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Review

Myalgia and chronic fatigue syndrome following immunization: macrophagic myofasciitis and animal studies support linkage to aluminum adjuvant persistency and diffusion in the immune system

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ABSTRACT

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a multifactorial and poorly undersood disabling disease. We present epidemiological, clinical and experimental evidence that ME/CFS constitutes a major type of adverse effect of vaccines, especially those containing poorly degradable particulate aluminum adjuvants. Evidence has emerged very slowly due to the multiplicity, lack of specificity, delayed onset, and frequent medical underestimation of ME/CFS symptoms. It was supported by an epidemiological study comparing vaccinated *vs* unvaccinated militaries that remained undeployed during Gulf War II. Affected patients suffer from cognitive dysfunction affecting attention, memory and inter-hemispheric connexions, well correlated to brain perfusion defects and associated with a stereotyped and distinctive pattern of cerebral glucose hypometabolism. Deltoid muscle biopsy performed to investigate myalgia typically yields macrophagic myofasciitis (MMF), a histological biomarker assessing longstanding persistency of aluminum agglomerates within innate immune cells at site of previous immunization. MMF is seemingly linked to altered mineral particle detoxification by the xeno/autophagy machinery. Comparing toxicology of different forms of aluminum and different types of exposure is misleading and inadequate and small animal experiments have turned old dogma upside down. Instead of being rapidly solubilized in the extracellular space, injected aluminum particles are quickly captured by immune cells and transported to distant organs and the brain where they elicit an inflammatory response and exert selective low dose long-term neurotoxicity. Clinical observations and experiments in sheep, a large animal like humans, confirmed both systemic diffusion and neurotoxic effects of aluminum adjuvants. Post-immunization ME/CFS represents the core manifestation of "autoimmune/inflammatory syndrome induced by adjuvants" (ASIA).

1. Introduction

Vaccines and clean water have played major roles in fighting life-threatening infectious diseases. During the past century, vaccination allowed the eradication of smallpox, almost eradication of poliomyelitis and considerable decline of measles and mumps [1]. Large vaccination coverage has been shown to avoid the resurgence of several infectious diseases by reducing the number of people who can transmit the pathogens [1]. Vaccines represent the most cost-effective method of infectious disease control and appear as globally safe. However, the risk of adverse effects inherent to any effective pharmaceutical product exists for vaccines as well. Despite low signalling rates, adverse effects following immunization (AEFI) deserve special attention because (i) unlike conventional medicines, vaccines are administered to healthy

Abbreviations: AEFIs, Adverse Effects Following Immunization; Al, Aluminum; ASIA, Autoimmune/inflammatory Syndrome Induced by Adjuvants; BCG, Bacille Calmette-Guérin; CCC, Canadian Consensus Criteria; CRPS, Complex Regional Pain Syndrome; FDG-PET, FluoroDeoxyGlucose-Positron Emission Tomography; GWI, Gulf War Illness; HANS, HPV vaccination Associated Neuro-immunopathetic Syndrome; HAV, Hepatitis A Virus; HBV, Hepatitis B Virus; HPV, Human Papilloma Virus; IBS, Irritable Bowel Syndrome; ICD, International Classification of Diseases; i.m., intramuscular; LPS, LipoPolySaccharide; ME/CFS, Myalgic Encephalomyelitis/Chronic Fatigue Syndrome; ME-ICC, ME International Consensus Criteria; MMF, Macrophagic MyoFasciitis; MRL, Minimal Risk Level; MS, Multiple Sclerosis; PGW, Persian Gulf War; POTS, Postural Orthostatic Tachycardia Syndrome; s.c., subcutaneous; SEID, Systemic Exertion Intolerance Disease; SPECT, Single-Photon Emission Computerized Tomography; Th2, T helper 2; TT, Tetanus Toxoid

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subjects; (ii) an unprecedented expansion of vaccination programs has been announced by America's biopharmaceutical research companies, with more than 260 new vaccines currently being developed [2]; and (iii) because AEFI could probably be largely avoided by the optimization of both vaccinology products and practices based on the understanding of AEIs pathophysiological mechanisms and risk factors [3]. A main concern about vaccine safety relates to the adjuvant compounds that are used in most vaccines to elicit strong and lasting immunization [4,5]. Particular attention has been paid to aluminum salts that were empirically introduced in vaccines by Alexander Glenny in 1926 and constitute the main class of adjuvants licensed for human and animal use worldwide [6,7].

There are two main aluminium (Al) salts used as vaccine adjuvants. Al oxy-hydroxide (AlOOH, Alhydrogel®), commonly called Al hydroxide, is composed of nanoparticles of about 2.2 nm × 4.5 nm × 10 nm which spontaneously form micron-sized aggregates having a nano-fibrous appearance at electron microscopy; while Al hydroxyphosphate (AlOHPO4, Adju-Phos®), commonly called Al phosphate, is amorphous [8]. Both adjuvants strongly potentiate the production of antibodies with very little production of cytotoxic T lymphocytes. The mechanisms underlying their adjuvant effect have only been intensely explored in recent years and remain incompletely understood [9]. Al hydroxide is a stable hydrated gel with a positive surface charge and high antigen adsorption capacities driven by hydrostatic interactions and hydroxyl group exchanges with phosphate of the ligand. Al phosphate has a negative charge, fewer hydroxyl groups, and lower adsorption capacities. The biodisposition kinetics of the two adjuvants are also significantly different: Al hydroxide is solubilized at a much slower rate, and is more avidly internalized and less toxic to phagocytic cells than Al phosphate [8].

The present review will focus on the possible implication of Al adjuvant-containing vaccines in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). Our own experience in the field comes from clinical phenotyping of a large cohort of patients in whom an unusually long persistence of Al hydroxide is detected within immune cells at site of previous immunization, forming a specific lesion called macrophagic myofasciitis (MMF) [10–14]. In 2011, Yehuda Shoenfeld used this MMF-syndrome as one paradigm of the condition he named "autoimmune/inflammatory syndrome induced by adjuvants" (ASIA) [5].

2. ME/CFS definitions

ME/CFS is a common, often severely disabling, costly and still unexplained condition [15]. In the USA between 836,000 and 2.5 million people have ME/CFS at a cost estimated between 17 and 24 billion dollars annually [15,16]. ME/CFS ranks as "very poor" in terms of health-related quality of life [17]. ME/CFS follows a prolonged course over years, with relapses and remissions. Patients experience a substantial loss of physical and mental functional capacity and may become wheelchair dependent, housebound or bed-bound. ME/CSF is associated with an increased risk of developing B-cell non-Hodgkin lymphoma [18].

A variety of names and definitions have been proposed to designate and characterize a similar constellation of symptoms of unknown cause affecting all the major systems and organs of the body [19].

The term "Myalgic encephalomyelitis" was first included by the WHO in their International Classification of Diseases (ICD) in 1969 and ME is still listed as a neurologic disease under ICD G93.3. Besides muscle pain and exercise intolerance, ME patients often present with neurologic dysfunction [20] which was acknowledged by the Oxford criteria in 1991 that emphasized the mental fatigue and neurological background of the disease [21]. The so-called CFS was subjected to a revised definition from the CDC in 1994 [22], including profound incapacitating fatigue of unknown cause lasting more than 6 months with at least four concomitant symptoms including myalgia, arthralgia, headache, memory or concentration impairment, unrefreshing sleep, post-exertional malaise, and tender lymph nodes (Table 1) [22]. The 94 CDC (Fukuda) criteria are still widely used but they put exclusive emphasis on the trivial term "fatigue" that proved to be detrimental to patients being seen as suffering from a psychiatric or psychological illness. To address this limitation, the 2003 Canadian Consensus Criteria (CCC) document interchangeably used "ME" and "CFS", the illness being referred to as "ME/CFS" [23]. In this document, fatigue but also post-exertional malaise, sleep dysfunction and myalgia/arthralgia were included as major criteria. The symptoms lasting more than 6 months had to be associated with at least two cognitive/neurological manifestations, and at least one symptom from two of the following categories: (1) autonomic manifestations, (2) neuro-endocrine manifestations, or (3) immune manifestations [23]. In 2011, a novel classification derived from the CCC reported as ME International consensus criteria (ME-ICC), abandoned the term CFS [24], and the condition became recognized as a major health problem in children [25]. In 2015, the Institute of Medicine [15] also suggested to replace the confusing name CFS by Systemic Exertion Intolerance Disease (SEID), and proposed a case definition that included the following 4 symptoms: (1) substantial reduction or impairment in the ability to engage in pre-illness levels of […] activities; (2) post-exertional malaise, (3) unrefreshing sleep; (4) cognitive impairment and/or orthostatic intolerance. As a whole SEID diagnostic criteria are less specific than CDC 94, CCC and ME-ICC criteria [26], since they do not exclude psychiatric disorders "except in the unlikely event that all symptoms can be accounted for by them" [15].

Table 1
The 1994 CDC definition for chronic fatigue syndrome

CDC 1994 definition (Fukuda) criteria
1. Profound fatigue for 6 or more consecutive months that is not due to ongoing exertion or other medical conditions associated with fatigue
2. The fatigue significantly profoundly interferes with daily functioning and work
3. The individual concurrently has four or more of the following symptoms:
• Post-exertion malaise lasting more than 24 h
• Unrefreshing sleep
• Significant impairment of short-term memory or concentration
• Muscle pain
• Pain in the joints without swelling or redness
• Headache of a new type pattern or severity
• Tender lymph node in the neck or the armpit

In summary, the variety of proposed case definitions and their apparent discrepancies may indicate that no firm consensus on nomenclature or classification has yet been reached among different countries and researchers to designate and characterize this complex and heterogeneous neuroimmune condition with multisystemic dysregulation.

3. ME/CFS overlaps with other conditions

ME/CFS frequently overlaps with other syndromes of unknown etiology including fibromyalgia, irritable bowel syndrome (IBS), postural orthostatic tachycardia syndrome (POTS) and other syndromes [27].

Fibromyalgia share many clinical manifestations with ME/CFS, including myalgia, fatigue, headache, impaired memory, decreased concentration and sleep disturbances. This large clinical overlap has fuelled a debate on whether ME/CFS and fibromyalgia are distinct entities or merely represent phenotypic variations of a single disease including more severe fatigue in one side of spectrum (ME/CFS) and more myalgia in the other (fibromyalgia). To date, the question remains unsolved [27]. Until a global consensus is reached, and since WHO officially classifies fibromyalgia among musculoskeletal disorders (WHO ICD10M79.7) and not among neurologic diseases like ME/CFS, we still distinguish the two conditions by using the 1990 ACR criteria for fibromyalgia, that are based on tenderness on pressure (tender points) in at least 11 of 18 specified sites and the presence of widespread pain [28]. Thus, by using both 1994 CDC criteria for ME/CFS

(Fukuda) and 1990 ACR criteria for fibromyalgia [28], ME/CFS patients may exhibit, or not, fibromyalgia co-morbidity (see below). However, new criteria for fibromyalgia have been proposed with the support of Lilly Research Laboratories in 2010, revised in 2011 and 2016 [29], abandoning tender points testing and thus becoming much more inclusive [30]. The 2016 criteria include (1) generalized pain, defined as pain in at least 4 of 5 regions; (2) symptoms present at a similar level for > 3 months; (3) widespread pain and other symptoms of sufficient severity (according to a widespread pain index and a severity scale score); (4) diagnosis of fibromyalgia being valid irrespective of other diagnoses, i.e. diagnosis of fibromyalgia does not exclude the presence of other clinically important illnesses.

IBS is one of the most common functional bowel disorders. It is characterized by abdominal discomfort and disordered bowel habits. About 50% of IBS patients exhibit co-mobidity: 14% have ME/CFS, and 35-90% of ME/CFS patients have IBS [31,32].

POTS is characterized by excessive increase in heart rate (> 30 beats/min) with positional change from laying to standing up. It is linked to autonomic nervous system dysfunction. POTS is often co-morbid with ME/CFS, especially in young patients [33]. About 40% of ME/CFS patients may suffer from POTS [34] and 64% of patients with POTS fulfill criteria for ME/CFS [35].

Complex Regional Pain Syndrome (CRPS) is another chronic pain and dysautonomic condition. CRPS affects the arms, hands, legs, or feet, and manifests by intense burning pain with hyperalgesia, and dramatic changes in skin temperature, color, or texture which can no longer be explained by an initiating noxious event [36]. CRPS can be associated with body perception disturbances and movement disorders.

4. ME/CFS and vaccines

Idiopathic ME-CFS shares similarities with post-infectious fatigue syndromes [37], but no pathogen has been shown to be present in all affected patients [38], leading to the emerging view that similar symptoms could be triggered by a variety of different pathogens and toxic compounds [38–42]. Among possible triggering factors, vaccines and their multiple components have long been suspected to play a role [43,44].

4.1. First strong signal: HBV vaccine

Following the first campaign of immunization against Hepatitis B virus (HBV) in Canada, a nurse declared in 1992 on Canadian television she had acquired ME/CFS after receipt of the vaccine. Viewers were asked to report any similar experience. The name of 69 such individuals were forwarded to the Department of National Health and Welfare who committed an *ad hoc* working group to examine the question. The committee confirmed the temporal associations between immunization against HBV and ME/CFS onset but recommended to allocate no funds for research on a possible causal link on the basis of arguments such as: (1) the absence of tight time-clustering between immunization and onset of symptoms; (2) the small proportion of overall Canadian ME/CFS patients vaccinated against HBV within "*3 months*" prior to onset of symptoms; and (3) the lack of biological plausibility [45]. Since that time numerous patients with histories of ME/CFS occurring at various times after immunization against HBV have been reported in the literature [11,46,47], and the aluminium hydroxide adjuvant used in HBV vaccines was shown to be poorly degradable and to persist and disseminate in the immune system for much longer time than previously believed [7,48]. Retrospectively, these novel data and those collected for other Al-containing vaccines (see below) cast doubt on the validity of temporal and plausibility arguments used by the Canadian *ad hoc* committee.

4.2. Limitations of epidemiological studies in the assessment of ME/CFS as an AEFI

In 2012, the Institute of Medicine [49] indicated "the evidence was inadequate to accept or reject a causal relationship for the vast majority of vaccine adverse effects they examined", due to the limited number of satisfactory epidemiological studies. Admittedly, recognizing ME/CFS as an AEFI and assessing the causal role of multiple immunizations in ME/CFS are challenging tasks because all identified limitations of AEFI epidemiological studies are at play in this setting. According to IOM [50] these limitations (in italic letters) include:

- *Lack of long-term follow-up studies*, precluding detection of delayed effects;
- *Small sample sizes,* precluding detection of rare occurrences;
- *Lack of evaluation of multiple vaccinations as a whole*;
- *Lack of symptoms specific to vaccination;*
- *Multiple symptoms occurring in combination*, at high risk of being trivialized and not being recognized as forming an entity, as pointed in the lay press for ME/CFS symptoms in pre-licensure HPV vaccine safety trial [51];
- *Underreporting inherent to passive surveillance systems*, ME/CFS symptoms often being not considered dramatic enough to desserve reporting; time lapsed since exposure being highly variable and often very long, thus blurring the picture; ME/CFS being ignored/unbelieved to be possibly linked to vaccine [51]; and poor diligence in reporting being the general rule [52];
- *High vaccination rates and multiple vaccine administrations* precluding comparison with control groups that did not receive the vaccines;
- *Restricted population in vaccine trials* yielding results that may not be generalizable to the general population (e.g. trials including children or individuals without risk factors);
- *Changes in vaccine technology* precluding safety experience based on earlier vaccines to be generalizable to substantially different new vaccines (see novel Al adjuvants, below).

4.3. Insights from the Gulf War Illness in deployed and non-deployed veterans

Fortunately, privileged epidemiological studies linked to the Gulf War Illness (GWI) compared vaccinated *vs* un-vaccinated individuals, pointing out a link between multiple vaccine administration and ME/CFS [53]. At least one quarter of Gulf War Veterans returning from the Persian Gulf War (PGW) in February 1991 has reported a variety of chronic symptoms that vary somewhat among individuals but share striking similarities with ME/CFS [53,54]. As defined by the CDC, cases of multisymptom GWI must have chronic symptoms from at least two of the following three groups: (1) fatigue; (2) mood/cognition (feeling down or depressed, memory problems, difficulty concentrating, trouble finding words, problems falling or staying asleep); and (3) musculoskeletal (joint pain, muscle pain) [55]. Due to biases that regularly occurred in the course of investigations, there is still uncertainty over the exact causal factors of GWI but, among other factors, multiple vaccinations administered within a short period of time have been repeatedly suspected [56].

A cross-sectional study from UK showed strong association of CDC-defined GWI with multiple vaccinations administered during deployment (odds ratio OR 5.0; 95% confidence interval 2.5 to 9.8) [57]. Moreover, "consistent, specific, and credible relations" were found between the reported number of inoculations and health indices in UK Gulf war veterans [58]. Both vaccination against biological warfare agents (anthrax and plague with pertussis as an adjuvant to boost immune responses) and multiple routine vaccinations were associated with the CDC multisymptom syndrome in the UK Gulf War cohort [59]. In Canada, a significant association has been reported between "non-routine immunizations" and several symptom-defined outcomes in Gulf

war veterans [60]. In USA, declines in long-term subjective health of Gulf War veterans were associated with receipt of anthrax vaccine and veterans who reported more severe reactions to vaccines were more likely to report declines in subjective health [61]. The U.S. licensed anthrax vaccine (AVA; Biothrax®) is adsorbed on aluminum hydroxide and can induce MMF [62]. In addition, the vaccine has been suspected to contain squalene because of detection of circulating squalene antibodies in affected veterans [63] but no relationship was found by the Department of Defense between squalene antibodies and chronic symptoms [64]. Therefore, the role of vaccines in GWI has remained elusive, more focus being put on the role of wartime chemical exposures, such as pyridostigmine bromide used as prophylaxis against chemical warfare attacks or personal pesticide use [54].

Toxicant exposures specific to PGW, however, could hardly be incriminated in veterans that were not deployed or who served elsewhere than in the Persian Gulf, and developed, at lower rate, chronic symptoms similar to GWI. Lea Steele [53] specifically studied possible effects of vaccines in veterans from Kansas who did not serve in the PGW (non-PGW veterans): compared to unvaccinated veterans, non-PGW veterans reporting vaccine administration had significantly more fatigue/sleep problems, pain symptoms, neurologic/cognitive/mood symptoms, and gastrointestinal symptoms. Vaccinated (n = 208) *vs* unvaccinated (n = 187) non-PGW veterans had significantly much higher prevalence of GWI as defined by both Kansas criteria (*i.e.* chronic symptoms occurring in at least 3 domains: 11.5% *vs* 3.7%, OR 3.78 [1.50-9.54]) and CDC criteria (OR 2.04 [1.15–3.60]). In contrast to PGW veterans in whom GWI possibly resulted from “clusters of causes” and “combination of effects”, no other cause than self-reported receipt of vaccines was found in non-PGW veterans [53].

5. ME/CFS in Al adjuvant-induced macrophagic myofasciitis

Accuracy of self-reported receipt of vaccine is classically open to question [53], but this is not the case when vaccine files are available and when a highly specific histological marker can unambiguously assess previous immunization as it is the case in patients with MMF [10].

5.1. Macrophagic myofasciitis: a biomarker of Al adjuvant biopersistency

5.1.1. Characterization in humans

Macrophagic myofasciitis (MMF), first described in 1998 [65], is a specific Al hydroxide-induced granuloma detected at site of previous vaccine injection [10]. The lesion is characterized by sheets of large macrophages that constantly enclose submicron to micron-sized agglomerates of aluminum nano-crystals in their cytoplasm, intermingled with lymphocytic infiltrates [10]. This immunologically active lesion is not associated with giant cell formation [66].

The first 75 patients reviewed in 2003 [67] had mainly received Al hydroxide through HBV (84%), Tetanus Toxoid-containing vaccines (TT 58%) or Hepatitis A virus vaccines (HAV 19%), usually administered in combination. The proportion of vaccine types changed markedly with time: our last 70 patients, collected from january 2013 to june 2018, of whom 56 had complete vaccine files, had mainly received, as their last immunization or within 10 years before biopsy, TT-containing (86%, 48/56), followed by HBV (27%, 15/56), HAV (11%, 6/56), HPV (11%, 6/56), and meningitis C (< 1%, 1/56) vaccines. TT-containing vaccines (mainly Diphtheria-Tetanus-Poliomyelitis, Diphtheria-Tetanus-Acellular Pertussis, and Diphtheria-Tetanus-Acellular Pertussis-Poliomyelitis), administered alone or in various combinations with non-TT vaccines, was the most recently administered vaccine in 64% of cases. These data clearly indicate that MMF is not specifically associated with HBV vaccine.

MMF is rarely detected despite the huge number of immunized individuals, but, shortly after its initial description, striking increase of MMF detection rate was noted by all French specialized centers [10,68–70]. Prominent detection of MMF in France from 1993 was likely due to: (1) the recommended replacement at the beginning of the decade of the subcutaneous (s.c.) by the intramuscular (i.m) route of immunization; (2) the huge immunization campaign against hepatitis B conducted in France, with 90 millions of doses sold in ten years, two thirds of which being administered to adults; and (3) the usual choice of the deltoid muscle for biopsy in adults in France while this muscle is not preferentially chosen in other countries [10]. For example, only 10% of adult muscle biopsies have been performed in the deltoid muscle in one US center, MMF being detected in 1% of these biopsies [14]. Despite the usual routine choice of an incongruent biopsy site precluding detection of most MMF cases, the lesion could be occasionally documented in Australia [71], Brazil [72], Germany [73–76], India [77], Ireland [78], Israel [79], Italy [80,81], Portugal [13,82,83], Saudi Arabia [84], Spain [85], UK [86,87], USA [13,62,88,89], and several other countries (personal communications to RK Gherardi), thus indicating that MMF is not specific to France.

5.1.2. Clearance of the lesion in animal models

The lesion has been reproduced experimentally in mice, rats, monkeys and sheep [10,90–92]. In sharp contrast to the quick elimination of soluble Al injected intravenously [93], intramuscular injection of isotopic Al hydroxide is associated with much slower elimination of Al in urine, accounting for 6% of the injected dose 28 days after injection in rabbits [94]. Based on data of this unique experimental toxicokinetic study, the duration for complete translocation of solubilized Al ions from the injected site to blood was calculated to be 5.5 months for Al hydroxide [95]. Consistently, experimental MMF invariably shrinks over time [91]. In monkeys, MMF induced by DTP vaccine injection - corresponding to 14- to 21-fold the human DTP-equivalent dose of adjuvant- was entirely cleared out from the injected muscle before 6 months (Al phosphate 100%, Al hydroxide 25%) and between 6 and 12 months (Al hydroxide 25%) after immunization [90]. Similarly to these animal models the vast majority of humans appear to clear out the adjuvant from the injected muscle within months, but in a small proportion of them MMF may be observed up to > 15 years after vaccination (see below).

Thus, longstanding MMF should be considered as a biomarker assessing difficulty of some individuals to clear out the adjuvant from their body [96]. For this reason we recommend to perform biopsy seeking MMF at least 18 months after the last immunization to allow the assessment of the unusually long persistence time of the lesion.

5.1.3. Genetic susceptibility factors

It is generally assumed that subpopulations of humans exist that are more sensitive to certain chemical or particulate exposures than the average population. Individual susceptibility factors usually reflecting specific “genes x environment” interactions likely explain why only a small proportion of vaccinees develop adverse effects. The exact ground of such individual susceptibility may include: (1) genetically-determined impairment of cellular defenses against the prooxidant effects of Al [70]; (2) HLA subgroups, such as HLA-DRB1*0, known to favour autoimmune responses [98]; (3) ageing and/or genetically- based inter-individual differences in production of the chemoattractant MCP1/CCL2 necessary for dissemination of immune cells loaded with Al adjuvant particles [99,100, 101]; (4) individual difficulty to clear out Al adjuvant from immune cells [48]. The size of experimental MMF lesions in rats markedly differs according to the genetic background [91] and in humans conspicuous inter-subject variability exists for aluminum elimination [97].

An intracellular mechanism called xeno/autophagy is instrumental in solubilisation and biodisposition of internalized mineral particles [102], as well as in metal toxicity [103] and many crucial functions in the immune and central nervous system. Promising preliminary data have been obtained by DNA screening of 34 genes directly involved in the xeno-autophagy machinery (in collaboration with Baharia Mograbi, IRCAN, Nice University, France), suggesting that MMF may reflect

genetically-determined inability of some individuals to efficiently dispose of injected aluminum adjuvants (patent deposited), paving the way for development of genetic tests predicting an increased risk of intolerance linked to adjuvant retention.

5.2. Macrophagic myofasciitis syndrome: a post-vaccinal ME/CFS

5.2.1. Clinical characterization

From 1994 to 2012, we have seen 583 adult patients with MMF in the Paris Est University Hospital Henri Mondor, Créteil, France [104], including patients duely registered in the Henri Mondor hospital clinical database (350 registered from 1994 to 2018) and additional patients followed by other centers but punctually referred to us for testing, biopsy, biobanking, certificate delivery, or legal expertise. There is no national MMF registry, but a total number of 445 MMF cases had been officially notified to the French national pharmacogilance system on October 31, 2015. Except cognitive dysfunction data which were specifically collected in our center but not captured at the national level, local and national sources showed similar characteristics of MMF patients, as also confirmed by series from Portugal [13] and USA [14].

The Henri Mondor hospital 1994-2018 MMF database indicates the following characteristics of patients: mean age 52.5 years, female predominance (71%), mean of 5 (range 1–12) Al-containing vaccine shots in the 10 years preceding biopsy (vaccine files available in 236/350), mean Al adjuvant persistence time 71 months (range 9–237 months, assessed by histology and Morin stain for Al), mean delay from clinical onset to biopsy 67 months, main symptoms including myalgia/arthralgia (92%, 309/335), fatigue (86%, 298/347) and cognitive complaints (82%, 154/187). Other symptoms include headaches (49%), dyspnea (51%), abdominal pain (30%), ocular symptoms (34%), thoracic pain (32%), urinary symptoms (21%), and fever (23%) [67].

Patients had received intramuscular administration of Al-containing vaccine prior to the onset of muscular symptoms [13,67]. The delay before first symptoms could substantially vary, ranging from 0 to 72 months in our initial series. Median delay from last vaccination to first myalgia was 11 months, 30% of patients complaining of myalgias within 3 months, 31% from 3 to 12 months, 19% from 12 to 24 months, and 20% after 24 months [67].

In our center, one third of myalgic patients that have received Al hydroxide-containing vaccines in the past 10 years had MMF at deltoid muscle biopsy [105]. Myalgia onset commonly occured after an exercise of unusual intensity, often in lower limbs with progressive extension upward, becoming diffuse at time of biopsy. Myopathic electromyogram and CK elevation are found in less than one half of patients [67].

Fatigue usually had deep impact on the daily life and most of affected patients had rapidly get out of their work after a few months. Cognitive alterations were found to be stereotyped at systematic testing, impacting attention, working and visual memory, and inter-hemispheric connexions, and were neither attributable to chronic pain nor to depression [106–108]. Standard brain MRI was usually normal but functional neuroimaging alterations were conspicuously found, including: (1) focal brain perfusion defects assessed by SPECT (single-photon emission computerized tomography), well correlated to both attention/memory alterations and inter-hemispheric dysconnexion [109]; (2) a characteristic pattern of posterior cerebral glucose hypometabolism assessed by FDG-PET (fluorodeoxyglucose -positron emission tomography) scanner, involving occipital cortex, hippocampus and cerebellum, and predictive of MMF detection at muscle biopsy [110–114].

The main symptoms of patients with MMF, *i.e.* arthromyalgia, fatigue and cognitive complaints, could occasionally occur in isolation for some time. However, a large majority of patients have multiple symptoms with international criteria for ME/CFS being fulfilled in at least 50% of cases [11,13]. The condition also meets the CDC criteria of multistymptom GWI [5].

5.2.2. Consistency analysis

Occurrence of myalgia/arthralgia, chronic fatigue and cognitive alterations in patients with longstanding MMF is very unlikely to represent chance association and rather forms a consistent syndrome. This is assessed by:

(1) *post-immunization* onset of symptoms;
(2) *similar structure of symptoms observed in independent French and foreign MMF series* [10,13,14,70];
(3) *significant association between "myalgia" and "MMF"* in patients who had deltoid muscle biopsy performed in French myopathologic centers before publication of the cause of MMF [10];
(4) *significant clinical differences depending on the presence/absence of MMF in deltoid muscle biopsy among myalgic vaccinees:* in sharp contrast to non-MMF myalgic patients, only a minority of MMF patients had fibromyalgia according to ACR 1990 criteria ($\geq$11 tender fibromyalgic points) (55.5 *vs* 16.6%, $p < 0.04$), and MMF patients had much more CNS involvement as assessed by delayed evoked potentials (38.5 *vs* 5.7%, $p < 0.01$). These data indicate that MMF and non-MMF patients differ by more than the simple detection of MMF [105];
(5) *significant association between "chronic fatigue" and "MMF" in a case-control study* ordered by the French drug agency: fatigue was both "more frequent and more severe in patients with MMF than without MMF in deltoid muscle" [115]; in sharp contrast to diseased controls, MMF patients had strikingly little medical antecedents, further indicating that cases differ from controls by more than the simple detection of MMF [115];
(6) *highly consistent functional neuroimaging changes (SPECT and FDG-PET scanner) in MMF patients, not attributable to chronic pain or depression,* indicating that MMF is detected in an homogeneous subset of patients with stereotyped condition [109–114];
(7) *perfect similarity of the MMF syndrome with the GWI multisymptom complex* [116] defined by CDC [55] which has been uniquely associated with vaccine exposure in military personnel non-deployed in the Persian gulf [53].

In summary, MMF is typically detected in adult patients with a homogeneous subset of ME/CFS of post-vaccinal onset.

6. ME/CFS and related conditions following HPV vaccines administrations

6.1. Current debates

In the absence of deltoid muscle biopsy that could determine if longstanding MMF was present or not, a number of papers have reported combinations of myalgia, arthralgia, chronic fatigue, cognitive dysfunction, unrefreshing sleep and neurovegetative alterations (meeting international criteria for ME/CFS, fibromyalgia, POTS, CRPS, or described as somatoform manifestations) temporally associated with administration of multiple injections of Al adjuvant-containing vaccines, in Australia [117], Canada [118], Denmark [119,120], Italy [121], Israël [47,122], Japan [123–125], Mexico [126], and USA [127]. Whether or not, such temporal associations may indicate possible causal link has been the matter of continuous controversy.

Controversy first emerged following the HBV vaccine campaign, running in parallel for ME/CFS [47] and multiple sclerosis (MS) [128–130], which occasionally occurred in combination [131] following immunization. Most epidemiological studies failed to substantiate the unprecedented increase of post-HBV immunization MS claims [129,130], but these short term studies overlooked the "t" factor. The possibility of delayed onset of clinical symptoms after HBV immunization was pointed out by Hernán [128]: in a unique case–-control study conducted on the long-term in the British population he found an increased risk of MS (OR 3.1; CI 1.5–6.3) in the 3 years

following HB vaccination. Interestingly, there was no increased risk during the first year after immunization (OR 1.8; CI 0.5–6.3), as reported in previous short-term studies, but the increased risk of developing MS became obvious at 2 and 3 years after immunization (OR 4.1; CI 1.3–13.6). This result is in keeping with the reported increase of overall incidence of MS in France following the HBV immunization campaign [129].

Then, controversy culminated with the debate about HPV vaccines safety [132]. Similarly to HBV vaccine that was associated to a disproportionately high level of AEFIs signalling compared to other vaccines, e.g. 5 fold for MS signaling [130], HPV vaccine programs in different parts of the world were associated to a 10 fold higher incidence of AEFIs signalling compared to other vaccines [125,133]. Post-HPV vaccine AEFIs approximately accounted for 1 per 1000 inoculations in Spain [132] and 1 per 1000 vaccinees in Canada [134]. However, HPV vaccine security has been endorsed by international regulatory health agencies [135,136] (EMA, WHO) and the Cochrane collaboration [137]. Nevertheless, criticisms were made, pointing out conflicts of interest with the industry and disclosing numerous methodological flaws in both HPV vaccine safety studies themselves and the systematic reviews grounding institutional reassuring claims [138-142].

6.2. Limitations of epidemiological studies on ME/CFS in HPV vaccine receivers

For the present review, we examined in detail the two studies indicating no evidence that the overall occurrence of CFS in HPV vaccinated girls was different from that expected in the same age groups [144,145].

Donegan *et al.* [144] analyzed the occurrence of ME/CFS in UK girls immunized the bivalent HPV vaccine (Cervarix®) that contains Al hydroxide mixed with 3-O-desacyl-4'-monophosphoryl lipid A.

In the first part of their study, *i.e.* "observed *vs.* expected analysis", Donegan *et al.* [144] considered that underreporting levels could range from 0% to 90%. However underreporting of adverse drug reactions is higher, with a median rate of 94% found across 37 studies in a systematic review, including a maximal level of 98% in UK [146], similar underreporting levels also applying to vaccines [147]. Moreover, the highest (90%) underreporting hypothesis tested by Donegan *et al.* [144] was associated with striking above-the-threshold signal of ME/CFS in Cervarix^{R} receivers. The authors did not retain this result and preferred to focus on lower ($\leq$75%) levels of underreporting that were not associated with increased signal. This is a highly debatable choice given the documented failure in the assessment of ME/CFS symptoms in HPV vaccine receivers [51], vaccine damage and ME/CFS being concepts to which the medical establishment remains generally hostile.

In the second part of their study, *i.e.* "self-controlled case series", Donegan *et al.* [144] has estimated the risk of ME/CFS in the year after the *first* Cervarix^{R} injection (first of three given in 6 months) and paid no attention to other Al-containing vaccines. However, the reported time to adverse effects after the first HPV vaccine dose ranged from 1 day to 51 months (mean 10.7 $\pm$ 11.6 months) in a series of 72 Japanese girls [125] and from 1 day to 43 months (mean 14.0 + 11.6 months) in another series of 35 girls [148]. In the same way, median time of first symptom onset was "11 to 12 months after the *last* Al hydroxide-containing vaccine administration" in our ME/CFS cases [105], making likely that a substantial number of ME/CFS cases possibly linked to Al hydroxide-containing Cervarix^{R} injection have been missed in the Donegan study, even when the risk window was extended to 18 months (*i.e.* about 12 months after the *last* injection, a time after which nearly 40 % of our MMF patients developed their first symptoms). At last, the authors felt there was "*no reason to suspect that girls with fatigue syndrome would be less likely to receive HPV vaccination*", thus precluding a healthy vaccinee bias. This does not respect good practices in the field of vaccine safety science. It is well established that underlying conditions that predispose to medical outcomes suspected to be vaccine adverse effects are linked to lower vaccine uptake [149]. For example, uptake of HPV vaccine dropped in from 82.3% to 39.4% when norvegian girls were diagnosed with ME/CFS prior to immunization [145]. This is an important bias, emphasized by experts from both the Japan Institute of Pharmaco-vigilance [138] and from the CDC who stated "*studies that do not control adequately* [healthy vaccinnee bias] *will tend to underestimate any real risks associated with vaccination*" [149].

Feiring *et al.* [145] studied the quadrivalent HPV vaccine (Gardasil®) which is adjuvanted by amorphous Al hydroxy-phosphate sulfate (Merck proprietary AAHS) that significantly differs from Al hydroxide [150]. The study found that people more at risk of developing ME/CFS tended to avoid the vaccine. Despite this healthy vaccinee bias, the authors remained confident in the reliability of their finding of no increase of ME/CFS in vaccinated *vs* non-vaccinated girls (after adjustment for age), because there was no higher increase of ME/CFS in girls than in boys while only girls had received the HPV vaccine. Indeed, a similar increased rate of ME/CFS was found in girls and boys during the studied years, and remained unexplained. Possible implication of other vaccines administered to both genders in this increase has apparently not been evaluated. Another critical point resided in the challenge of discriminating comorbid conditions from ME/CFS. Reported Gardasil® adverse effects have been fragmented into multiple subcategories, such as POTS, CRPS, somatoform syndrome, dysautonomic syndrome, ME/CFS, fibromyalgia, HPV vaccine syndrome, and HPV vaccination associated neuro-immunopathetic syndrome (HANS). It may be, therefore, misleading to compare idiopathic ME/CFS to the HPV vaccine syndrome since its symptoms are only partially co-morbid with ME/CFS. The variety and inaccurate designations of HPV vaccine adverse events has been viewed as a major obstacle in reporting [143,151].

Of note, MMF syndrome, which is caused by an adjuvant substantially different from AAHS, is much less polymorphic than the HPV vaccine syndrome, both POTS and CRPS being nearly never documented in the setting of MMF. In the setting of HPV vaccine, the traditional observational epidemiological approach has been complicated by the lack of a case definition for the multiple symptoms that constitute the signal, making highly desirable novel epidemiological approaches [151].

For example, the Uppsala Monitoring Center developed a novel data-driven cluster analysis of HPV vaccine reports in Vigibase®, the WHO international database, that identified natural groupings based on terms used to report AEFI. The analysis revealed clusters of serious AEFI more frequently reported in HPV vaccine reports compared to non-HPV vaccine reports in the same sex and age band. They included headache, dizziness, fatigue and syncope that sometimes contained diagnostic labels of POTS, CRPS and CFS but most often lacked explicit diagnoses, pointing out marked underestimation of the signal by traditional post-marketing safety evaluation [151].

In Japan, Osawa analysed temporality of the AEFI on the population level, and showed that the peak of post-vaccination syndrome onset followed the peak period of HPV vaccination and that novel cases were not seen after 14 months from withdrawal of the government recommendation for HPV vaccination [125].

Another approach was based on careful analysis of HPV vaccine randomized trials by Martinez-Lavin [140], who among several disquieting results, pointed out the shocking fact that pre-licensure randomized trials were almost always made against Al adjuvants-containing comparators - not inert placebos. The only one quadrivalent HPV vaccine double blind trial using inert saline placebo showed 0.4% (5/1165) of serious adverse events in HPV-vaccinated subjects *vs* none (0/584) in the inert placebo group [140]. None of these effects were considered vaccine-related, but a potential role of Al adjuvants was further suggested by the largest Gardasil® trial in which receipt of the 9-valent vaccine that contains 500 μg Al adjuvant AAHS was associated with significantly higher rates of both local and systemic compared to

the 4-valent vaccine that only contains 225 μg AAHS [140]. Such a safety imbalance between the two Gardasil® vaccines suggesting a dose-effect was recently confirmed by a FDA report showing higher rates of injection-site reactions, multiple sclerosis and spontaneous abortions with the 9-valent vaccine [152].

7. Insights from experimental studies

In addition to limitations of epidemiological approaches, inadequate understanding of biologic mechanisms underlying vaccine adverse effects is a major factor hindering assessment of causality [49]. This led the Institute of Medicine to declare "*the value of dialogue between both epidemiologic and mechanisms approaches cannot be overstated. These conversations between different types of research can be difficult, but the results are worth it*" [49].

Indeed, the history of vaccines has been largely built on an empirical basis during the last century. This was specifically the case for the Al adjuvants that were first introduced in vaccines in 1926 but remained administered a very low rate to the general population until 1985 when they began to be massively injected along with the introduction of Al-containing DTP, HiB, HBV, HAV, pneumoccocus, meningoccocus, HPV and other vaccines [153]. This was done without clear knowledge of the injected Al adjuvant fate and, since that time, very little effort has been made to clarify the question [7].

Therefore, the classical hypotheses on the injected Al adjuvant fate were tested in mouse models in our lab.

7.1. Old dogma turned upside down

It was classically believed that once injected in the tissue, Al adjuvants and the vaccine antigens adsorbed at their surface form an extracellular depot at site of injection, then progressive solubilisation of the particulate adjuvant was thought to take place, mediated by Al chelating acids present in the interstitial fluid, causing gradual desorption of the vaccine antigen and the observed adjuvant effect [94]. In the frame of this pre-conception it was claimed that Al adjuvant inocuity could be inferred from the little amount of injected Al and rapid elimination of soluble Al in the urine [94]. None of these dogmatic hypotheses proved to be correct when experimentally tested in our lab.

We first showed that, in contrast to previous belief, Al hydroxide particles injected in muscle are not solubilized in the interstitial fluid, and vaccine derived Al is not quickly eliminated in urine: instead, this nearly insoluble particulate compound is quickly captured by monocyte/macrophage lineage cells [99] and persists within these cells from many months after injection in animals [91] to up to > 15 -years in some human beings with MMF. As stated above (Section 5.1.2) and below (Section 7.2.2.) results of the sole experimental study on the toxicokinetics of Al adjuvants [94] are incompatible with rapid biodisposition and renal elimination of vaccine-derived Al [7]. In addition, theoretical models based on Flarend's pre-conceptions and short-term results are flawed [7]. For example, Mitkus *et al.* [95] proposed a model to assess the risk of Al vaccines in infants, by reference to an oral minimal risk level (MRL) extrapolated from animal studies. They only considered solubilized Al, with erroneous calculations of absorption duration. Systemic Al particle diffusion and neuro-inflammatory effects were omitted. The MRL they used was both inappropriate (oral Al lactate *vs.* injected Al adjuvant) and too high regarding recent animal studies indicating that MRL should be reduced by at least 7 fold [7]. In summary, systematic analysis of the available "reference" studies has revealed complete failure to support their reassuring claims, and make mandatory novel experimental studies of Al adjuvant toxicokinetics conducted on the long term and in a sufficient number of animals, under the aegis of health agencies [7].

We also showed that, in contrast to the classical depot formation hypothesis, Al particles do not stay entirely localized in the injected tissue in mice, but, instead, can disseminate within immune cells to the regional lymph nodes and then to more distant sites and to the brain [99,154] where they persist as long as in the injected muscle [155]. The distant organs showing collections of Al particle-loaded cells include the regional lymph nodes, spleen and liver, and the brain in which they enter in using a CCL2-dependent Trojan horse mechanism and from which they do not recirculate [99]. In line with our studies, it has been shown that removal of the vaccine injection site as early as 2 hours after administration has no appreciable effect on the immunological response in rats, thus indicating that the adjuvant exerts its effect remote from the injection site which invalidates the depot formation theory [156]. This is an important point since there appears to be a fine balance between the efficacy of Al adjuvants and their potential toxicity, and these may be one and the same effect [157]. Obviously, the potential toxicity of Al adjuvants depend on whether the bioactive nanomaterial remains localized at injection points or rather scatters and accumulates in distant organs and tissues [99,154]. The latter appears to take place since systemic diffusion of Al adjuvants reported in mice [99,154,155] was also documented in sheep that developed Al-induced granulomas persisting at injection sites associated to similar large macrophage infiltrates with increased Al levels in the draining lymph nodes [92].

We finally showed that, in contrast to previous belief that innocuity of Al adjuvants can be inferred from the low quantities of Al^{3+} injected with vaccines ("the dose makes the poison" paradigm), neurotoxic effects of Al hydroxide particles (Alhydrogel®) respond to a non-linear dose response curve with selective toxicity of the lowest tested dose [158]. Compared to high concentrations, that were associated with spontaneous formation of large particle aggregates and surprisingly caused no toxicity, the lowest Alhydrogel® concentration selectively caused cerebral Al accumulation, microglial activation and long-term neurotoxicity in mouse. Interestingly, the toxic low concentration uniquely formed small 'bacteria-size' agglomerates that were presumably easier to capture and to transport to distant sites [158]. It is, therefore, likely that toxicity of particulate adjuvants taken up by immune cells obeys the specific rules of small particle toxicology rather than any simplistic dose-response relationship.

In summary, our experimental results suggest that capture and long-term Al hydroxide biopersistency within phagocytic cells is a prerequisite for its neuromigration and neurotoxicity in mouse.

7.2. Comparing toxicology of different forms of Al and different types of exposure is incorrect

It is often stated that the intake of oral Al is higher than the quantity of Al injected with vaccines which, therefore, could cause no harm. This superficial statment ignores the marked differences of Al fate in the two situations.

7.2.1. Oral Al (initial value 100%)

In case of healthy intestinal barrier, 99.7% of oral Al is eliminated in faeces and only 0.3% can cross the barrier, in an atomic form, and become bound to blood transporters like transferrin, albumin and citrate. Then > 0,2% is quickly eliminated in the urine [93,159] and the remaining < 0.1% is distributed to the whole body. Of note the body spaces comprise 41% of cells (35 trillions cells) and 59% of extracellular spaces [160]. Preferential Al deposition occurs in bone extracellular matrix, but other organs may show deposits, mainly extracellular deposits [161]. Intoxication may occur on the long term especially in case of combination of high intake with intestinal barrier alteration and/or renal insufficiency.

7.2.2. Injected Al hydroxide (initial value 100%)

In contrast to oral Al, 100% of the initial adjuvant dose crosses the natural barrier with the needle and reaches the internal milieu. In case of Al hydroxide, Al is in a poorly soluble particulate form [8], and more than 6% of the injected Al is quickly eliminated in the urine [94], the remaining 94% being avidly captured by macrophages and transported

to distant organs where Al particles remain mainly intracellular [99]. Thus, in contrast to oral Al, very little of the injected Al diffuses in the extracellular spaces, the bulk of it being selectively and highly concentrated in a small fraction of the phagocytic cells (one of the 200 cell types of the body), representing about 3% of the body weight [162]. This incorporation in phagocytic cells limits extracellular solubilization of the particles, and induces long-term cell survival [163]. The very slow solubilization rate of Al adjuvant particles, especially Al hydroxide [7], makes determination of Al blood levels nearly useless to assess Al adjuvant toxicity. When a single dose of adjuvant corresponding to 0.85 mg Al is administered i.m. to an adult human, an increase in the plasma Al concentration of 0.8% is expected, that would be masked by the Al background if an isototpic ^{26}Al-labelled adjuvant is not used [94]. For the same reason, the spontaneous cumulative urinary excretion of Al is quasi-flat for Al hydroxide a few days after injection [7]. Thus, Al adjuvants do not usually cause massive intoxication by soluble Al similarly to what was previously documented in patients with renal failure undergoing dialysis with Al-containing water. Instead, the particulate Al adjuvants exclusively concentrate in immune cells, a very small part of the human body, in which they chronically exert their immunostimulatory adjuvant effects [164,165], until eventual dispostion. If one estimates that the diffusion space of a locally i.m. injected adjuvant could hardly exceeds 1% of the body space before solubilization of the particle, rough calculation indicates that an oral dose of Al should be about 1 million fold higher than the vaccine dose to induce the same level of Al in phagoctic antigen presenting cells.

Specialized toxicologists are now aware that comparing toxicological properties of different forms of Al (soluble *vs* particulate) administered by different routes (oral *vs* i.m.) is incorrect and, therefore, inadmissible [166]. This constitutes another reason to dismiss "the dose makes the poison" rule to adress toxicity of Al hydroxide adjuvant particles.

7.3. From Al toxicity to chronic immune stimulation

Several experimental studies of the literature have documented the potential neurotoxicity of Al adjuvants. In a seminal study, Alhydrogel® adjuvant, subcutaneously injected in mice at doses relevant to the dose received by US veterans with GWI, induced motor deficits and cognitive alterations associated with motor neuron death and a significant increase of reactive astrocytes indicative of an inflammatory process [167]. Subsequently, toxicity on the adult or developing mouse brain of either Al adjuvant or whole Al-containing vaccines has been reported in Canada [168–170], Israël [171–172] and France [158]. Of note, small animal studies showing toxic effects of Al adjuvants are often suspected to be irrelevant to the human situation but this is not the case of large animal models. Therefore, it should be emphasized that Spanish veterinarians have reported that multiple Al-containing vaccine administrations in sheep can induce a biphasic neurologic disease including initial meningo-encephalomyelitis with behavioral alterations followed by progressive spinal neurodegenerative changes, offering an invaluable model to understand the human ASIA [173–174]. Moreover, multiple injections of the adjuvant alone (Al hydroxide), compared to saline placebo, was sufficient to induce both diffusion of Al and granulomas to draining lymph nodes [92], and the behavioral changes observed in sheep ASIA, including restlessness, aggressiveness, stereotypies, dissociation from the group, and lethargic states (Asin J & Llujan L, personal communication). Whole Al vaccine injections resulted in even more pronounced immunological effects than Al adjuvant alone [92,175]. Both the Al adjuvant alone and the whole vaccine groups showed increased biologic unwellness markers, such as high circulating levels of cortisol, the stress hormone, in winter time.

Pathophysiology of ME/CFS remains poorly understood, but the classical hypothesis that ME/CFS patients may suffer from an inappropriate clearance of either pathogens or toxic compounds with immuno-stimulating effects [41] causing "protracted immune stimulation that fails to switch off" [176] and leading to eventual immune system "burnout" fits well with recent evidence that ME/CFS patients are flush with cytokines until around the three-year mark, at which point the immune system becomes exhausted and cytokine levels drop [177]. Consistently with patients with longstanding ME/CFS studied by Hornig *et al.* [177], MMF patients typically exhibit immune system "burnout" assessed by significant drop of blood IL1b, IL1ra, IL4, IL10, IL12, IL17 and FGFb, at the exception of the major monocyte chemoattractant CCL2 which is selectively increased [101].

It has been demonstrated that, even in the absence of initial CNS inflammation, brain microglia respond to peripheral inflammation by increasing their production of MCP-1/CCL2 which attracts circulating CCR2-expressing monocytes [178]. This monocyte influx drives peripheral inflammatory states-associated sickness behaviour, manifesting by fatigue, mood disorders, cognitive dysfunction and sleep disturbances [179]. In the setting of immunization, MCP1/CCL2 expression is upregulated by Al hydroxide [180], which likely polarizes response to vaccine towards T helper 2 immune responses [181], and favour Al adjuvant-loaded cells incorporation to the brain [99]. Al hydroxide particles elicit inflammation by activating the so-called NALP3 inflammasome [182] and NALP3 inflamasome activation mediates fatigue-like behaviour in mice via neuroinflammation [183]. The hallmark of this activation is the release of IL1beta, which was detected in both brain immune cells and neurons loaded with Al hydroxide particles in our mouse experiments [99]. In the same way, chronic pain syndromes arise from hypersensitization within the dorsal horn of the spinal cord and microglia activated by an adjuvant like CFA administered at the periphery has been shown to initiate hypersensitization through release of IL1b and other inflammatory cytokine [184]. Thus, Al adjuvants that enter the CNS can amplify activation of microglial cells triggered by peripheral inflammation which is known to elicit fatigue and pain. The immune system also plays a pivotal role in modulating learning and memory, and hippocampal synaptic plasticity is particularly sensitive to neuroinflammation [185]. It has been consistently shown that neonatal administration of Al hydroxide-containing HBV vaccine induces a T helper 2 (Th2) immune response in the periphery, while increasing IL-1β, IL-6, and TNF-α in the hippocampus and hampering hippocampal synaptic plasticity, whereas neonatal Bacille Calmette-Guérin (BCG) vaccination induces opposite effects [186]. Of note, Al hydroxide and Al phosphate are strong Th2 adjuvants that can likely act in synergy with known factors of a Th1 to Th2 shift of the adaptive T cell responses, including mental stress, excess sympathetic stimulation, excess glucocorticoids, high female hormones levels, immunosuppression, chronic infection or overwhelming microbial burden [187–189]. Long-term Th2 shift has long been suspected to underpin clinical manifestations of GWI [190], and, consistently, immune activation with a Th2 shift has been documented in the cerebrospinal fluid of ME/CFS patients [191].

7.4. Future directions deserving investigation: innate immune memory and microbiome

7.4.1. Innate immune memory

In almost all MMF patients, ME/CFS manifests after multiple immunizations. The impact of multiple vaccinations on the immune system has been rarely investigated but represents a critical question [3]. Increasing attention is currently paid to memory-like characteristics of innate immune cells, including peripheral monocytes/macrophages and brain microglia, called trained innate immunity [192]. It was long believed that, in contrast to cells of the adaptive immune system, monocytes and macrophages do not have immunological memory, mounting an identical naïve response each time they are stimulated. Recent studies have demonstrated that, in fact, the innate immune system can adopt a long-term activated phenotype by previous encounters with various microbial or vaccine stimuli. Thus upon infection or vaccination, monocytes/macrophages can be functionally

reprogrammed so as to display an enhanced response against unrelated infections [193]. For example, as also described above [186], BCG vaccination prevents tuberculosis but also induces non-specific beneficial effects, against certain forms of malignancy and unrelated pathogens and autophagy plays a key role in these nonspecific effects [194]. Besides the beneficial effects of trained innate memory, however, deleterious effects may well occur through sequential immune stimuli causing microglial priming favouring neurodegeneration [195], or through the induction or maintenance of autoimmune and auto-inflammatory diseases in case of inappropriate activation or individual susceptibility [196]. To our knowledge, this question has not been investigated yet in ME/CFS and ASIA.

7.4.2. Microbiome dysbiosis

Immunosuppression that is typically associated with longstanding ME/CFS, as confirmed in MMF patients (see Section 7.3), makes possible that opportunistic development of as yet unidentified pathogens or, more likely, microbiote dysregulation could contribute to or perpetuate ME/CFS [197]. As stated above (Section 5.1.3), longstanding MMF reflects limitation of cellular disposition of particles by the auto/xenophagy machinery [48]. Individual limitation of auto/xenophagy processing, linked to genetic traits or to aging [198], may impede macrophage clearance of adjuvant particles and increase the inflammatory response but it may similarly affect clearance and immune response to intracellular microbes, as previously documented in intestinal epithelial cells of patients with Crohn's disease and other IBDs linked to microbiome dysbiosis [199–201]. In keeping with this view, patients with MMF often suffer from abdominal discomfort and IBD and their general symptoms may occasionally improve after antibiotic therapy [202]. Both compassionate L-carnitine administration used to stimulate mitochondrial function and dietetic measures with probiotics intake seem to be of some benefit in many cases. The fact that clinical symptoms typically occur after immunization in both humans and sheep suggests that vaccines and their adjuvants, similarly to the different pathogens previously implicated at the origin of ME/CFS cases, may interact with various stressors to trigger cascading events that compromise immunologic, metabolic, neuroendocrine and neuro-vegetative functions and push the body toward a state of illness (see Fig. 1). Possible implication of microbiome dysbiosis in these events is suggested by epipharyngitis documented in Japanese girls with HPV vaccine-induced ME/CFS [124], and by epidemiological evidence that French girls immunized against HPV have a slight but significant increased risk of developing IBD [203]. Of note, Al *per se* [204–205] and mental stress [206] are established factors of chronic intestinal inflammation. Moreover, it has been clearly shown in mice that given microbiome strains are important providers of natural adjuvants necessary to elicit immune response to influenza vaccine [207]. Therefore, it is not excluded that persistent microbiome species could induce the immunological alterations previously reported in ME/CFS patients [197], and search for an imbalance of microbiome communities in ASIA patients with or without biopsy-proven MMF could well prove contributory in the future [208].

8. Post-immunization ME/CFS as a core manifestation of ASIA

Yehuda Shoenfeld had the great merit to coin the concept of ASIA in 2011 [5]. Striking clinical similarities between GWI, MMF syndrome and ME/CFS had been previously reported [4], but Yehuda Shoenfeld extended the concept to the deleterious effects of every compound with adjuvant properties, including pathogens themselves and non-vaccinal adjuvant particles. Microbial adjuvants naturally present in pathogens were among the first vaccine adjuvants, including mycobacterial walls used in Freund's complete adjuvant which immunostimulating molecules are muramyl dipeptide and the tréhalose dimycolate, and gram negative bacteria the endotoxin of which is called lipopolysaccharide (LPS) and its adjuvant derivative monophosphoryl lipid A. Silicone particles represent the main non-vaccinal mineral particles with adjuvant properties [209]. Patients with leaky silicone breast implants develop siliconosis consisting in release of silicone particles that allows them, similarly to microbial or vaccine adjuvant particles, to be taken up by macrophages and transported to lymphoid organs and manifesting by a disease complex similar to ME/CFS [40,210,211].

Yehuda Shoenfeld has admitted that most ASIA patients have ME/CFS [19]. There has been a tendancy, however, to extend the ASIA concept to immune diseases beyond ME/CFS, to include autoimmune diseases of post-vaccinal onset, such as Sjogren syndrome [212], narcolepsy [213], antiphospholipid syndrome [214], and primary ovarian failure [215], as well as lymphoma [216]. It is true that idiopathic ME/CFS (up to around 60 %) may suffer from autoimmune responses [217,218] and that ASIA shares similarities with undifferentiated connective tissue disease [219]. It is also true that ME/CFS is associated with an increased risk of lymphoma [18], and that the dramatic rate of immune disorders observed in italian militaries, including lymphomas, leukemias and autoimmune diseases, has been linked to suboptimal vaccine practices, such as injecting 5 vaccine shots simultaneously [3]. In this setting, the Italian Senate committee has calculated that the cumulated amount of non-antigenic vaccine compounds received by Italian militaries-including Al adjuvants (7.65 mg corresponding to 2.57 mg Al), 44 excipients and 47 contaminants- was always above the official security threshold [3]. However, extending too much the scope of ASIA is at high risk of blurring the core picture. The initially proposed definition of ASIA is probably too loose and, therefore, remains a matter of debate despite the extreme practical usefulness of recognizing that similar clinical presentations may be observed in patients exposed to a variety of immunostimulatory compounds.

Whether post-vaccinal ME/CFS represents an authentic autoimmune disease as suggested by the term ASIA is also still incompletely settled. On the one hand, a number of autoantibodies have been reported in patients with idiopathic POTS and ME/CFS [218]. In a subset of these patients auto-antibodies may be specifically directed against neurotransmitter receptors present in the sympathetic nervous system, including ß2 adrenergic receptors, and muscarinic 3 and 4 acetylcholine receptors, and may likely play a role in clinical manifestations as suggested by immunoabsorption studies [220]. In the same way, prolonged B-cell depletion with anti CD20 rituximab has been associated with sustained clinical responses in a subgroup of patients with idiopathic ME/CFS [221]. On the other hand, we found no mention in the literature of detection of specific anti-neuroceptor auto-antibodies in post-vaccinal ME/CFS and very little in post-vaccinal POTS [222]. Our MMF patients inconstantly presented with low titers of common circulating autoantibodies, mainly antinuclear antibodies that were detected in about 30% of patients, assessing low grade autoimmunity. In addition, a minority of MMF patients (10-20%) had a well-defined concurrent autoimmune disorder (MS, thyroiditis, dermatomyositis, etc) [67]. We do not remember to have seen MMF cases evolving from pure initial ME/CFS to full blown specific autoimmune disease. It is therefore not excluded that specific autoimmunity only occurs in a subset of post-immunization ME/CFS patients, presumably due to either individual susceptibility to develop an autoimmune disease or to a specific, possibly opportunistic, antigen challenge. For example, it has been suggested that persistent microbiome pathogens could induce immunological alterations previously reported in idiopathic ME/CFS patients, including altered NK cell functions, clonal T-cells, and autoantibodies [197]. Todate, the role of specific auto-antibodies against neurotransmitter receptors, though representing a fascinating new issue in ME/CFS, remains elusive in post-immunization ME/CSF.

Nevertheless, the ASIA concept has gained growing popularity in human and veterinary medical communities, with more than 4000 reported cases in the literature [223], pointing out that a critical need has been met by ASIA in routine practice of human and veterinary medicine [173,223].

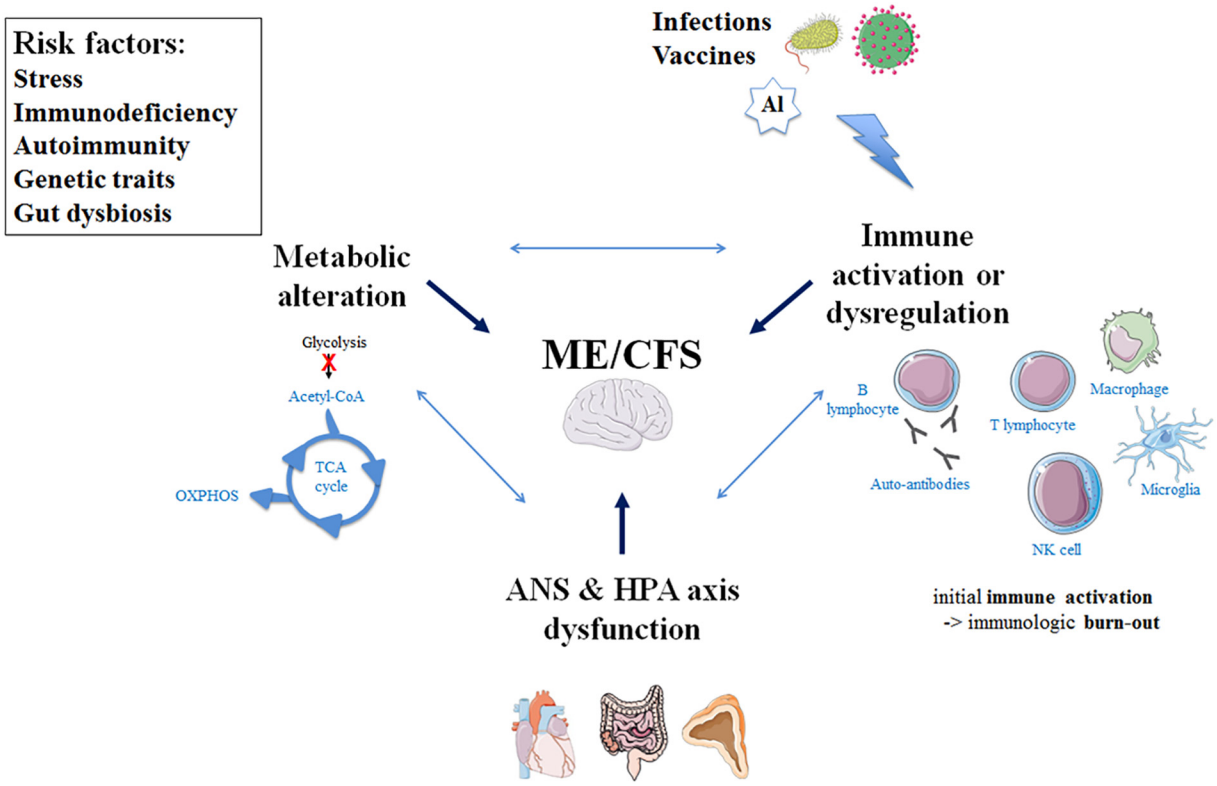

Fig. 1. Schematic representation of the ME/CFS pathophysiology following challenge with infectious or adjuvanted vaccine particles (redrawn from Sotzny *et al.*, 2018 [207]).
Aluminum adjuvant-containing vaccines, similarly to persistent natural pathogens, can induce immune system dysregulation and metabolic, neuroendocrine and autonomic nervous system disturbances at the origin of ME/CFS symptoms. Contribution of a variety of risk factors is likely.
Abbreviations: Acetyl-CoA: acetyl coenzyme A, ANS: autonomic nervous system, ME/CFS: Myalgic Encephalomyelitis/Chronic Fatigue Syndrome, NK: natural killer, OXPHOS: oxidative phosphorylation, TCA: tricarboxylic acid.
Drawings by Servier medical art® https://smart.servier.com/

9. A tentative pathophysiological model

Post-infectious fatigue is recognized to occur in about one in ten people infected with Epstein-Barr virus or *Coxiella burnetti*, the causative agent of Q fever, and in a number of patients infected by enteroviruses, *Borrelia burddorferi*, and other infectious agents. Long-term persistency of the infectious agent has been repeatedly shown to cause prolonged immune activation and ME/CFS-like syndrome [224–227]. Both human MMF [10] and relevant small and large animal models [92,99,154,155,173] indicate that the same holds true for ME/CFS following administration of Al adjuvants that persist unusually long within immune cells throughout the immune system.

ME/CFS has an extremely complex pathophysiology affecting multiple systems. The reader is referred to excellent extensive papers on each of the impacted systems [228–231] and on their interplays [232]. Basically ME/CFS is associated with: (1) immune system abnormalities including impaired natural killer cell function and/or T cell function, increased and then decreased production of inflammatory cytokines [177], and occasional increase in some autoantibodies [218]; (2) cellular metabolism abnormalities with impaired ability to produce energy from oxygen, glucose, fatty acids, and amino acids, associated with mitochondrial dysfunction and reduced oxidative metabolism; these changes cause abnormal response to exercise and mimick an hibernation state [233]; (3) neuroendocrine and neurovegetative disturbances including dysregulation of the hypothalamic-pituitary-adrenal axis (HPA axis) and, particularly in adolescents, orthostatic intolerance with blood pressure or heart rate regulation abnormalities.

Fig. 1, redrawn from Sotzny *et al.* 2018 [218], summarizes the differents changes driven by immune dysregulation that may be caused by vaccine or natural adjuvants in susceptible individuals and likely form the core ASIA pathophysiology (Fig. 1).

10. Conclusion

Adjuvant safety is an “important and neglected field” [234], suffering from both misconception of Al adjuvant toxicokinetics [7] and lack of population-based studies evaluating associations between exposure to Al adjuvants and clinical outcomes [235].

ME/CFS is a multifactorial condition of major public health and clinical importance. Evidence that ME/CFS may represent an important type of AEFI has emerged very slowly due to the multiplicity, apparent lack of specificity, delayed onset, and frequent medical underestimation of symptoms, all characteristics ranging among the main explanations for the “inherent methodological limitations of epidemiological studies” in the field of vaccine safety (see above Section 4.2.). Fortunately, however, a well-conducted epidemiological study comparing vaccinated vs unvaccinated individuals has provided strong evidence of post-immunization ME/CFS [53]. In depth clinical analysis of patients with post-immunization ME/CFS has revealed highly consistent cognitive and functional neuroimaging alterations. Biologic plausibility of an association between particulate adjuvant administration and ME/CFS was supported by long-term Al adjuvant persistency in immune cells of affected individuals (assessed by MMF detection at muscle biopsy), and by Al adjuvant transportation to distant organs documented in small and large animal models, with long-standing immunostimulating and low dose neurotoxic effects.

These data, fitting the ASIA concept, have already grounded right to compensation for damages in USA and France where the highest

administrative court ruled compensation for 8 of our patients that had received mandatory vaccination for professional reasons. We hope they will trigger solid additional epidemiologic and basic research studies on long-term Al adjuvant fate and toxicity, individual susceptibility factors, and satisfactory alternatives to Al adjuvants. Several efficient and biodegradable adjuvants devoid of noxious metals have been already identified [236–238].

Competing interests statement

This review paper compiles previous results from our INSERM group obtained with the help of public sources of funding (Région Ile-de-France, Agence Nationale de Sécurité du Médicament) and from patients associations and charities including Association Française contre les Myopathies (AFM), Entraide aux Malades de Myofasciite à Macrophages (E3M) and Children's Medical Safety Research Institute (CMSRI/Dwoskin Foundation). Neither AFM nor E3M nor CMSRI played any role in the design, data analysis, interpretation of results and writing of this or any other papers from our group. None of the authors received payment from these non-profit organizations. Romain Gherardi and François-Jérôme Authier have occasionally done expert testimony and have deposited one patent relevant to the field of vaccine safety.

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