

Behavioral abnormalities in female mice following administration of aluminum adjuvants and the human papillomavirus (HPV) vaccine Gardasil

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Abstract Vaccine adjuvants and vaccines may induce autoimmune and inflammatory manifestations in susceptible individuals. To date most human vaccine trials utilize aluminum (Al) adjuvants as placebos despite much evidence showing that Al in vaccine-relevant exposures can be toxic to humans and animals. We sought to evaluate the effects of Al adjuvant and the HPV vaccine Gardasil versus the true placebo on behavioral and inflammatory parameters in female mice. Six-week-old C57BL/6 female mice were injected with either, Gardasil, Gardasil + pertussis toxin (Pt), Al hydroxide, or, vehicle control in amounts equivalent to human exposure. At 7.5 months of age, Gardasil and Al-injected mice spent significantly more time floating in the forced swimming test (FST) in comparison with vehicle-injected mice (Al, $p = 0.009$; Gardasil, $p = 0.025$; Gardasil + Pt, $p = 0.005$). The increase in floating time was already highly significant at 4.5 months of age for the Gardasil and Gardasil + Pt group ($p \leq 0.0001$). No significant differences were observed in the number of stairs climbed in the staircase test which measures locomotor activity. These results indicate that differences observed in the FST were unlikely due to locomotor dysfunction, but rather due to depression. Moreover, anti-HPV antibodies from the sera of Gardasil and Gardasil + Pt-injected mice showed cross-reactivity with the mouse brain protein extract. Immunohistochemistry analysis revealed microglial activation in the CA1 area of the hippocampus of Gardasil-injected mice. It appears that Gardasil via its Al adjuvant and HPV antigens has the ability to trigger neuroinflammation and autoimmune reactions, further leading to behavioral changes.

Keywords Gardasil · Aluminum · ASIA syndrome · Autoantibodies · Autoimmunity · Neuroinflammation

Abbreviations

Al Aluminum
ASIA Autoimmune/autoinflammatory syndrome induced by adjuvants

β 2-GPI β 2-Glycoprotein I
FST Forced swimming test
HPV Human papilloma virus
Pt Pertussis toxin
U. S. FDA United States Food and Drug Administration

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Introduction

Like other drugs, vaccines can cause adverse events, but unlike conventional medicines, which are prescribed to people who are ill, vaccines are administered to healthy individuals. Hence, there is an added concern regarding risks associated with vaccinations. While most reported side effects from vaccines are mild and transient, serious adverse events do occur and can even be fatal [1, 2].

There are currently major stumbling blocks in our understanding of the exact mechanisms by which such events can be triggered. The main reason for this is the poor methodological quality of many clinical studies that evaluate vaccine safety and the lack of in-depth research into adverse phenomena [3]. In addition, adverse events may not fit into a well-defined category of an autoimmune disease but rather, present themselves as a constellation of non-specific symptoms (i.e., arthralgia, myalgia, fatigue, nausea, weakness, paresthesia, depression, mild cognitive disturbances) [2]. Another complicating factor in researching vaccine-related adverse events is that the latency period between vaccination and the development of an overt and diagnosable autoimmune and/or neurological disease can range from days to many months [4–6], likely depending on individuals' genetic predispositions and other susceptibility factors (i.e., previous history of autoimmune disease or previous history of adverse reactions to vaccines).

From the above, it is clear that establishing a definite causal link between vaccinations and disease manifestations in humans remains a complex task. Thus, the potential risks from vaccines remain currently ill-understood and controversial. A further obfuscation to our understanding of potential risks from vaccinations stems from the persistent use of aluminum (Al) adjuvants-containing placebos in vaccine trials [7]. Indeed, contrary to popular *assumptions* of inherent safety of Al in vaccines, there is now compelling data from both human and animal studies which implicates this most widely used adjuvant in the pathogenesis of disabling neuroimmuno-inflammatory conditions [8–11].

Due to their capability of enhancing the immune response to foreign antigens, substances with adjuvant properties have been used for decades to enhance the immunogenicity of human and animal vaccines [12]. Because of their immune-potentiating capacity, adjuvants enable the usage of smaller amount of antigens in vaccine preparations and are thus attractive from a commercial standpoint. Nonetheless, enhanced immunogenicity also implies enhanced reactivity. Indeed, although Al acts as an effective vehicle for the presentation of antigens, this process is not always benign since the adjuvant itself is

intrinsically capable of stimulating pathological immune and neuro-inflammatory responses [9–11, 13–16]. In spite of these data, it is currently maintained by both the pharmaceutical industry and drug-regulating agencies that the concentrations at which Al is used in vaccines does not represent a health hazard [17].

Apart from potential hazards associated with adjuvant use, other ingredients in vaccines also have the capacity of provoking undesirable adverse events. Indeed, since the mechanisms by which the host's immune system responds to vaccination resemble the ones involved in the response to infectious agents, a recombinant or a live attenuated infectious antigen used for vaccination, may inflict a range of immune and autoimmune responses similar to its parallel infectious agent [18, 19].

The HPV vaccine Gardasil is one of many vaccines currently on the market that is adjuvanted with Al. Since the licensure by the US Food and Drug Administration (FDA) and subsequent introduction on the market in 2009, the HPV vaccine has been linked to a variety of serious neurological and autoimmune manifestations. Notably, out of 152 total cases identified via PubMed 129 (85 %) are related to neuro-ophthalmologic disorders (Table 1). It should be noted that the pattern of adverse manifestations emerging from HPV vaccine case reports, matches that reported through various vaccine safety surveillance systems worldwide, with nervous system and autoimmune disorders being the most frequently reported [20].

Like most other vaccine safety trials, the trials for the HPV Gardasil vaccine utilized an Al-containing placebo [21, 22] and hence the safety profile of the vaccine remains obscured by the use of a potentially toxic placebo [7]. Thus, in order to investigate better, the safety profile of Gardasil, as well as the Al adjuvant, in the current study, we evaluated and compared the effects of Al and whole HPV vaccine formulation versus that of a true placebo on behavioral, neurohistological and autoimmune parameters in young female C57BL/6 mice.

Materials and methods

Mice husbandry

Six-week-old C57BL/6 female mice were obtained from Harlan Laboratories (Jerusalem, Israel) and were housed in the animal facility at Sheba Medical Center. The mice were raised under standard conditions, 23 ± 1 °C, 12-light cycle (6:30 am–6:30 pm) with ad libitum access to food and water. The Sheba Medical Center Animal Welfare Committee approved all procedures.

Table 1 Summary of cases of autoimmune and inflammatory manifestations following HPV vaccination reported in the peer-reviewed medical literature

Number of case reports	Age	Symptoms/main clinical features	Final diagnosis	References
2	17	Visual impairments	ADEM	[52]
	20	Headache, nausea, vomiting, diplopia		[53]
5	16	Upper limb pseudoathetosis	CIS/MS/	[54]
	16	Acute hemiparesis	Clinically definite MS	
	21	Incomplete TM, left optic neuritis		
	25	Headache, incomplete TM		
	26	Incomplete TM, brainstem syndrome		
2	19	Leg numbness, mid-thoracic back pain	Demyelinating disease unspecified	[55]
	18	Blurriness, paresthesia, optic neuritis		
1	11	Mood swings, abnormal eye movements, dizziness, leg weakness, myoclonic jerks	Opsoclonus myoclonus	[56]
4	17	Back pain, progressing spastic paraparesis, right arm weakness, left eye visual loss	Neuromyelitis optica	[57]
	14	Back pain, right thigh dysesthesias, left optic neuritis		
	13	TM with flaccid paraplegia		
	18	Back pain and leg weakness, complete loss of monocular vision		
2	16	Visual loss, headaches, left hemiparesis	Optic neuritis	[58]
	17	Visual disturbances, demyelinating lesions		[59]
2	27	Paresthesia, demyelinating lesions	TM fitting the criteria for MS	[59]
	26	Progressive paresthesia, demyelinating lesions		
1	15	Facial paralysis	Bell's palsy	[59]
1	12	Nausea, vertigo, severe limb and truncal ataxia, and persistent nystagmus	Cerebellar ataxia	[60]
1	19	Chronic (3 months) disabling shoulder pain	Brachial neuritis	[61]
53	12–39	Orthostatic intolerance, severe non-migraine-like headache, excessive fatigue, cognitive dysfunction, gastrointestinal discomfort, widespread neuropathic pain	Dysautonomia, POTS, orthostatic intolerance and CRPS	[62]
40	11–17	Headaches, general fatigue, coldness of the legs, limb pain and weakness, orthostatic intolerance, tremors, persistent asthenia		[63]
6	20	Weight loss, dizziness, fatigue, exercise intolerance		[64]
	22	Diarrhea, weight loss, fatigue, dizziness, syncope		
	12	Syncope, pre-syncope, dizziness, small fiber neuropathy		
	15	Dizziness, headache, pre-syncope, syncope		
		Paresthesia, tachycardia, fatigue, headache,		
	14	diarrhea, weight loss		
	18	Paresthesia, leg pain, orthostatic intolerance, Fatigue, dizziness		
4	16	Paresthesia, numbness, limb paralysis, pain		[65]
	13	Allodynia, numbness, severe pain		
	15	Paresthesia, numbness, severe pain		
	12	Paresthesia, muscle weakness, pain		
1	14	Headaches, dizziness, recurrent syncope, orthostatic intolerance, fatigue, myalgias, tachycardia, dyspnea, visual disturbances, phonophobia, cognitive impairment, insomnia, gastrointestinal disturbances, weight loss		[66]
2	11	Widespread neuropathic pain, paresthesia, insomnia, profound fatigue	Fibromyalgia	[67]
	14	Widespread neuropathic pain and paresthesia		
1	32	Paresthesia, muscle twitching, myalgia, fatigue, hyperhidrosis, and tachycardia, exercise intolerance	Autoimmune myotonia	[68]

Table 1 continued

Number of case reports	Age	Symptoms/main clinical features	Final diagnosis	References
3	14	Skin rash, fever, nausea, stomach aches, headache, insomnia, night sweats, arthralgia, anxiety, depression, amenorrhea, elevated serum levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and low levels of estradiol	POF	[69]
	13	Depression, sleep disturbance, light-headedness, tremulousness, anxiety, cognitive dysfunction, amenorrhea, high serum levels of FSH and LH with undetectable estradiol		[70]
	21	A menorrhoea preceded by oligomenorrhoea, high serum levels of FSH and LH and low estradiol		
3	16	5 months amenorrhoea preceded by 12 months oligomenorrhoea, hot flashes, low serum levels of estradiol and Anti-Müllerian hormone		
	18	6 months amenorrhoea, low serum levels of estradiol and Anti-Müllerian hormone		
	15	3 months amenorrhoea preceded by 9 months oligomenorrhoea, hot flashes, low serum levels of estradiol and undetectable Anti-Müllerian hormone		
2	15	Vasculitic rash, soft tissue swellings of ankles and forearms, arthralgia, lethargy, epistaxis	Vasculitis	[71]
	15	Severe flare of cutaneous vasculitis		
1	16	Fatigue associated with prolonged menorrhagia, antiplatelet autoantibodies	Thrombocytopenic purpura	[72]
1	11	Jaundice, hepatosplenomegaly elevated serum aminotransferases	Autoimmune hepatitis	[73]
1	26	Severe constant epigastric pain, vomiting, fever	Pancreatitis	[74]
3	17	Arthralgias, pruritic rashes on lower extremities, bipedal edema, livedo reticularis, proteinuria, positive ANA and anti-dsDNA antibodies	SLE	[75]
	45	Intermittent fever, generalized weakness, oral ulcers, alopecia, malar rash, photosensitivity, arthritis, intestinal pseudo-obstruction, ascites, positive ANA, anti-dsDNA, anti-Ro/SSA and anti- La/SSB antibodies		
	58	Malar and scalp rashes, fever, easy fatigability, cervical lymph nodes, gross hematuria and pallor, severe anemia and thrombocytopenia, active nephritis, patient expired a day after hospital admission		
6	32	Fatigue, severe myalgia, polyarthralgia, anorexia, severe skin rash, malar rash, aphthous stomatitis, pharyngodynia, cervical lymphadenopathy, alopecia, severe weight loss, anemia, positive ANA and anti-dsDNA antibodies		[76]
	29	Weakness, diarrhea, malar rash, photosensitivity, arthritis, alopecia, severe weight loss, proteinuria, positive ANA and anti-dsDNA antibodies		
	16	High-grade fever, generalized asthenia, diffuse polyarthralgia, multiple erythematous annular cutaneous lesions on the face, trunk, and lower limbs, positive ANA and lupus anticoagulant		
	16	Fever, pharyngodynia, erythematous skin lesions of elbows and knees, generalized asthenia, anorexia, polyarthralgia, anti-cardiolipin and lupus anticoagulant		
	19	Mild arthralgia, dyspnea, cervical lymphadenopathy, skin rash, positive ANA and anti-dsDNA antibodies		
1	13	Erythematous facial rash, fever, periorbital edema, weight loss, malaise, fatigue, alopecia, cervical, axillary and inguinal lymphadenopathy, anemia, thrombocytopenia, positive ANA, anti-RNP, anti-Smith and anti-RO/SSA antibodies		[77]
	19	Myalgia, arthralgia, generalized weakness, oral ulcers, Raynaud's phenomenon, alopecia, headache, dyspnea, tachycardia, positive ANA, anti-Sm, anti-Ro, anti-RNP, anti-dsDNA, leukopenia, and complement consumption		
1	20	Myalgias, arthralgias, livedo reticularis, Raynaud's phenomenon, headache, tinnitus, positive ANA, lupus anticoagulant and anti-CCP	Rheumatoid arthritis	[77]

Table 1 continued

Number of case reports	Age	Symptoms/main clinical features	Final diagnosis	References
1	16	Knee joint swelling, low back, buttock and chest wall pain, elevated leukocyte count in the synovial fluid, elevated C-reactive protein	Juvenile spondyloarthritis	[77]

Out of 152 reported cases, 129 (85 %) relate to neuro-ophthalmic disorders

ANA antinuclear antibodies; *ADEM* acute disseminated encephalomyelitis; *CIS* clinically isolated syndrome; *CRPS* complex regional pain syndrome; *MS* multiple sclerosis; *POF* primary ovarian failure; *POTS* postural orthostatic tachycardia syndrome (disorder of the autonomic nervous system); *SLE* systemic lupus erythematosus; *TM* transverse myelitis

Injection procedures and experimental design

Six-week-old C57BL/6 female mice received three injections (spaced 1 day apart) of either (a) quadrivalent HPV vaccine Gardasil, (b) Gardasil + pertussis toxin (Pt), (c) Al hydroxide or (d) vehicle control (19.12 mg/mL NaCl, 1.56 mg/mL L-histidine). The number of injected animals was 19 per experimental group. Gardasil, Al and vehicle were injected intramuscularly (i.m.), while the Pt was given intraperitoneally (ip). The amount of injected Al and the HPV vaccine was the equivalent of human exposure. In particular, each mouse in the Gardasil and Gardasil + Pt group received 0.25 μ l of Gardasil (dissolved in 20 μ l of vehicle solution). 0.25 μ l of Gardasil is the equivalent of a human dose since the average weight of a six-week-old mouse is approximately 20 g. Gardasil is given as a 0.5-mL dose to teenage girls of cca 40 kg. Thus, a 20-g mouse receives cca 2000 \times less of the vaccine suspension than a human. Similarly, each mouse in the Al adjuvant group received 5.6 μ g/kg body weights Al hydroxide dissolved in 20 μ l vehicle solution. A single Gardasil dose contains 225 μ g of Al and is given to a cca 40-kg female. This equates to 5.6 μ g Al hydroxide/kg body weight. The mice in the Pt group received 250 ng of Pt with each injection of Gardasil. Pt was added to this group for the purpose of damaging the blood-brain barrier. Since the actual adjuvant form used in Gardasil, amorphous Al hydroxyphosphate sulfate (AAHS), is a proprietary brand of the vaccine manufacturer and is not commercially available, we used Alhydrogel as a substitute.

Five out of 19 animals from each of the four experimental groups were used for sera collection purposes. These animals were not subjected to behavioral testing as sera were collected via retro-orbital bleeding which is a stressful procedure that in addition often leads to vision deficits. The behavior of mice was evaluated at three and 6 months post-immunization for (1) locomotor function and depression by the forced swimming test (FST), (2) locomotor and explorative activity by the staircase test and (3) cognitive functions by the novel object recognition test. Following the first round of behavioral testing at

4.5 months of age, five mice from each of the four experimental groups were killed and brain tissues were collected and processed for histological examinations. Blood specimens were also collected at this time for serological analysis.

Behavioral tests

Forced swimming test

The FST is the most widely used model of depression in rodents. It is commonly used for evaluation of antidepressant drugs, and experiments aimed at inducing and examining depressive-like states in basic and pre-clinical research [23, 24]. Nonetheless, it should be noted that increased floating time in the FST apart from being indicative of depressive behavior can also indicate locomotor dysfunction. For the purpose of this test, mice were placed in individual glass beakers (height 39 cm, diameter 21.7 cm) with water 15 cm deep at 25 °C. On the first day, mice were placed in the cylinder for a pretest session of 10 min, and later were removed from the cylinder, and then returned to their home cages. Twenty-four hours later (day 2), the mice were subjected to a test session for 6 min. The behavioral measure scored was the duration (in seconds) of immobility or floating, defined as the absence of escape-oriented behaviors, such as swimming, jumping, rearing, sniffing or diving, recorded during the 6-min test.

Staircase test

Locomotor, explorative activity and anxiety were evaluated by the staircase test, as described previously by Katzav et al. [25]. In this test, stair-climbing and rearing frequency are recorded as measures of general locomotor function, exploratory activity and anxiety/attention. The staircase maze consisted of a polyvinyl chloride enclosure with five identical steps, 2.5 \times 10 \times 7.5 cm. The inner height of the walls was constant (12.5 cm) along the whole length of the staircase. The box was placed in a room with constant lighting and isolated from external noise. Each

mouse was tested individually. The animal was placed on the floor of the staircase with its back to the staircase. The number of stairs climbed and the number of rears were recorded during a 3-min period. Climbing was defined as each stair on which the mouse placed all four paws; rearing was defined as each instance the mouse rose on hind legs (to sniff the air), either on the stair or against the wall. The number of stairs descended was not taken into account. Before each test, the animal was removed and the box cleaned with a diluted alcohol solution to eliminate smells.

Novel object recognition test

This is a visual recognition memory test based on a method described by Tordera et al. [24]. The apparatus, an open-field box (50 × 50 × 20 cm), was constructed from plywood painted white. Three phases (habituation, training and retention) were conducted on three separate test days. Before the training session, the mice were individually habituated by allowing them to explore the box for 10 min (day 1). No data were collected at this phase. During training sessions (day 2), two identical objects were placed into the box in the northwest and southeast corners (approximately 5 cm from the walls), 20 cm away from each other (symmetrically) and then the individual animal was allowed to explore them for 5 min. Exploration of an object was defined as directing the nose to the object at a distance of ≤ 1 cm and/or touching it with the nose and rearing at the object; turning around or sitting near the object was not considered as exploratory behavior. The time spent in exploring each object was recorded as well as the number of interactions with both objects. The animals were returned to their home cages immediately after training. During the retention test (day 3), one of the familiar objects used during the training session was replaced by a novel object. Then, the animals were placed back into the box and allowed to explore the objects for 5 min. The same parameters were measured as during the training session, namely the time spent in exploring each of the two objects and the number of interactions with them. All objects were balanced in terms of physical complexity and were emotionally neutral. The box and the objects were thoroughly cleaned by 70 % alcohol before each session to avoid possible instinctive odorant cues. A preference index, a ratio of the amount of time spent exploring any one of the two items (old and new in the retention session) over the total time spent exploring both objects, was used to measure recognition memory.

Statistical analysis

Results are expressed as the mean \pm SEM. The differences in mean for average immobility time in the FST, the

staircase test parameters (number of rearing and stair-climbing events) and novel object recognition were evaluated by ANOVA and Tuckey for multiple comparisons in the post hoc analysis. Significant results were determined as $p < 0.05$.

Brain perfusion and fixation

The mice were anesthetized by an i.p. injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) and killed by transcardiac perfusion with phosphate-buffered saline (PBS) followed by perfusion with 4 % paraformaldehyde (PFA, Sigma-Aldrich Israel Ltd., Rehovot Israel) in phosphate buffer (PO₄, pH 7.4). After perfusion, the brain was quickly removed and fixed overnight in 4 % PFA (in PO₄, pH 7.4) at 4 °C. On the following day, the brain was cryoprotected by immersion in 30 % sucrose in 0.1 M PO₄ (pH 7.4) for 24–48 h at 4 °C before brain cutting. Frozen coronal Sects. (30–50 μ m) were cut on a sliding microtome (Leica Microsystems GmbH, Wetzlar, Germany), collected serially and kept in a cryoprotectant at -20 °C until staining.

Detection of autoantibodies in the sera

The levels of autoantibodies in the mice sera were tested by a homemade ELISA 1 month post-injection. Briefly, ELISA plates (M9410, Sigma-Aldrich) were coated separately with 20 μ g/well of different antigens: Gardasil which contains the HPV L1 major capsid protein of HPV types 6, 11, 16 and 18, mouse brain protein extract, mouse brain phospholipid extract, Al hydroxide, dsDNA and β 2glycoprotein-I (β 2GPI). The plates were incubated overnight at 4 °C, washed and blocked with 3 %BSA in PBS 1 h at 37 °C. Sera were added at dilution of 1:200 for 2 h at room temperature. The binding was probed with goat anti-mouse IgG conjugated to alkaline phosphatase at concentration of 1:5000 for 1 h at 37C. Following appropriate substrate, the data were read by ELISA reader at 405 nm.

Inhibition assay

Brain protein extracts were prepared by lysis of brains from five healthy C57BL/6 mice, using ice-cold lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10 % glycerol, 1 % Triton X-100, 1 mM EDTA, 1 mM PMSF, 1 mM sodium vanadate, 0.1 % protease inhibitor mixture (Sigma-Aldrich L-4391 St Louis, MO, USA) for 30 min on ice and centrifuged at 13,000 rpm for 20 min. The lysate was dialyzed against PBS. Protein concentration was determined by BCA Protein Assay Kit (Pierce, Thermo scientific, Rockford, IL, USA).

ELISA plates were coated with the HPV vaccine Gardasil which contains the HPV L1 major capsid protein of HPV types 6, 11, 16 and 18. Following blocking with 5 % skim milk powder, sera from the immunized mice, at different dilutions 1:200–1:10,000, were added to the plates in order to define 50 % binding of the sera to the HPV. Next, dilutions of sera which showed 50 % binding to HPV were incubated overnight at 4 °C with different concentrations of mouse brain protein extract (10–50 µg/ml) as the inhibitor. The following day, the mixtures were subjected to ELISA plates coated with HPV for 2 h at room temperature. The binding of the antibodies which did not create complex with the brain protein extract was probed with anti-mouse IgG conjugated to alkaline phosphatase, followed by the appropriate substrate. The percentage of inhibition was calculated as follows: % inhibition = $100 - [(OD \text{ of tested sample without inhibitor} - OD \text{ of tested sample with inhibitor}) / (OD \text{ of tested sample without inhibitor})] \times 100$.

Brain tissue immunostaining

Brain sections were stained free-floating, incubated with the first antibodies overnight at 4 °C. The slices were then washed in PBS + 0.1 % Triton X-100 and incubated at room temperature for 1 h with the corresponding fluorescent chromogens-conjugated secondary antibody. Sections were stained for specific antigens with antibodies against activated microglia (anti-Iba-1, polyclonal, Abcam, Cambridge, UK) and astrocytes (anti-GFAP monoclonal, Dako, Carpinteria, CA, USA). Counter staining was performed with Hoechst (Sigma-Aldrich Israel Ltd., Rehovot Israel).

Image acquisition, quantification and statistical analyses

Iba-1 and GFAP immunostaining was visualized using $\times 4/0.1$ NA, $\times 10/0.25$ NA and $\times 40/0.65$ NA objective lenses on a Nikon eclipse 50i fluorescence microscope equipped with a Nikon DS Fi1 camera. In order to minimize bleaching of the fluorescence, images were obtained by serially moving the slide with no fluorescence and then acquiring the images in a standard manner. All sections were then studied quantitatively for differences in immunostaining density among the groups, using Image J software (NIH, USA). Region of interests (ROIs) was drawn manually using the 'Polygon selection' tool. Brain regions were identified using a mouse brain atlas. ROIs were chosen to represent anatomical regions previously shown to be involved in cognition and/or to exhibit variable sensitivity to neuroinflammation in other models. The mean intensity of the specific ROIs ($\times 10$ magnification) was recorded for each individual animal recorded

(Analyze \gg Measure), and data were analyzed using SPSS statistical software (version 15.0). Univariate analysis was conducted for each ROI/Antibody separately using 'group' as a fixed factor and 'experiment' as a Covariate. Post hoc analysis, one-way ANOVA, Student's *t* test, simple regression or correlation analysis was used when appropriate, according to the experimental design. Significance level was determined in one-tailed and two-tailed tests. The level of statistical significance of differences is $p < 0.05$.

Results

Behavioral tests

The ANOVA analysis showed significant differences in the performance of the mice in the forced swimming and the staircase tests 3 months after injection (Fig. 1). The specific differences were detected by the post hoc test which showed that the two groups injected with the Gardasil vaccine spent significantly more time floating compared to control mice and Al-injected mice (Fig. 1a). No significant differences were found between the groups in the overall memory skills (measured by the novel object recognition test), locomotor function, exploratory activity and anxiety which were measured in the staircase apparatus (Fig. 1c).

The analysis after the behavioral testing at 6 months post-injection demonstrated that the alterations in the FST performance were sustained in the group injected with Gardasil + Pt compared to control mice ($p = 0.024$; Fig. 1b), indicating that the effect of Gardasil + Pt exposure was long-lasting. Moreover, at 6 months post-injection, the Al-injected group likewise spent significantly more time floating compared to the control group ($p = 0.044$, Fig. 1b). Although the Gardasil group showed increased floating time compared to the vehicle-injected control group, the observed difference was not statistically significant. Given that after the first round of testing at 3 months post-injection, we killed five animals from each of the four experimental groups; it is possible that our experiment was insufficiently powered to detect milder adverse effects arising from the different treatments. Significant differences were also observed in the rearing frequency in the staircase test. Namely, the Al-injected mice showed a significantly lower frequency of rearing compared to the group injected with Gardasil + Pt in the staircase test ($p = 0.021$; Fig. 1d). A lower frequency of rearing is an indication of a reduced exploratory response to a novel environment, and, it can also indicate a non-selective attention deficit. There was no statistically significant difference in the number of stairs climbed in the staircase test between the groups (not shown). In the FST,

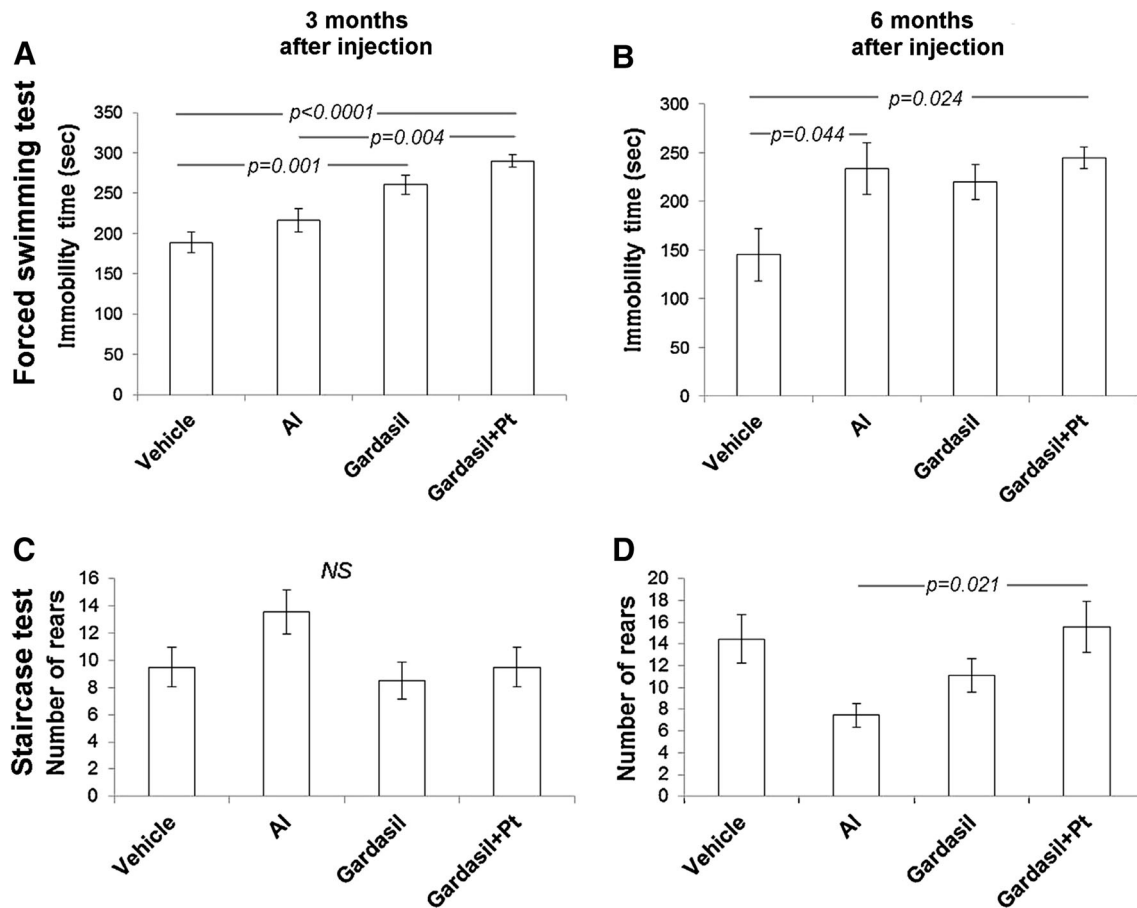


Fig. 1 Effects of AI, Gardasil and Gardasil + Pt toxin injections on behavioral tests. **a** and **b** show the floating time in C57BL/6 female mice as evaluated by the forced swimming test (FST). Results are presented as duration in seconds (mean \pm SEM) of immobility, defined as the absence of escape-oriented behaviors, such as swimming, jumping, rearing, sniffing or diving, recorded during the 6-min test. **a** Three months post-injection ($n = 14$ per treatment

group); **b** Six months post-injection ($n = 9$ per treatment group). **b**, **c** show the reduced exploratory activity in C57BL/6 female mice as evaluated by the rearing frequency in the staircase test. Results are presented as the number of rears (mean \pm SEM) during a 3-min testing period. **a** Three months post-injection ($n = 14$ per treatment group); **b** Six months post-injection ($n = 9$ per treatment group)

however, the changes were still significant despite the lower number of animals. No significant differences in behavior were observed in the novel object recognition test.

Autoantibody profile and inhibition assay

One month post-injection of either AI, Gardasil and Gardasil + Pt, the profile of serum antibodies was analyzed at dilution of 1:200. Elevated levels of antibodies recognizing the HPV L1 capsid protein of HPV types 6, 11, 16 and 18 ($p < 0.002$), as well as anti-brain protein extract ($p < 0.002$) and anti-brain phospholipid extract antibodies ($p < 0.001$) were observed in the two groups of mice that received the HPV vaccine (Fig. 2). The titers of anti-HPV antibodies, anti-brain protein extract and anti-brain phospholipid extract antibodies were reduced after 2 months (data not shown). No elevation in the titers of anti-AI-

hydroxide, anti-dsDNA and anti- β 2GPI antibodies, was detected in the sera of any of the four treatment groups of mice (Fig. 2).

The binding of anti-HPV antibodies from the sera from the two treatment groups immunized with Gardasil to HPV L1 antigens was significantly inhibited by the mouse brain protein extract in a dose-dependent manner in comparison with AI-injected mice whose sera were negative for anti-HPV antibodies (Fig. 3).

Brain tissue immunostaining

Following the behavioral tests at 4.5 months of age, five animals were killed from each of the four experimental groups and used for brain immunostaining procedures. With this relatively small group size, there were no clear changes between the groups in both astrocyte and

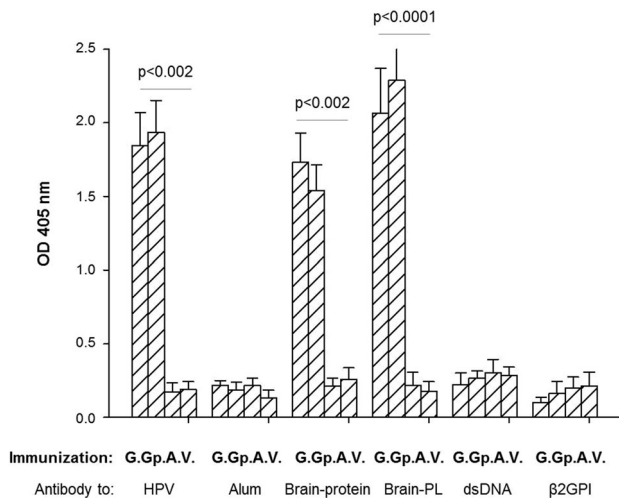


Fig. 2 Titers of serum antibodies 1 month post-injection with either AI (A), Gardasil (G), Gardasil + Pt toxin (Gp) and vehicle (V). A homemade ELISA was used to detect the levels of anti-HPV, anti-AI hydroxide (Alum), anti-mouse brain protein extract, anti-mouse brain phospholipid (PL) extract, anti-dsDNA and anti-β2glycoprotein-I (β2GPI) antibodies in the sera of immunized mice. Pools of sera ($n = 5$ per treatment group) were used as samples. All sera samples were assayed in triplicate. Data are presented as mean OD 405 ± SEM

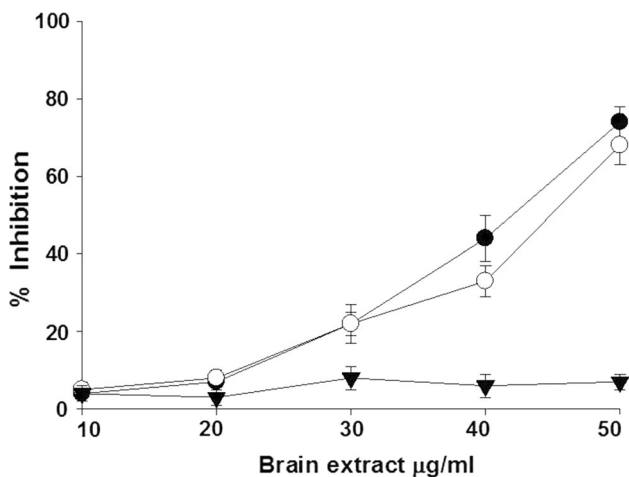


Fig. 3 Inhibition of the binding of antibodies from the sera of Gardasil-injected mice to components of the vaccine (presumably the HPV antigens) by the mouse protein extract. Pools of sera ($n = 5$ per treatment group) were used as samples. All sera samples were assayed by duplicates in independent experiments. Data are presented as mean (% Inhibition) ± SEM where % inhibition = $100 - [(OD \text{ of tested sample without inhibitor} - OD \text{ of tested sample with inhibitor}) / (OD \text{ of tested sample without inhibitor})] \times 100$ (inverted triangle) Al, (filled black circle) Gardasil, (open circle) Gardasil + Pt

microglia staining in any of the regions of interests we investigated (CA1, CA3, dentate gyrus and the striatum). Nonetheless, there was a significant difference between the groups in the density of Iba-1 immunostaining using one-tailed analysis ($p = 0.046$). Further post hoc analysis revealed significant increase in Iba-1 density in the CA1 of

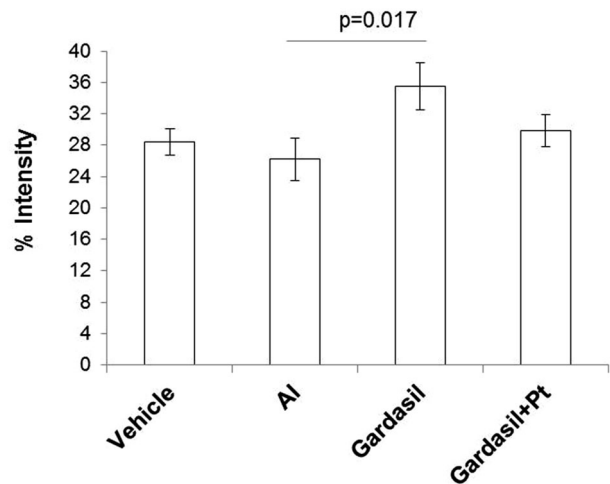


Fig. 4 Iba-1 immunostaining in the CA1 area of the hippocampus of C57BL/6 female mice injected with AI, Gardasil and Gardasil + Pt toxin. Brain sections from five animals out of each group were examined quantitatively for differences in immunostaining density using Image J software (NIH, USA) as described in “Materials and methods”. The data are presented as % mean (% Intensity) ± SEM

Gardasil-immunized mice compared to AI-injected mice ($p = 0.017$; Fig. 4). These results suggest that the CA1 might be vulnerable to small changes in neuroinflammation as a result of Gardasil immunization.

Discussion

The present results show alteration of behavioral responses and neuro-inflammatory changes in mice as a result of AI and Gardasil vaccine injection in exposure doses which are equivalent to those in vaccinated human subjects. In particular, mice injected with AI and Gardasil spent significantly more time floating in the FST test (measure indicative either of locomotor dysfunction or depressive behavior), compared to control animals (Fig. 1a, b). In contrast, no significant differences were observed in the number of stairs climbed in the staircase test which is a measure of locomotor activity.

In addition, the AI-injected group showed abnormal responses to a novel environment, which was manifested in reduced rearing frequency in the staircase test, which indicates a reduction in exploratory behavior (Fig. 1d). The number of stairs and rears in this test is normally used to provide measures of general physical motor abilities and level of interest in the novelty of the environment. Rearing in response to environmental change (i.e., removing a mouse from the home cage and placing the animal in an open box or a staircase apparatus) is also considered an index of non-selective attention in rodents, while rearing

during object investigation likely reflects selective attention [26].

We further observed significant increase in levels of anti-HPV antibodies, and antibodies targeting the brain protein and the brain phospholipid extract components in the two groups of mice that received the Gardasil injection (Fig. 2). Moreover, the recognition of vaccine components (presumably the HPV L1 capsid protein species) by the antibodies from the sera of Gardasil-immunized mice was inhibited in a dose-dependent manner by the mouse brain protein extract (Fig. 3). On the basis of these results, it would appear that the anti-HPV antibodies from Gardasil-vaccinated mice have the capacity to target not only the HPV antigens but also brain antigen(s), either directly or via negatively charged phospholipids. Finally, we observed significant inflammatory changes in the Gardasil-injected mice, namely the presence of activated microglia in the CA1 area of the hippocampus (Fig. 4).

Possible mechanisms of vaccine-induced injury

The role of adjuvants

It is interesting to note that, in our hands, the extent of adverse neurological manifestations was similar in the three treatment groups whose only common denominator was the Al compound. As we noted above, the clinical trials for both HPV vaccines, Gardasil and Cervarix, used an Al-containing placebo and the safety of the vaccines was thus presumed on the finding that there was an equal number of adverse events in the vaccine and the alleged placebo group [21, 22, 27–31]. The HPV vaccines, like many other vaccines, are adjuvanted with Al in spite of well-documented evidence that Al can be both neuro- and immuno-toxic [10, 11, 13, 32–35] and hence does not constitute an appropriate placebo choice.

The appearance of diverse adverse neurological and immuno-inflammatory manifestations following routine vaccinations is well documented in the medical literature (Table 1). Although the classical explanations for these phenomena have largely centered on vaccine antigens, in recent years attention has shifted to Al adjuvants. Consequently, in the last decade, studies on animal models and humans have indicated that Al adjuvants have an intrinsic ability to inflict adverse immune and neuro-inflammatory responses [9–11, 13, 14, 33, 35–37]. This research culminated in delineation of ASIA-‘autoimmune/inflammatory syndrome induced by adjuvants’, which encompasses the wide spectrum of adjuvant-triggered medical conditions characterized by a misregulated immune response [2]. Notably, the vast majority of adverse manifestations experimentally triggered by Al in animal models and those

associated with administration of adjuvanted vaccines in humans are neurological and neuropsychiatric [2]. These observations should not be particularly surprising given Al’s well-established neurotoxic properties [38, 39]. What has, however, been argued is that the concentrations at which Al is used in vaccines are not sufficient to cause neurotoxicity [17, 40]. This argument, however, is not supported by recent evidence.

It should be noted that the long-term biodistribution of nanomaterials used in medicine is largely unknown. This is likewise the case with the Al vaccine adjuvant, which is a nanocrystalline compound spontaneously forming micron/submicron-sized agglomerates. It has been recently demonstrated that Al adjuvant compounds from vaccines, as well as Al-surrogate fluorescent nanomaterials, have a unique capacity to cross the blood–brain and blood–cerebrospinal fluid barriers and incite deleterious immuno-inflammatory responses in neural tissues [10, 13, 41]. Thus, a proportion of Al particles escapes the injected muscle, mainly within immune cells, travels to regional draining lymph nodes, then exits the lymphatic system to reach the bloodstream eventually gaining access to distant organs, including the spleen and the brain. Moreover, the Trojan horse mechanism by which Al loaded in macrophages enters the brain, results in the slow accumulation of this metal, due to lack of recirculation [10, 41]. The sustained presence of Al in central nervous system tissues is likely responsible for the myriad of cognitive deficits associated with administration of Al-containing vaccines in patients suffering from post-vaccination chronic systemic disease syndromes including macrophagic myofasciitis (MMF) [9, 11, 35].

Thus, contrary to prevalent assumptions, Al in the adjuvant form is not rapidly excreted but rather, tends to persist in the body long-term. As demonstrated by Khan et al. [41], intramuscular injection of Al-containing vaccine in mice is associated with the appearance of Al deposits in distant organs, such as spleen and brain, which were still detected 1 year after injection. Similarly, Al-particle fluorescent surrogate nanomaterials injected into muscle were found to translocate to draining lymph nodes and thereafter were detected associated with phagocytes in blood and spleen. Particles linearly accumulated in the brain up to the 6-month end point. They were first found in perivascular CD11b + cells and then in microglia and other neural cells. The ablation of draining lymph nodes dramatically reduced the biodistribution of injected Al-fluorescent surrogate nanocompounds. In addition, the nanoparticle delivery into the brain was found to be critically dependent on the major monocyte chemoattractant protein MCP-1/CCL2 as intramuscular injection of murine rCCL2 strongly increased particle incorporation into intact brain while CCL2-deficient mice had decreased neurodelivery [41].

In the ASIA syndrome, there could be a the prolonged hyperactivation of the immune system and chronic inflammation triggered by repeated exposure and unexpectedly long persistence of AI adjuvants in the human body (up to years post-vaccination) [6, 42]. It is probable that one of the reasons why AI adjuvants are retained long-term in bodily compartments including systemic circulation is due to their tight association with vaccine antigens or other vaccine excipients [43]. Even dietary AI has been shown to accumulate in the central nervous system over-time, producing Alzheimer's disease type outcomes in experimental animals given dietary equivalent amounts of AI to what humans consume through a typical Western diet [44].

The ability of AI adjuvant nanoparticles to cross the blood–brain barrier via a macrophage-dependent Trojan horse mechanism may explain in part why some vaccines have a predilection to affect the central nervous system [8, 10, 33, 35, 39]. Another explanation comes from the fact that AI nanomaterials can on their own damage the blood–brain barrier and induce neurovascular injury [16, 45]. Collectively, these studies [16, 41, 45] show that nano-AI can accumulate in brain cells, inducing nerve and blood vessel damage and protein degradation in the brain. Persistent accumulation of nano-AI compounds regardless the source (i.e., vaccines, dietary) in the central nervous system may thus increase the likelihood of the development of acute and/or chronic neurological disorders.

With respect to the particular AI compounds used in HPV vaccines, AAHS in Gardasil and ASO4 (3-*O*-desacyl-4'-monophosphoryl lipid A (MPL) adsorbed onto AI hydroxide) in Cervarix, it should be noted that these new adjuvants induce a much stronger immune response than conventional AI adjuvants used in other vaccines (i.e., AI hydroxide and AI phosphate) [46]. Stronger immunogenicity of an adjuvant formulation also implies by default stronger reactogenicity and risk of adverse reactions. Because of the differences in immune-stimulating properties between different AI adjuvant compounds, safety of a particular adjuvant formulation cannot be a priori assumed on the basis of the allegedly good historical track record of other formulations. Rather, they need to be thoroughly evaluated case by case.

According to the US FDA, a placebo is, '*an inactive pill, liquid, or powder that has no treatment value*' [47]. From the literature cited above as well as the present study, it is obvious that AI in adjuvant form is neither inactive nor harmless and hence cannot constitute as a valid placebo. Commenting on the routine practice of using AI-based adjuvants as placebos in vaccine trials Exley recently stated that it is necessary to make a very strong scientific case for using a placebo which is itself known to result in side effects and that no scientific vindication for such practice is

found in the relevant human vaccination literature [7]. Conceivably, there is even less justification for using a novel and more potent AI formulation than those that have been in standard use (AI phosphate and hydroxide). The only aim that this practice achieves is to give potentially misleading data on vaccine safety. Moreover, it is unethical to give a placebo to healthy clinical trial subjects that has no benefit but rather, may cause harm.

The role of vaccine-induced antigens: immune cross-reaction

As noted above, we observed significant elevation of antibodies recognizing Gardasil components, most likely the HPV L1 capsid protein of HPV types 6, 11, 16 and 18 ($p < 0.002$) and of antibodies targeting the mouse brain protein ($p < 0.002$) and phospholipid extracts ($p < 0.001$) in the sera of Gardasil-immunized mice (Fig. 2). The binding of anti-HPV antibodies from the sera of mice injected with Gardasil to components of the HPV vaccine, presumably the HPV L1 antigens, was inhibited in a dose-dependent manner by using mouse brain protein extract as the inhibitor (Fig. 3). Taken together, these results suggest that antibodies from Gardasil-vaccinated mice have the capacity to target not only the HPV L1 antigens but also brain antigen(s), either directly or via negatively charged phospholipids.

This interpretation is consistent with the findings of Kanduc [48] who showed that antigen present in both HPV vaccines Gardasil and Cervarix (the major capsid L1 protein of HPV-16) shares amino acid sequence similarity with numerous human proteins, including cardiac and neuronal antigens, human cell-adhesion molecules, enzymes and transcription factors. Moreover, such contention is also supported by a case of severe acute cerebellar ataxia (ACA) following HPV vaccination where combined immunosuppressive therapy with methylprednisolone pulse and intravenous immunoglobulin (IVIg) therapies as well as immunoadsorption plasmapheresis resulted in complete recovery of the patient. In this particular case, the patient (12-year-old girl) developed symptoms of ACA, including nausea, vertigo, severe limb and truncal ataxia, and bilateral spontaneous continuous horizontal nystagmus with irregular rhythm, 12 days after administration of the HPV vaccine. Severe ACA symptoms did not improve after methylprednisolone pulse and IVIg therapies, but the patient recovered completely after immunoadsorption plasmapheresis [49]. Although no significant antibodies were detected in this patient, the remarkable effectiveness of immunoadsorption plasmapheresis strongly suggested that some unidentified antibodies were involved in the pathophysiology of ACA [49]. Citing the work of Kanduc [50], the authors of this case

have stated that further research on molecular mimicry between human proteins and HPV16 L1-derived peptide is needed to determine the exact pathologic mechanism of ACA [49]. Altogether, these observations suggest that possible immune cross-reactions derived from utilization of HPV L1 antigens in current HPV vaccines might be a risk for cardiovascular and neurological autoimmune abnormalities [48, 50]. Our observation that nearly 85 % (129/152) of HPV vaccine adverse case reports in the current scientific literature relate to neuro-ophthalmic abnormalities may lend further support for this conclusion (Table 1).

Conclusions

In summary, both AI and Gardasil vaccine injections resulted in behavioral abnormalities in mice (Figs. 1, 2, 3). Furthermore, immunostaining analysis showed an increase in the Iba-1 density in the CA1 area of the hippocampus in Gardasil-immunized mice in comparison with AI-injected mice, thus suggesting that CA1 might be vulnerable to neuroinflammation as a result of Gardasil immunization (Fig. 4).

In addition, we observed that the brain protein extract significantly inhibited in a dose-dependent manner, the binding of total IgG isolated from the sera of Gardasil-immunized mice to components of the vaccine, most likely, the HPV L1 capsid antigenic component (Fig. 3). Therefore, it is likely that mice immunized with the HPV vaccine developed cross-reactive anti-HPV antibodies which in addition to binding to the HPV L1 capsid protein may also bind to brain auto-antigens. The putative target antigen(s) should be further identified by immunoprecipitation and proteomics analyses.

In light of these findings, this study highlights the necessity of proceeding with caution with respect to further mass-immunization practices with a vaccine of yet unproven long-term clinical benefit in cervical cancer prevention [20, 51] and which in the other hand is capable of inducing immune-mediated cross-reactions with neural antigens of the human host. This note of caution becomes even more relevant when considering the continually increasing number of serious disabling neurological adverse events linked to HPV vaccination reported in the current medical literature (Table 1) and in vaccine surveillance databases [20].

Finally, in light of the data presented in this manuscript, new guidelines should be requested on the use of appropriate placebos in vaccine safety trials [7].

Compliance with ethical standards

Conflict of interest Yehuda Shoenfeld has acted as a consultant for the no-fault US National Vaccine Injury Compensation Program. L.T. has served as an expert witness in cases involving adverse reactions following qHPV vaccine administration. The other co-authors declare no competing interests.

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