understand you are unable to travel to Japan in such a short notice next week.**
Is this correct!? We are happy to invite you (we will cover the travel cost) in case you happen
to be able to travel!!

Grateful for your response at your earliest opportunity.

Warm regards,

Koji Nabae
Deputy Director
Division of Tuberculosis and Infectious Disease Control
Ministry of Health, Labour & Welfare
Government of Japan-
Tel: [redacted]
Fax: +81-3-3581-6251
email: nabae-koji@mhlw.go.jp

From: Helen Petousis-Harris [mailto:h.petousis-harris@auckland.ac.nz]
Sent: Tuesday, February 18, 2014 5:19 AM
To: 'Robert Pless'
Cc: Robert Pless (Robert.Pless@phac-aspc.gc.ca); 難波江 功二(nabae-koji); ZUBER, Patrick Louis F.; Wharton, Melinda (CDC/OID/NCIRD)
Subject: RE: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

Dear Rob

Oh dear! I am so saddened to hear how extensive the impact of Lee, Shaw and Tomljenovic's activities has become.

I will certainly do anything I can to assist. To the best of my knowledge the rebuttal on our website is the only attempt to address this particular issue which Shaw and Lee presented at a coronal enquiry here. Placing the rebuttal in the public domain was the only means of providing the information to the crown representatives involved in that process at the 11th hour. Prof David Gorsky has written prolifically on some of the experiments in his science blog over the past few years so I assume he has also given the material some thought.

I do not know if I am expert on this but certainly have some experience in considering aluminium in vaccines and its role in inflammatory responses and local AEFI as part of my PhD some years ago. I assume you are referring to the VLP tightly bound to the adjuvant and the Shaw and Tomljenovic 'hypothesis' that it somehow finds its way to the brain carried by macrophage?

A phone call would probably be useful. It is a little after 9am in NZ.

Kind regards.

Helen

Helen Petousis-Harris. PhD, MRSNZ
Senior Lecturer, General Practice and Primary Health Care
Director of Immunsiation Research and Vaccinology
Immunisation Advisory Centre
University of Auckland
DDI +64 9 923 2078 Fax 9 3737030 Mob [redacted]
Building 734, Level 3, Tamaki Campus, Morrin Rd, Glen Innes
Private Bag 92019, Victoria St West, Auckland 1142, New Zealand

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From: Robert Pless [mailto:rpless2@gmail.com]
Sent: Tuesday, 18 February 2014 6:20 a.m.
To: Helen Petousis-Harris
Cc: Robert Pless (Robert.Pless@phac-aspc.gc.ca); "難波江 功二(nabae-koji)"; ZUBER, Patrick Louis F.; Wharton, Melinda (CDC/OID/NCIRD)
Subject: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

Dear Dr. Petousis-Harris,

I am writing you with an urgent request outlined below, having read the Immunization Advisory Centre's Commentary on coronial inquiry expert witness testimony that was prepared in response to allegations by Chris Shaw and Sing Hang Lee. I am a current member of the WHO Global Advisory Committee on Vaccine Safety and am writing in part on behalf of Dr. Koji Nabae of the Japanese Ministry of Health, for assistance. The GACVS has been looking at this issue from the global perspective and have released several statements over the last two years to address concerns around aluminum and autoimmune disease.

As you may be aware, there have been ongoing concerns in Japan regarding the HPV vaccine, where cases of chronic pain and complex regional pain syndrome allegedly linked to the vaccine have led to a partial suspension of their national vaccination program and there has been a great deal of public interest. An expert advisory group has met several times but have not reached a conclusion about the restart of the program. A meeting has recently been organized in Tokyo for February 26th, where Dr. Lee will present his findings. It is likely that Dr. Shaw's co-investigator, Lucija Tomljenovic will be present as well. There will be a second presentation on Macrophagic Myofasciitis and the HPV vaccine, a stretch of the MMF story first related to the hepatitis B vaccine.

We are seeking your advice on someone who may be able to address the more detailed questions around HPV DNA - specifically the hypotheses you have address in your statement regarding the alleged role of aluminum binding to DNA fragments and subsequent effects. While the issue of whether the fragments constitute "contamination" has been dealt with, your statement was the only one to address the more obscure alleged consequences of the presence of those fragments. The GACVS has not yet had a chance to delve into the DNA question.

While we appreciate the short notice, the meeting and even the date were very recently confirmed. That said, the ideal would be someone who would be available to travel to the meeting in Japan and address the issue as it arises in

person. Please let me know if I can clarify anything by phone, and indeed if this is at all possible and who could be contacted and provided with further details.

Best regards,
Rob

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Robert Pless, MD, MSc
Medical Advisor
Health Security Infrastructure Branch
Public Health Agency of Canada
Ottawa, Ontario K1A 0K9
Tel./Mobile: [redacted]
Email: robert.pless@phac-aspc.gc.ca

Landon James Watt

From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Monday, 24 February 2014 5:26 p.m.
To: Helen Petousis-Harris
Cc: 難波江 功二(nabae-koji)
Subject: Trail Video Conference

Dear Helen,

If you happen to be available, please log in at the following website for a trial.

https://mhlw-web.webex.com/mw0307l/mywebex/default.do?service=1&siteurl=mhlw-web&nomenu=true&main_url=%2Fmc0806l%2Fmeetingcenter%2Fmeetinginfo%2Fmeetinginfo.do%3Fsiteurl%3Dmhlw-web%26confID%3D1619468164%26Action%3DMI%26MTID%3Dm5452882331666f9e0b71fc7f1c7d29e1%26FrameSet%3D2%26Host%3D17f3378036031c2c0e5e44%26UID%3D0&UUIDFromJAction=0

Meeting Number: 861 675 442
Password (if required): KEKKAKU20%

Koji

Landon James Watt

From: Robert Pless <rpless2@gmail.com>
Sent: Tuesday, 18 February 2014 6:20 a.m.
To: Helen Petousis-Harris
Cc: Robert Pless (Robert.Pless@phac-aspc.gc.ca); "難波江 功二(nabae-koji)"; ZUBER, Patrick Louis F.; Wharton, Melinda (CDC/OID/NCIRD)
Subject: URGENT: Regarding the posted commentary on the coronial inquiry expert witness testimony

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Best regards,
Rob

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Robert Pless, MD, MSc
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Public Health Agency of Canada
Ottawa, Ontario K1A 0K9
Tel./Mobile: [redacted]
Email: robert.pless@phac-aspc.gc.ca

Landon James Watt

From: 難波江 功二(nabae-koji) <nabae-koji@mhlw.go.jp>
Sent: Wednesday, 26 February 2014 4:48 a.m.
To: Helen Petousis-Harris
Cc: Koji Nabae (k-nabae-@nifty.com)
Subject: Video Conf

Dear Helen,

We will send you an URL and Meeting Number tomorrow morning
around at 13:15 NZ time. Unfortunately, it will be written in Japanese,
but I hope and I am sure you can find the URL and meeting number (when necessary).

Since I will be at a conference venue, please call my cell phone
[redacted]
in case of emergencies.

Talk to you soon!

Koji