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Neuroinflammation in acute and chronic brain injury: Cerebellar Hemorrhage and Alzheimer's Disease

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ABSTRACT (ENGLISH)

Neuroinflammation has been known as a weapon of defense against any type of injury in the Central Nervous System, comprising both acute and chronic inflammatory conditions. Increasing evidence suggests that inflammation is linked not only with acute direct neurological injury but also chronic neurodegenerative diseases. Nevertheless, its exact role and the ways it exerts functions are poorly understood in different brain disorders. In this thesis, we took collagenase microinjection induced cerebellar hemorrhage mice as an acute brain injury model to investigate acute neuroinflammatory response. Depending on molecular and behavioral tests, resident innate immune cells, i.e. microglia, were found to be activated and to play a detrimental role after bleeding, initiated through the release of pro-inflammatory cytokine and chemokine C-C Motif Chemokine Ligand 2 (CCL2), following by recruitment of peripheral circulating monocytes and macrophages into the brain lesion site. Alzheimer's disease (AD) is viewed as a chronic brain injury model, along with persistent immune responses in aging brains. Chronic and recurrent infections could explain this inflammatory state. We identified a relationship between infection and AD in a systematic review and meta-analysis of observational studies in AD patients. We found that the presence of Herpesviridae, its alpha subfamily, and gram negative bacteria, particularly Herpes Simplex Virus Type 1 (HSV-1), Epstein-Barr virus (EBV) and Chlamydia pneumoniae (*Cpn*), increased the risk of AD. When the herpes virus infected host who also carried the allele ε4 of apolipoprotein E4 (*APOE* ε4), risk due to infection was further increased. Taken together, the protective effects of neuroinflammation can become harmful, resulting in significant injury in different brain diseases. A deeper understanding of the mechanisms of neuroinflammation in acute and chronic brain injury may help us better understand the stage-dependent role of the immune response and thus to develop better therapeutic strategies for our patients.

ZUSAMMENFASSUNG (DEUTSCH)

Neuroinflammation ist als Verteidigungswaffe gegen jede Art von Verletzung im ZNS bekannt, die bei einer Reihe von akuten oder chronischen Entzündungszuständen eingreift. Immer mehr Hinweise deuten darauf hin, dass Inflammation nicht nur mit akuten neurologischen Verletzungen, sondern auch mit chronischen neurodegenerativen Erkrankungen in Verbindung steht. Die genaue Rolle der Inflammation und und die Art und Weise, wie sie ihre Funktionen ausübt, sind jedoch bei verschiedenen Hirnverletzungen kaum verstanden. In dieser Arbeit verwendeten wir Kollagenase-Mikroinjektions-induzierte Kleinhirnblutung-Mäuse als Modell für akute Hirnverletzungen, um die akute neuroinflammatorische Reaktion zu untersuchen. In Abhängigkeit von molekularen und Verhaltenstests wurde festgestellt, dass residente angeborene Immunzellen, sog. Mikroglia, aktiviert werden und nach Blutungen eine schädliche Rolle spielen, indem sie eine übermäßige Freisetzung des proinflammatorischen Zytokins und Chemokins CCL2 einleiten und anschließend periphere zirkulierende Monozyten und Makrophagen in die Hirnläsionsstelle rekrutieren. Die Alzheimer-Krankheit wird als ein Modell für chronische Hirnverletzungen angesehen, unter Beteiligung anhaltender Immunreaktionen in alternden Gehirnen. Chronische und wiederkehrende Infektionen könnten diesen Entzündungszustand erklären. Wir identifizierten einen Zusammenhang zwischen Infektion und Alzheimer durch eine systamtische Literaturübersicht und Meta-Analyse in Observationsstudien an Patienten mit Alzeimer. Das Vorhandensein von Herpesviren sowie der Alpha-Unterfamilie gramnegativer Bakterien erhöhte das Alzheimer-Risiko, insbesondere HSV-1, EBV und *Cpn*. Wenn der mit dem Herpesvirus infizierte Wirt auch Träger von *APOE* ε4 war, war das Alzheimer-Risiko noch starker erhöht. Wenn die neuroinflammatorischen Reaktionen überschießen, ändert sich anscheinend die Schutzwirkung zu einer schädlichen Wirkung, was zu nicht zu vernachlässigenden Schäden und manifesten Krankheiten des ZNS führt. Die eingehende Erforschung der Neuroinflammation bei akuten und chronischen Hirnverletzungen hilft uns, Immunreaktionen zu verschiedenen Zeitpunkten im ZNS besser zu verstehen und eine optimale therapeutische Strategie für entsprechende Patienten bereitzustellen.

LIST OF ABBREVIATIONS

Aβ	Amyloid β
AD	Alzheimer's disease
APOE	apolipoprotein
CCL2	C-C Motif Chemokine Ligand 2
CCR2	C-C Motif Chemokine Receptor 2
CH	cerebellar hemorrhage
CI	confidence interval
CMA	Comprehensive Meta-Analysis
CMV	Cytomegalovirus
CNS	central nervous system
Cpn	*Chlamydia pneumoniae*
CRP	C-reactive protein
CSF-1R	colony stimulating factor-1 receptor
EBV	Epstein–Barr virus
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
G-CSF	colony stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
HHV6	Human Herpes Virus Type 6
Hp	*Helicobacter pylori*
HSV-1	Herpes Simplex Virus Type 1
ICH	intracerebral hemorrhage
IFN-γ	interferon gamma
i.g.	intragastric gavage
IL-1α	interleukin-1α
IL-1β	interleukin-1β
IL-2	interleukin-2

IL-4	interleukin-4
IL-6	interleukin-6
IL-10	interleukin-10
IL-12	interleukin-12
i-NOS	inducible nitric oxide synthase
i.p.	intraperitoneal administration
JHBRC	Johns Hopkins Brain Resource Center
LPS	lipopolysaccharide
MSBB	Mount Sinai Brain Bank
NSAIDs	non-steroidal anti-inflammatory drugs
OR	odds ratio
PBS	Phosphate-buffered saline
PBST	PBS containing 0.1% Triton X-100
PCR	Polymerase Chain Reaction
qPCR	Quantitative Real-time RT-PCR
PRISMA	Preferred Reporting Items for Systematic Review and Meta-Analysis
PRISMA-P	Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols
RCT	Randomized Controlled Trial
RevMan	Review Manager
ROSMAP	Religious Orders Study, Memory and Aging Project
SCA1	Spinocerebellar Ataxia Type 1
SE	standard error
SND	standard normal deviation
TGF-β	transforming growth factor-β
TNF-α	Tumor Necrosis Factor-α
VZV	varicella zoster virus
WBC	white blood cell

GENERAL INTRODUCTION

Neuroinflammation is an ongoing hot topic among generations of scientists. When inflammatory reactions occur within the brain and spinal cord, “neuroinflammation” is generally adopted [1]. In the past, researchers did not use this term to describe the immune regulations in the Central Nervous System (CNS), instead they used the term “reactive gliosis”. This referred to the increased number of glia cells that were found as soon as the CNS suffered internal or external injuries [2]. However, as the understanding of this complicated inflammatory process deepened, much more than the actions of glia cells found involved, “neuroinflammation” is currently widely used.

Inflammation has been known as the body’s weapon against every sort of stimuli, which is also the same in the CNS. An appropriate immune reaction promotes tissue repair while alleviating structural and functional damages. However, if the inflammatory response becomes improper, a detrimental effect will replace the protective role, causing non-negligible injury or even disease [3]. Thus, to explore the neuroinflammation in different situations and to regulate relative immune responses as optional therapeutic strategies are essential healthcare issues.

Neuroinflammation is regarded to participate in a series of acute or chronic inflammatory conditions in the CNS. A growing body of evidence suggests it is associated with acute and direct local injury, for instance the hemorrhagic stroke and traumatic brain injury, while links with chronic neurodegenerative diseases, such as Alzheimer’s disease and Parkinson’s disease, also strongly exist [4]. Cerebellar hemorrhage (CH) is one of hemorrhagic stroke, which is a fatal and urgent disease, with a considerable rate of lifelong disability, without dependable treatments. The acute neuroinflammatory reaction gets involved in the secondary brain injury and subsequent recovery following hematoma formation, contributing to the functional outcomes [5]. However, its exact effect on disease manifestation and progression is poorly understood. On the other hand, the hypothesis that chronic neuroinflammation is connected with the onset of aging-related cognitive impairment, especially Alzheimer’s disease (AD) [6], which is the most prevalent dementia manifesting with progressive spatio-temporal disorientation, memory loss and decline in the ability to live independently, has been proposed

in the past years. The persistent neuroimmune responses result in plaques deposition and synaptic dysfunction in aging brains [3]. Intriguingly, the abnormal neuropathological structures, senile plaques and neurofibrillary tangles, were found not only in AD but also in some neurological impairment with chronic infections [7]. The robust and repeatedly reported relationship between chronic neuroinflammation with long-term infectious stimuli and Alzheimer's disease has not been well established.

Hence, in the thesis, I investigated the mechanisms of acute neuroinflammatory response in CH using collagenase microinjected cerebellum hemorrhagic mice models, and also explored the relationship between chronic infection and AD through a systematic literature review and meta-analysis in AD patients, aiming to better understand the role of neuroinflammation in acute and chronic brain disorders.

Part 1. Neuroinflammation in acute brain injury: Cerebellar hemorrhage

1. INTRODUCTION

1.1. CEREBELLAR HEMORRHAGE

Spontaneous bleeding that suddenly occurs in the brain is a catastrophic medical event. As the Global Burden of Diseases, Injuries, and Risk Factors Study estimated, including 119 studies from different low, middle, and high-income countries, the health burden of brain bleeding shows a growing trend, rising by 47% within two decades from the 1990s [8, 9]. Of them, cerebellar hemorrhage is one of the most dangerous types. It affects about 10% of all the hemorrhagic strokes, with a high incidence, around 50 per 100,000 in elderly populations develop CH [10]. In addition, American researchers have calculated that there are approximately 10,000 new patients suffering CH every year in their country [11]. The more common age of onset is around 50 years old, with no sex difference [12, 13]. In view of etiology, hypertension is the major risk factor for hemorrhages in cerebellum, so controlling blood pressure within the appropriate range is an effective and preventative measure for CH [13]. Clinical symptoms of cerebellar hemorrhage are always sudden at the beginning of the attack, especially posterior headache. It is frequently regarded as an onset symptom [14]. Other symptoms include vertigo, dysmetria and hypotonia. If the patients have larger cerebellar hematoma, they are more likely to enter a coma [12]. In addition, more than 66.7% of the patients have ataxia and even lifelong motor deficits, because cerebellum is known as the subcortical motor structure [15]. Because of the specificity of cerebellar location, any expansion in the narrow posterior fossa would influence nearby structures of tissues, even extending into the fourth ventricle [12]. Moreover, we lack data of sufficiently large sample size and comparable clinical trials to satisfactorily analyze the prognosis of CH patients receiving surgical intervention [16]. For a long time since hematoma evacuation was firstly reported for CH in 1906 [16], cerebellar hematoma was considered to be a surgical lesion [11]. However, one meta-analysis that involved 6,580 patients with CH treated in America and Germany, indicated patients that experienced surgical intervention did not have significant beneficial effects on functional outcomes, when compared with patients receiving conservative treatment

[17]. The majority of patients cannot have complete functional recovery and still need long-term care on account of disability [18].

Hence, CH is not only viewed as a public health issue but also a great social and economic burden for human beings.

1.2. ACUTE NEUROINFLAMMATION AND CH

Brain injury following intracerebral hemorrhage (ICH) generally contains two parts, primary and secondary brain injury. The initial bleeding rapidly causes hematoma formation, and disrupts the patients' brain architecture, that is called primary brain injury. As we described above and some other studies reported, functional recovery is not optimal when people focused on treating primary brain injury after ICH with initial surgical hematoma evacuation [19, 20]. Two Randomized Clinical Trials targeting multiple sclerosis and traumatic brain injury suggested inhibition of inflammatory responses provided a beneficial effect on functional recovery [21, 22]. The clinical evidence makes us realize the possibility of success, mediating inflammation may improve neurological diseases. In hemorrhagic stroke, neuroinflammation has been proven to play a crucial role in secondary brain injury and occurs immediately following hematoma formation [23]. Acute neuroinflammation after ICH contains a cascade of events, activation of microglia and relative inflammatory signaling pathways, releasing of cytokines, chemokines and toxic chemicals, infiltration of leukocytes into the brain [24]. Microglia is widely accepted to be the earliest reactive inflammatory cell after hemorrhagic stroke. Resident microglia are activated by various stimuli depending on distinct receptors and contribute to the release of cytokines, chemokines, ferrous iron, and so forth [25]. Some clinicians retrospectively analyzed admitted ICH patients, and they found that the initial number of peripheral white blood cells (WBC >10,000/mL3) was significantly associated with more serious early neurological deterioration within 72 hours [26]. Moreover, the more apparent changes in initial WBC count also predicted a worse degree of functional recovery at 3 months following ICH [27]. Thus, the acute inflammatory events exacerbate brain injury and further lead to poor prognosis post ICH.

To modulate the hematoma induced inflammatory process may bring a new therapeutic target with longer treatment time window. However, the mechanism underlying CH remains uncertain until now.

In order to explore CH, we need to establish a mouse model better reflecting the actual clinical situation in humans. There are two most commonly used models by researchers: induction with autologous blood or bacterial collagenase, of which infusion of collagenase makes blood vessels burst and blood leaks out. Regarding operation, autologous blood injection sometimes cannot mimic the lesion as we expected, because a large volume of blood easily flows back into the needle. To avoid this issue, researchers tend to perform two or more injections, which makes the bleeding look like a recurrent attack [28]. Whereas collagenase infusion is convenient for dose change and is well localized [29]. As for the degree of simulating the human condition, the hematoma in the bacterial collagenase induced hemorrhagic model generates spontaneously, expands gradually, maintains tissue loss and presents neurobehavioral deficit that are similar to clinical settings [30, 31]. On the other hand, bacterial collagenase operations also bring about a similar inflammation condition around the injured site, including microglia is activated within 1 hour, pro-inflammatory cytokines: Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α) were significantly enhanced within 72 hours [25, 32-34]. Moreover, the effective and predictive collagenase induced cerebellar hemorrhage model, which was successfully established in rodent, can also be used as a reference, especially their operative procedure [35].

We therefore applied bacterial collagenase microinjected mouse model to investigate the acute neuroinflammation of CH.

1.3. MICROGLIA

Resident microglia can be activated by different brain injuries. After cerebral bleeding, microglia have been reported to be the fastest reacting immune cell type in the acute neuroinflammation [36]. They play vital roles in the inflammatory response and exhibit

biphasic functions after ICH. In some cases, microglia take responsibility to remove cell debris, damaged neurons and red blood cells, clean up hematoma following hemorrhage, releasing anti-inflammatory cytokines, such as Interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), so as to protect nervous system and promote recovery [37]. While they also probably polarize into pro-inflammatory phenotype, becoming the primary source of cytotoxic cytokines, such as TNF-α, IL-1β, IL-6, C-X-C Motif Chemokine Ligand 2, proteases and ferrous iron, infiltrating leukocytes into the brain, which contribute to aggravate neuroinflammation [38]. The exact roles of reactive microglia may alter depending on the time, stimuli and surrounding environment.

Notably, microglia have spatial difference in the brain. Compared with microglial density of other brain regions, whose mean value is 70 cells/mm2, microglia in the cerebellum have less than half (30 cells/mm2). Of them, gray matter of the deep cerebellar nuclei is the most microglia accumulated region [39]. Considering the sparse distribution of microglia in the cerebellum, it seems to become the most vulnerable brain region. However, the results about transcriptome of cerebellar microglia suggested immune alertness associated genes were more expressed in them, indicating that they were in a hyper-vigilant state [40], possibly owing to the longer developmental time of the cerebellum, beginning in embryos and continuing throughout the first year after birth. During this developmental period, cerebellar microglia may fall into a habit, to be more particularly high in reaction to the changing environment [41]. Recently, the characteristics of cerebellar microglia have been proven to contribute to loss of Purkinje cells with aging and thereby increase susceptibility to ataxia [41]. Nevertheless, we cannot simply conclude cerebellar microglia are more sensitive or dull to extrinsic and intrinsic threatening due to the cerebellum having the lowest microglial density with unique immune-alert state.

The exact role of microglia in the acute neuroinflammation after CH has not yet been defined before. Thus, we firstly explore reactive cerebellar microglia and relative inflammatory responses in our study, and expect to provide new insights into the therapeutic strategy.

2. MATERIALS AND METHODS

2.1. EXPERIMENTAL ANIMALS

We purchased C57BL/6J male mice (weeks 8-10) from Experimental Animal Center, Nanjing Medical University, Nanjing, China. They were carefully raised under constant temperature (24 ± 2 °C) and humidity (60 ± 5%) environment. Then in order to maintain their internal clocks, the lights were turned on at 8:00 am and turned off at 8:00 pm every day regularly. All the groups of mice were allowed to get food and water without any restrictions. Concerning the conservation of experimental animals, we followed the Guide for the Care and Use of Laboratory Animals that issued by National Institutes of Health, United States (Eighth Edition) to design, conduct experiments as well as report data. Our experiments with mice were approved by the Animal Ethical and Welfare Committee of Nanjing University.

2.2. ESTABLISHMENT OF CEREBELLAR HEMORRHAGE MOUSE MODEL

During surgical operations, the body temperature of mice was maintained at 37°C ± 0.5°C with a heating pad. The male C57BL/6 mice were firstly anesthetized with sodium pentobarbital (50 mg/kg) and the head fixed on a stereotaxic frame (Kopf Instruments, Model 962, Tujunga, CA) in prone position. We carefully removed hair from the head and neck, sterilizing with 75% alcohol. Along the midline of the head, the scalp was cut from the front to the back by a scalpel. The extra tissue and blood were wiped by cotton balls. According to the accepted CH injury model [35] and mouse brain atlas [42], we drilled a 1mm borehole on the skull, and collagenase was infused into the cerebellar right paramedian white matter, the stereotaxic coordinates were A -6.24mm; L 1.5mm; H 2.7mm. With a syringe pump (Harvard Apparatus, Holliston, MA), the 0.01 unit collagenase (Type IV-S, Sigma, St Louis, MO) dissolved in 0.2 μL saline was microinjected at a flow rate of 0.1 μL/min. When the injection was finished, we still made the needle stay put for 10 min, in order to prevent leakage. After the operations, the borehole was closed with bone wax and the incision was sutured and disinfected.

For proper postoperative rehabilitation, the animals were placed separately to recover from the surgery and had free access to food and water after waking up. Mice in the Sham group received

an equal volume of saline alone. If the lesion area was not limited to cerebellum, we excluded the relative data for further analysis.

2.3. DRUG TREATMENT

All the drugs were obtained from MedChemExpress (Monmouth Junction, NJ).

Ki20227 (CAS No. 623142-96-1) was used to deplete microglia. It was dissolved in DMSO and diluted with corn oil, and given by intragastric gavage (i.g.). The dosage of medicine was 20mg/kg/day. After 14 days of continuous administration, a collagenase-induced CH model was prepared [43].

Minocycline (CAS No. 13614-98-7) was used to inhibit activation of microglia. Phosphate-buffered saline (PBS) was the solvent. Mice received 50mg/kg/day of minocycline by intraperitoneal administration (i.p.) when the CH model was successfully induced [44].

Bindarit (CAS No. 130641-38-2) was used to block CCL-2 synthesis. DMSO was the solvent and diluted with corn oil. After CH induction, it was given by i.g. with 40mg/kg/day [45].

In control groups, each drug was replaced by an equal volume of vehicle (10% DMSO in corn oil or PBS). The drugs were treated until the end of experiments, 3 days after CH induction.

2.4. HISTOLOGICAL STAINING

The experimental mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg), followed by thoracotomy and perfusion of 10 ml cold PBS from the left ventricle, subsequently slowly infusing 4% paraformaldehyde. After fixation, the skull of each mouse was carefully and quickly removed to get the cerebellum. It was then put into 4% paraformaldehyde PBS solution 12h for post-fixation at 4 ℃. The fixed tissues were successively dehydrated in 20% and 30% sucrose buffer at 4 ℃ for 48 h on average. Cerebellar tissue was embedded in O.C.T. and frozen at -20 ℃ for coronal sections (CM 3050S, Leica, Wetzlar, Germany).

In order to determine the size of brain injury, with help of the brain atlas, the targeted sections containing hematoma were cut with a thickness of 50μm and pasted on gelatin-coated slides. Slides of each mouse were obtained with a 200μm interval within the injured area and stained

with cresyl violet and luxol fast blue. The injury size of each section was measured by Image J (U.S. National Institutes of Health, Washington, DC). The total volume of lesion was calculated as the sum of the injured areas (all sections) × interval [46].

Regarding the assessment of microglial activation, the selected brain slices of 25μm thickness were washed with PBS containing 0.1% Triton X-100 (PBST) for 3 times, 5min each. And then they were incubated in PBST solution with 10% bovine serum (Millipore, Temecula, CA, USA) for 30min at room temperature. After washing, rabbit anti-Iba-1(1:500, Wako, Richmond, VA, Cat# 016–20001) was used to stain overnight at 4°C. Then PBST mixed with related secondary antibody (1:2000; Invitrogen, San Diego, CA) incubated slices at room temperature for 2h in a dark environment. Finally, slices were sealed in mounting medium with DAPI (Santa Cruz Biotech, Dallas, TX). Similar procedures were conducted in evaluation of CCL-2 expression. The primary antibody was goat anti-mCCL-2 (AF-479, 1:250; R&D Systems Minneapolis, MN, Cat# AF-479-NA).

Next is measurement of Purkinje cell degeneration, the brain slices were incubated with primary antibodies: mouse anti-caspase-3 (sc-56053, 1:250; Santa Cruz Biotech, Dallas, TX, Cat# sc-56053) and rabbit anti-calbindin (ProteinTech Group, Chicago, IL, Cat# 14479–1- AP). Purkinje cell linear density was calculated as the number of calbindin-labeled Purkinje cells ÷ the length of Purkinje cell layer, and analyzed by Image J software. In each group, there were 6-8 mice, and the final value of each mouse was the average of 5-8 brain sections [47].

The control group was incubated without a primary antibody, and the other steps remained consistent. High-resolution fluorescent images were observed and captured by an inverted laser confocal microscope TCS SP8 (Leica, Wetzlar, Germany).

2.5. WESTERN BLOT

The male mice were anesthetized as previously described. The tissues, including cerebrum, cerebellum and brainstem were homogenized in precooled lysis buffer (4 °C), which contains 50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM EDTA, 10 mM sodium fluoride and protease inhibitors. Adding by weight/volume ratio of 1:9. The brain tissue homogenates were centrifuged at a

speed of 12000rpm for 15min (4 ℃). Cellular debris became a pellet in each tube, we collected its supernatant as our tested sample. Pierce 660nm Protein Assay（Thermo Fisher Scientific, Waltham, MA）was applied to detect protein concentration. The proper concentration of each sample was obtained by lysis buffer dilution.

Equal amounts of protein (50 μg) were separated by 10% SDS polyacrylamide gel electrophoresis after lysates were boiled in loading buffer for 10 min to fully denaturation. Then making the electrodes correctly connected, the proteins were transferred to PVDF membranes (Millipore, Burlington, MA). For the purpose of reducing nonspecific binding, the immunoblots were blocked with 5 % skim milk powder that dissolved in TBS (tris buffered saline) for 60min. Then incubated with primary antibodies: rabbit anti-Iba-1 (1:500, Wako, Richmond, VA, Cat# 016–20001), mouse anti-CCL-2 (1:1000, ProteinTech Group, Chicago, IL, Cat# 66272-1-Ig) and mouse anti-β- actin (1:5000, Sigma, St Louis, MO, Cat# A1978), overnight at 4°C, of which β-actin was regarded as a reference. Washing the membranes 3 times with TBST (TBS containing 0.2% Tween-20). Primary antibodies were further labeled with corresponding HRP-conjugated secondary antibodies (1:1000). The Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA) was used for developing and fixing. The immunoreactive bands were visualized and exposed to Kodak medical x-ray film (Denville Scientific Inc., Metuchen, NJ). Semi-quantitative analysis of gray value, as for the optical densities of protein bands, was carried out by Image J software.

2.6. BRAIN WATER CONTENT EVALUATION

The measurement of water content in brain tissues reflected the degree of encephaledema. On day 3 post CH, the skulls of mice were immediately stripped for cerebrum and cerebellum isolations after euthanasia. Then placed each isolated brain section in 1.5ml EP tubes that had been measured in advance, and weighed the wet weight of the brain tissues by a microanalytical balance (ModelAE 100, Mettler Instrument Co., USA). Subsequently drying the wet samples at 100 °C for 24h in an oven, with opened lids, in order to obtain the dry weight. The values of exact wet and dry weight were obtained by minus tube weight. Brain water content was calculated as (wet weight–dry weight) ÷ wet weight × 100 [48].

2.7. QUANTITATIVE REAL-TIME RT-PCR

The cerebellum was gently isolated after cold PBS transcardial perfusion of mice.

Total RNA was extracted by using Trizol reagent (Vazyme, Nanjing, China). Adding chloroform (1/5 volume of Trizol) into the homogenized sample. Following the incubation, then the mixture was centrifuged at 12000 g for 15min at a low temperature of 4°C. Carefully transfer the upper aqueous phase into the new tubes. Depending on the calculated pipetting last step, the cold equal volume of isopropanol was added in each tube. Mix gently without any form of vortex. Washing the translucent gel-like pellet once with 75% ethanol. At last, resuspending the RNA pellet with 30-50μL nuclease-free water. The absorbance value of OD 260/280 and the RNA concentration of the sample were determined using a nucleic acid concentration analyzer (Nanodrop, Thermo Fisher, USA).

We transcribed RNA into cDNA using the Hifair® II 1st Strand cDNA Synthesis SuperMix (Yeasen, Shanghai, China). In the first step, the residual DNA genome was removed with reagents as follows in Table(1), incubate the mixture at 42°C for 2 min. The second step is to prepare reaction solution with the mixture in first step, set appropriate temperature and time for reverse transcription Table(2).

Table(1). Components of mixture for DNA removal.

Component	**Amount of usage**
Rnase free ddH2O	To 10ul
5×gDNA digester Buffer	2 μL
gDNA digester	1 μL
Template RNA	1 ng-500 ng

Table(2). Reverse Transcription.

A. Reaction System

Component	**Amount of usage**
reaction mixture in first step	10 μL
2×Hifair® II SuperMix plus	10 μL

B. Procedure Setting

Temperature	Time
25℃	5 min
42℃	30 min
85℃	5 min

Then mRNAs of Interleukin-1α (IL-1α), IL-1β, Interleukin-2 (IL-2), Interleukin-4 (IL-4), IL-6, IL-10, Interleukin-12 (IL-12)(P35), IL-12(P40), TNF-α, inducible nitric oxide synthase (i-NOS), interferon gamma (IFN-γ), colony stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF) and CCL-2 were detected with Hieff UNICON® qPCR SYBR Green Master Mix (Yeasen, Shanghai, China). The primers we used are shown in Table(3). Quantitative Real-time RT-PCR (qPCR) was performed in LightCycler480 real-time RT-PCR platform (Roche, Basel, Switzerland) [49].

Table(3) [50]. Primers that used in Quantitative Real-time RT-PCR (qPCR).

Gene	**Primer (Forward)**	**Primer (Reverse)**
Product		
IL-1α	CGCTTGAGTCGGCAAAGAAAT	CTTCCCGTTGCTTGACGTTG
IL-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
IL-2	CGGCATGTTCTGGATTT	AGGTACATAGTTATTGAGGGC
IL-4	GCTAGTTGTCATCCTGCTCTTC	GGCGTCCCTTCTCCTGTG
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IL-10	TTTAAGGGTTACTTGGGTTGCC	CCGCATCCTGAGGGTCTTC
IL-12/p35	TAACTAATGGGAGTTGCCTGGCCT	AGGGCCTGCATCAGCTCATCAATA
IL-12/p40	AGGGCCTGCATCAGCTCATCAATA	TTTCTCTCTTGCTCTTGCCCTGGA
TNF-α	TGTGCTCAGAGCTTTCAACAA	CTTGATGGTGGTGCATGAGA
IFN-γ	ACTAGGCAGCCAACCTAAGCAAGA	CATCAGGGTCACCTGACACATTCA
i-NOS	ACATGCAGAATGAGTACCGG	TCAACATCTCCTGGTGGAAC
G-CSF	ATGGCTCAACTTTCTGCCCAG	CTGACAGTGACCAGGGGAAC
GM-CSF	GGCCTTGGAAGCATGTAGAGG	GGAGAACTCGTTAGAGACGACTT
CCL2	TCTGTGCTGACCCCAAGAAGG	TGGTTGTGGAAAAGGTAGTGGAT
Gapdh	AACTTTGGCATTGTGGAAGGGCTCA	TTGGCAGCACCAGTGGATGCAGGGA

Interleukin-1α (IL-1α), IL-1β, Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (i-NOS), interferon gamma (IFN-γ), colony stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), C-C Motif Chemokine Ligand 2 (CCL2).

A single peak in melting curve was the symbol of specificity of each reaction. $Ct_{\text{target gene}} - Ct_{\text{reference gene}} = \triangle Ct$, $\triangle Ct_{\text{treatment}} - \triangle Ct_{\text{control}} = \triangle\triangle Ct$, of which the reference gene was GAPDH. The relative levels of target genes were analyzed by the $2^{-\triangle\triangle Ct}$ method [51].

2.8. ENZYME LINKED IMMUNOSORBENT ASSAY

The protein expression of TNF-α, IL-6, IL-1β and CCL-2 in brain homogenates was analyzed by sensitive sandwich Enzyme linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA) [52-54]. Initially, we incubated the 96 wells plate overnight at 4°C with each capture antibody, in order to coat the target protein in the wells. After blocking and washing, standard curves were made with standards that we have already known their concentration. Adding 100 μL/well of samples to the wells we needed. To seal the plate and incubate with the detection antibody and then the Avidin-HRP at room temperature for 1h and 30 minutes, respectively. Repeat 5-7 times washing, we added 100 μL/well of 1X TMB Solution to each tested well. After 15 minutes, a stop solution was used to terminate the reaction. Shaking was carried out for all incubation steps, so as to obtain more precise O.D. values. Finally, we read the plate at 450nm, 570nm (calibrated wavelength) and analyze data.

2.9. FLOW CYTOMETRY

At the beginning of the flow cytometry analysis, we need to obtain single-cell suspensions [48, 55]. In our experiments, they were prepared from the cerebrum, cerebellum and brainstem. We cut brain tissues into pieces with ophthalmic scissors. To avoid damage from the heat of mechanical friction, all the cutting steps were conducted on the ice and precooled solutions. The digestive process was performed in 1 mg/ml collagenase IV (Sigma, St. Louis, MO) Hank's balanced salt solution in a 37°C water bath for 1h. Then we collected the fluid that passed through a 40μm wire mesh (Sigma-Aldrich). For analyzing immune cells infiltration, after centrifuging at 300 g for 5 min, the pellet was re-suspended in 30% percoll (Sigma, St. Louis, MO) and 70% percoll was gently dropped at the bottom of tube with a needle. Another centrifugation without brake at 700 g for 30 min was performed to collect mononuclear cells, which were in the interfaces of previous density gradient. Counting the cells by a

hemocytometer. Next, we diluted 1*10^6 cells/100ul in each tube. Cells were stained for various surface markers that we purchased from BD Bioscience, Inc (San Jose, CA) or Biolegend, Inc (San Diego, CA) as follows: CD3 (17A2), NK1.1 (PK136), CD8 (53–6.72), CD45 (30-F11), CD11b (M1/70), CD4 (GK1.4), F4/80 (BM8), Ly6G (1A8), Ly6C (HK1.4), CD192 (SA203G11). In each experiment, isotype was regarded as a negative control, while 7-AAD was used to distinguish live or dead cells.

For analyzing microglia cytokine expression, intracellular staining, tested cells were collected by re-suspending in 30% percoll. Firstly the membrane markers were stained. Then cells were fixed, washed and permeabilized with a commercial solution, which contains polyformaldehyde and saponin (MultiSciences Biotech, Hangzhou, China). TNF-α (MP6-XT22, Biolegend, Inc, San Diego, CA), the intracellular staining was then performed.

The raw data was acquired by an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA) and further analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

2.10. BEHAVIORAL ANALYSIS

2.10.1 Gait test

A gait test was used to evaluate the walking pattern of mice, thereby reflecting gait abnormality of CH mice. A black plastic tunnel with a length of 100 cm, width of 10 cm and height of 10 cm, ending with a dark box was used to perform experiments. A sheet of white absorbent paper (100 cm × 10 cm) was placed at the bottom of the tunnel to record footprints. The day before the trial, we let each mouse walk on the tunnel and adapt to the test environment. On the day of the test, nontoxic dye was applied to paint hind limbs of each mouse. Subsequently, we put them at the beginning of the tunnel and allowed them spontaneously move forward to the end. The white papers with footprints were carefully dried and then the stride length and width were measured. To ensure the reliability and consistency of the data, we selected three successive footprints from the phase of a smooth walk for analysis [56].

2.10.2 Rota-rod test

The rota-rod machine (Model 47650, Ugo Basile, Varese, Italy) was used to assess motor

coordination and balance of mice. The rotating beam is divided into five independent compartments. At the beginning of the experiments, each mouse walked on its own cylinder section at a low rotation speed of 5 rpm for 30 s to adapt to the movements. Then the rotating speed was evenly accelerated from 5 rpm to 50 rpm within 5 min as we set before placing the mouse in position. The mice had to walk and avoid losing their balance, falling off on the plate below. The endurance time that each mouse walking on the beam was recorded. It is positively correlated with animal's motor coordination. For the test, six trials with a 10 min resting interval were conducted to minimize stress and fatigue [57].

2.10.3 Open field test

An open field arena, in which the length, width and height are all 50 cm, was used to evaluate spontaneous locomotor activity in our experiments. Two hours before each trial, we placed mice cages in the tested environment to let them adapt to lighting, temperature and humidity. When the experiment started, ensuring there was no noise and peculiar smell in the room. Then we put the mice in the center of the arena. The locomotor activity, including distance and velocity during 10 min, was recorded by a video camera and analyzed by Clever TopScan (Clever Sys Inc., Reston, VA) [58]. At the end of each mouse's experiment, the bottom and sides of the opening equipment were scrubbed with 75% alcohol, and thoroughly dried, so as to prevent odors interference that was left by other mice.

In general, all the behavioral tests mentioned above were used to evaluate cerebellar ataxic motor symptoms following CH.

2.11. STATISTICAL ANALYSIS

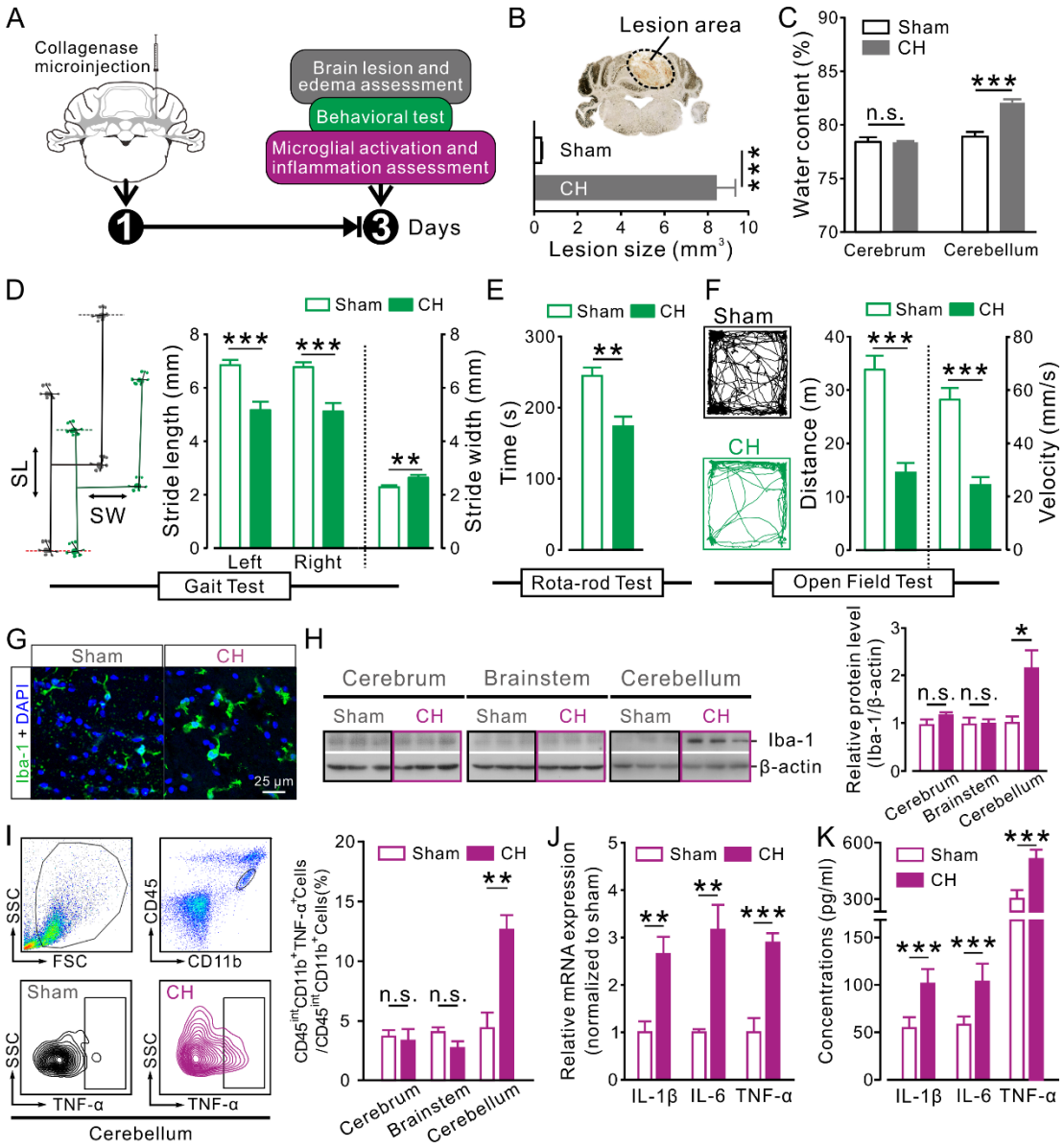
All data were presented as mean ± SEM, and analyzed by GraphPad Prism 8.0 (GraphPad Software, San Diego, California USA). Unpaired 2-tailed Student's t test and one-way analysis of variance (ANOVA) with Newman-Keuls post hoc test were used for statistical analysis. The definition of statistical significance was P-value < 0.05.

3. RESULTS

3.1. CH MODEL ESTABLISHMENT

As shown in Fig(1) A, unilateral microinjection of collagenase into the cerebellar paramedian white matter was applied to induce the formation of hematoma, and establishment of CH model [35]. The studies of ICH suggested a significant inflammatory reaction started in a few hours and peaked at 3 days [59-61], therefore, we selected this peaking time point to explore the acute neuroinflammation of CH mice model in our experiments. Compared with the Sham group, the cerebellar hemispheres injected with collagenase showed obvious brain damage with larger lesion size ($n=9$, $P<0.001$) Fig(1) B. In addition, the hematoma in our CH model led to local cerebellar edema, an increased degree of water content that was limited to the cerebellum (Sham group: $n=9$, CH group: $n=10$, $P< 0.001$), not influencing the cerebrum ($P> 0.05$) Fig(1) C. These results confirmed that the collagenase induced model simulated clinical pathological characteristics of CH [62].

It is worth noting that CH patients have different levels of ataxic symptoms, including gait abnormalities, postural instability and dyskinesia, which are important reasons for the high disability prevalence of CH [63, 64]. Therefore, behavioral tests were performed to evaluate the motor abilities of mice. In the gait test, CH mice developed a significant gait abnormality with shortened stride length (Sham group: $n=12$, CH group: $n=13$. Left stride length: $P< 0.001$; Right stride length: $P< 0.001$) and increased stride width ($P<0.01$) Fig(1) D, when we measured their spatial distributions of footprints. The endurance time of CH mice on the rotating machine was decreased (Sham group: $n = 16$, CH group: $n = 18$, $P < 0.01$) Fig(1) E, suggesting impaired motor coordination. In addition, the results of movement distance (Sham group: $n=19$, CH group: $n=17$, $P < 0.001$) and velocity ($P < 0.001$) in the open field analysis indicated the remarkably reduced locomotor ability of the collagenase injected mice Fig(1) F. In general, the CH mice model could mimic clinical ataxic symptoms of CH patients, such as gait disturbance and motor deficiency.



Fig(1) [50].CH model establishment and relative acute neuroinflammation. A. diagrammatic drawing. CH was induced by unilateral collagenase microinjecting into the right paramedian white matter in the cerebellum. Experiments about brain lesion and edema assessment, behavioral test, microglial activation and inflammation assessment were performed 3 days after CH. B-C. Lesion size and water content. CH led to a significantly larger lesion size (B) and great edema (C) in the cerebellum. D-F. Motor deficiency following CH, testing by gait analysis (D), rota-rod (E) and open field (F). G-I. Activation of microglia. Immunofluorescence staining in blue color (DAPI), green color (Iba-1), Scale bar=25μm (G). Immunoblots for Iba-1 protein expression of cerebrum, brainstem and cerebellum (H). Flow cytometry for measuring the percentage of TNF-α positive CD45intCD11b+ subset in the cerebrum, brainstem and cerebellum (I). J-K. Pro-inflammatory cytokines production. The relative mRNA expressions were detected by qPCR (J), and protein levels were further confirmed by Enzyme-linked immunosorbent assay (ELISA) (K). The values are expressed as mean ± SEM. Compared with Sham group, n.s. represents no significance. *P < 0.05, **P < 0.01, ***P < 0.001. Others' contributions: Shutao Xie and Bo Song contributed to Fig(1) A-C. Shutao Xie, Bo Song and I finish Fig(1) D-G together. ("Together" represents equal contribution.)

3.2. ACUTE NEUROINFLAMMATION AFTER CH

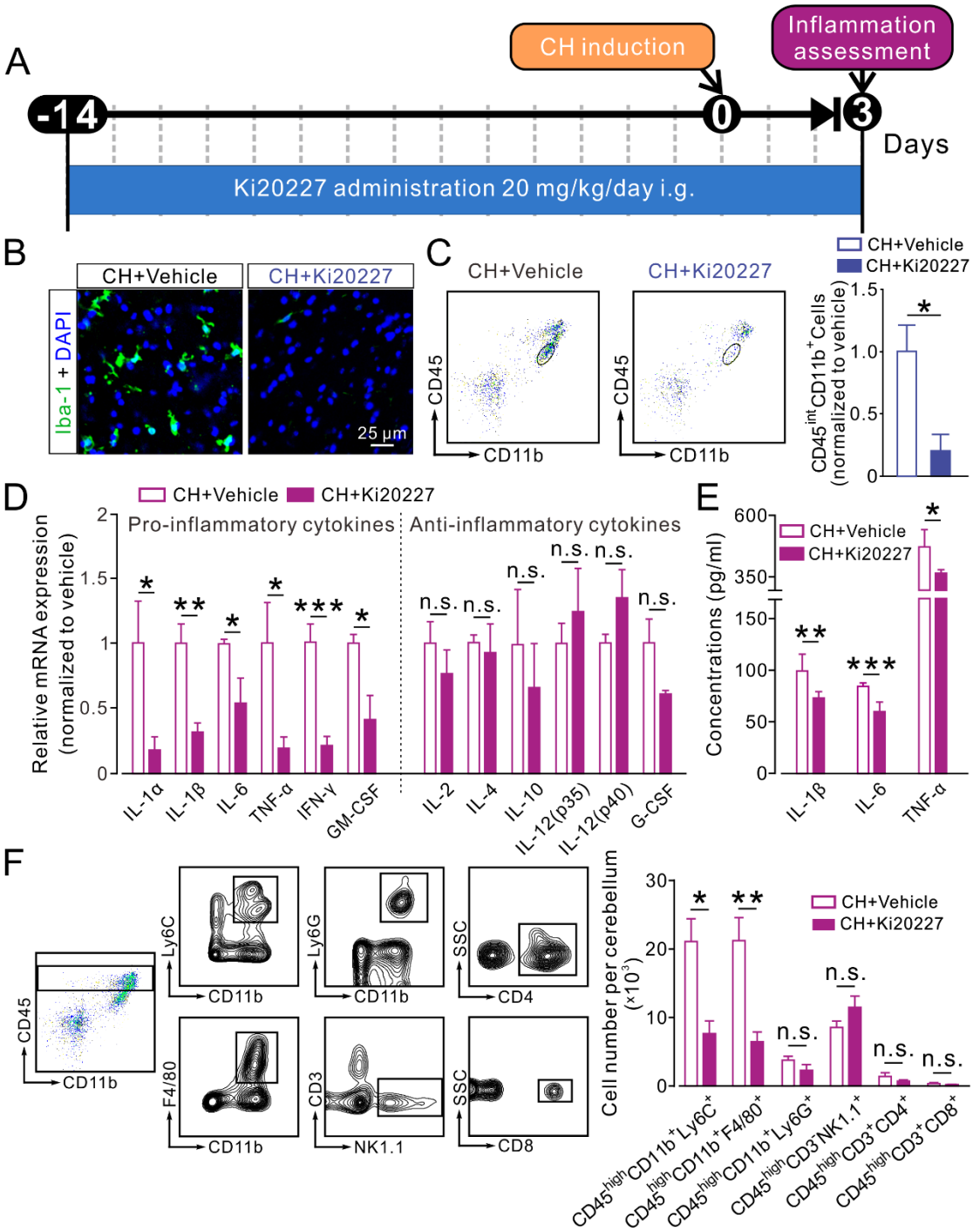
We next determined the acute neuroinflammation condition of CH. On day 3, our results of the immunofluorescence histochemistry showed Iba-1 (microglia marker) positive cells in the CH group with an amoeboid shape, that is, the evidence of microglial activation [65] Fig(1) G. β-actin was used as internal reference protein, in the cerebellum, the relative expression of Iba-1 in CH mice was significantly higher than that in the Sham group ($n = 6$, $P < 0.05$). While relative Iba-1 expression restricted in the injured cerebellum, not involving the cerebrum and brain stem ($P > 0.05$) Fig(1) H. Besides, according to the gating strategy Fig(1) I, in the CD45intCD11b+ microglia subgroup, the ratio of TNF-α-positive cells increased in the cerebellum ($n = 4$, $P < 0.01$), rather than cerebrum and brain stem ($P > 0.05$). Microglial activation and relative expression of inflammatory mediators of CH mice were region specific. Next, we used qPCR and ELISA to further detect the expression of typical cytokines following CH in the cerebellum. As shown in Fig(1) J, compared with the Sham group, hematoma resulted in increased mRNA levels of cerebellar IL-1β (Sham group: $n = 6$, CH group: $n=8$, $P<0.01$), IL-6 ($P<0.01$), and TNF-α ($P<0.001$). The protein levels of IL-1β ($n = 6$, $P < 0.001$), IL-6 ($P < 0.001$), and TNF-α ($P<0.001$) were elevated as well Fig(1) K. Our findings indicated CH induced acute neuroinflammation, activating cerebellar microglia and producing a large amount of pro-inflammatory cytokines.

3.3. MICROGLIAL ELIMINATION AMELIORATED ACUTE NEUROINFLAMMATION FOLLOWING CH

Previous studies suggested microglia play a crucial role in the neuroinflammation [66, 67]. In order to explore the mechanism of acute neuroinflammation after CH, we further focused on microglia. The work of Nynke Oosterhof indicated that colony stimulating factor-1 receptor (CSF-1R) primarily regulates the survival of microglia, especially their density [68, 69]. We administered Ki20227, a selective antagonist of CSF-1R, to treat microglia. As illustrated in Fig(2) A, mice were given saline as vehicle or Ki20227 by intragastric gavage for 14 consecutive days before CH induction to eliminate microglia. Subsequently, 3 days following the injection of collagenase into the cerebellum, we evaluated the neuroinflammation condition

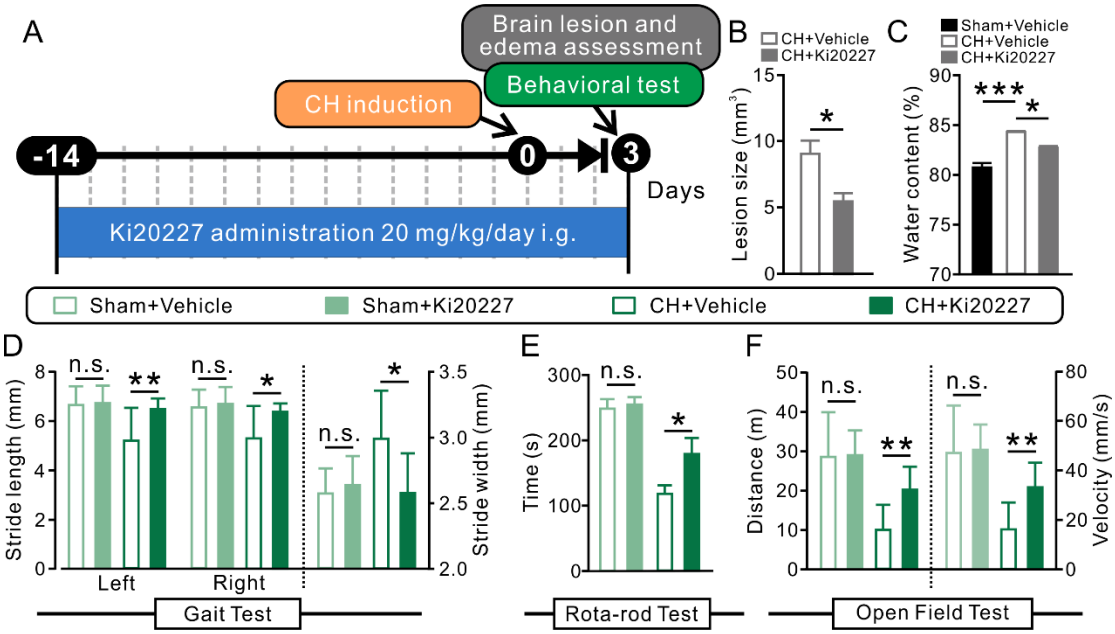
with microglia depletion. Immunofluorescence staining for Iba-1 and flow cytometry were used to determine the clearance efficiency of Ki20227 treatment on microglia. Compared with CH + Vehicle group, the results of staining showed dramatically reduced signals of Iba-1 in the cerebellum in the CH + Ki20227 group Fig(2) B. Meanwhile, the number of cerebellar microglia (CD45int CD11b+) significantly decreased when received Ki20227 Fig(2) C ($n = 4$, $P<0.05$). Thus, the cerebellar microglia of CH mice were effectively eliminated.

Microglia can exhibit neuroprotective features, such as clearing cellular debris. However, on the other hand, microglia can also release a group of pro-inflammatory cytokines that damages CNS repair, even leads to neurological impairment [70]. The dual role of microglia was also found in cerebral hemorrhage [71, 72]. Thus, we tried to explore the effect of reactive cerebellar microglia post CH induction. Compared with CH mice, elimination of microglia with Ki20227 remarkably decreased pro-inflammatory cytokines expression: IL-1α ($P < 0.05$), IL-1β ($P < 0.01$), IL-6 ($P < 0.05$), TNF-α ($P < 0.05$), IFN-γ ($P < 0.001$) and GM-CSF ($P < 0.05$), while didn't significantly influence anti-inflammatory cytokines expression at mRNA level: IL-2 ($P > 0.05$), IL-4 ($P > 0.05$), IL-10 ($P > 0.05$), IL-12 (p35) ($P > 0.05$), IL-12 (p40) ($P > 0.05$) and G-CSF ($P > 0.05$) Fig(2) D ($n = 6$). The protein levels of IL-1β ($P < 0.01$), IL-6 ($P < 0.001$) and TNF-α ($P < 0.05$) were detected by ELISA, striking reductions were found in these typical pro-inflammatory cytokines Fig(2) E ($n = 6$). Rapid activation of microglia involved leukocytes infiltration, which is regarded as a key inflammatory cascade event after intracerebral hemorrhage has been reported [73-75]. Following the formation of cerebellar hematoma, we used flow cytometry to analyze the number of monocytes (CD45highCD11b+Ly-6C+), macrophages (CD45highCD11b+F4/80+), neutrophils (CD45highCD11b+Ly-6G+), natural killer cells (CD45highCD3−NK1.1+) and T cells (CD45highCD3+CD4+ or CD8+) depending on corresponding gating strategy, of which monocytes ($P < 0.05$) and macrophages ($P < 0.01$) significantly decreased when received Ki20227 Fig(2) F. Our results suggested cerebellar microglia may play pro-inflammatory roles in acute neuroinflammation of CH, and depletion of microglia could alleviate strong inflammatory responses, including lower levels of pro-inflammatory cytokines, fewer monocytes and macrophage infiltration.



Fig(2) [50]. Depletion of microglia alleviates acute neuroinflammation in CH mice. A. diagrammatic drawing. C57BL/6 mice received 20 mg/kg/day Ki20227 for 14 days and 3 days after CH by inductionintragastric gavage (i.g.), then the CH mice in both Ki20227 treatment and vehicle group were sacrificed for inflammation assessment. B. Immunofluorescence staining in blue color (DAPI), green color (Iba-1), Scale bar = 25 μm. C. Flow cytometry analysis for the number of cerebellar microglia (CD45intCD11b+), with gating strategy and normalized data. D. mRNA expression of pro-inflammatory cytokines: IL-1α, IL-1β, IL-6, TNF-α, IFN-γ, GM-CSF, and anti-inflammatory cytokines: IL-2, IL-4, IL-10, IL-12 (p35), IL-12 (p40), G-CSF. E. Protein expression of representative pro-inflammatory cytokines: IL-1β, IL-6 TNF-α, were analyzed by ELISA. F. Flow cytometry analysis for leukocytes infiltration in the cerebellum, the number of monocyte (CD45high CD11b+Ly6C+), macrophage

(CD45highCD11b+F4/80+), neutrophil (CD45highCD11b+Ly- 6G+), NK cell (CD45highCD3−NK1.1+), CD4+T cell (CD45highCD3+CD4+) and CD8+T cell (CD45highCD3+CD8+). The values are expressed as mean ± SEM. Compared with Sham group, n.s. represents no significance. *P < 0.05, **P < 0.01, ***P < 0.001. Others' contribution: I assisted Shutao Xie to finish Fig(2) A. ("Assist" means I participated in all steps, but others did more contributions.) Shutao Xie contributed to Fig(2) B.



Fig(3) [50]. Depletion of microglia improves pathological injury and behavioral symptoms of CH mice. A. diagrammatic drawing. C57BL/6 mice received 20 mg/kg/day Ki20227 for 14 days and 3 days after CH induction, then the mice in Sham + Vehicle, Sham + Ki20227, CH + Vehicle and CH + Ki20227 group were sacrificed for brain lesion, edema assessment and behavioral test as required. B. Lesion size C. water content D-F. The behavioral symptoms were evaluated by gait test, including stride lengths and width of mice (D), rota-rod (E), and open field test, including movement distance and velocity (F). The values are expressed as mean ± SEM. Compared with Sham group, n.s. represents no significance. *P < 0.05, **P < 0.01, ***P < 0.001. Others' contribution: Shutao Xie contributed to Fig(3) B. Shutao Xie, Bo Song and I finish Fig(3) A, D-F together.

3.4. MICROGLIAL ELIMINATION IMPROVED CEREBELLAR INJURY AND MOTOR DEFICIENCY OF CH MICE

Considering the fact that microglial clearance can reduce cerebellar acute neuroinflammation, we further determined its effect on cerebellar injury and functional recovery after the occurrence of CH Fig(3) A. Compared with the CH + Vehicle group, the cerebellar lesion size was reduced with Ki20227 treatment (n = 9, P<0.05) Fig(3) B. Besides, when the edema was induced by collagenase microinjection (Sham: Vehicle group: n = 9, CH: Vehicle group: n = 8, P < 0.001), microglia

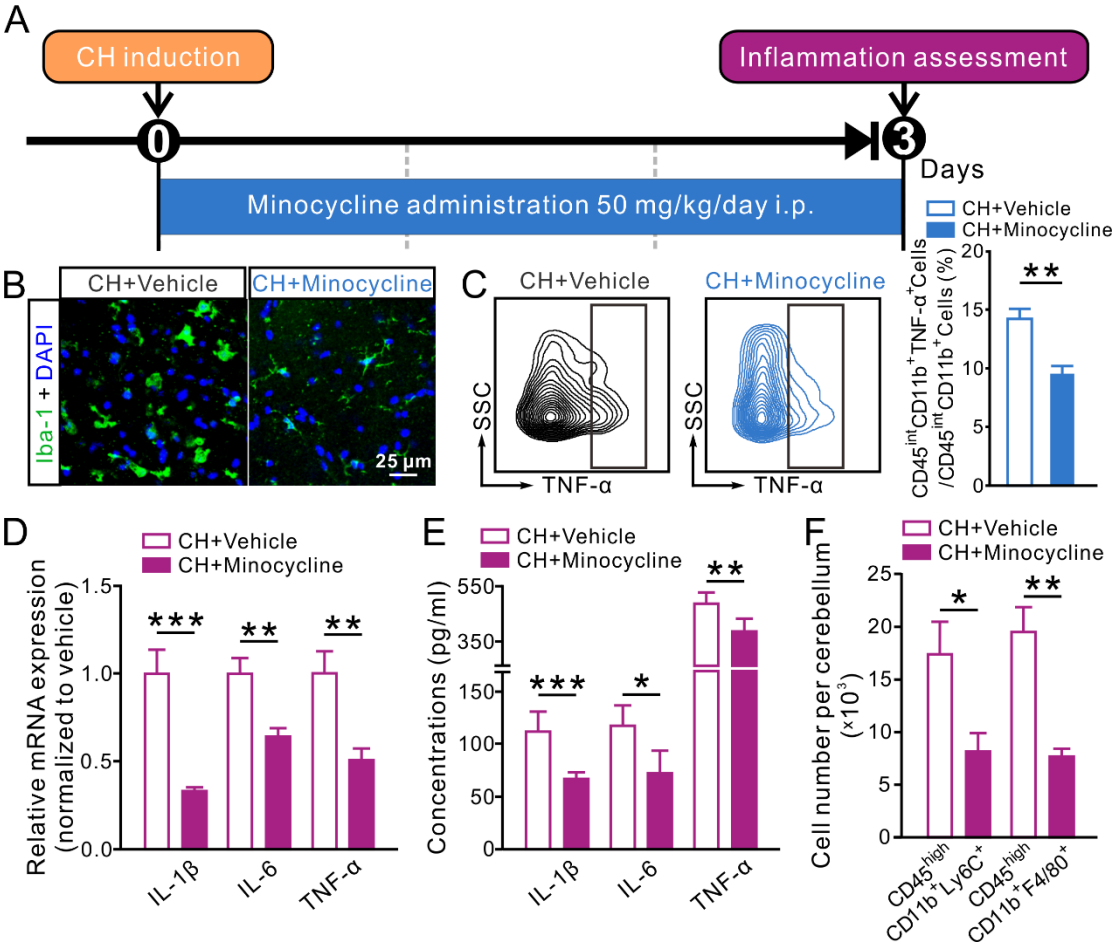
elimination significantly alleviated cerebellar water content as well (CH: Ki20227 group: n = 8, $P < 0.05$) Fig(3) C. In regard to ataxia-like motor symptoms, we evaluated them with gait test, rota-rod test and open field test. All the behavioral results between Sham groups (Sham: Gait test: n = 11, Rota-rod test: Vehicle group: n=10; Ki20227 group: n=11, Open field test: n = 11) indicated no significant influence on motor performances was found, when Sham-operated mice experienced microglia depletion Fig(3) D-F. However, if CH mice were given Ki20227, they exhibited longer stride lengths (n = 11, Stride length: left: $P < 0.01$; right: $P < 0.05$) and a shorter stride width ($P < 0.05$) Fig(3) D than injured mice. The endurance time on the rota-rod was also prolonged by treatments (CH: Vehicle group: n=12; Ki20227 group: n=11, $P < 0.05$) Fig(3) E. In addition, the overall movement distance ($P < 0.01$) and velocity ($P < 0.01$) in the open field test were both improved (n = 11) Fig(3) F. Hence, our data suggested that clearing microglia could promote the histological and locomotor functional recovery after CH.

3.5. INHIBITION OF MICROGLIA ACTIVATION ALLEVIATED ACUTE NEUROINFLAMMATION IN CH MICE

Minocycline is known as the tetracycline antibiotics and can easily cross the blood brain barrier [76]. As K Kobayashi reported, minocycline can selectively suppress the activation of microglia, not polarizing into pro-inflammatory phenotype [77]. In order to preserve other basic functions of cerebellar microglia, we used minocycline and further observe the effect of microglia activation on CH-induced acute neuroinflammation. After successful model establishment, CH mice were treated with 50 mg/kg/day minocycline and normal saline for 3 days Fig(4) A. By fluorescence labeling for microglia marker, Iba-1, we found that the cells expressing Iba-1 presented a ramified morphology, which is the distinctive morphology of resting microglia, in the cerebellum in minocycline treatment group Fig(4) B. Besides, flow cytometry analysis also demonstrated the percentage of TNF-α- expressing cells were significantly decreased in the cerebellar microglia (n = 4, $P < 0.01$) Fig(4) C. An effective suppression of pro-inflammatory microglia polarization after CH was achieved by minocycline.

Next, we detected the influence of minocycline on CH-induced acute neuroinflammation. The results of qPCR showed mRNA expression of representative pro-inflammatory cytokine: IL-1β

(P < 0.001), IL-6 (P <0.01), TNF-α (P <0.01), significantly declined with drug treatment (n = 6) Fig(4) D. The similar reduced phenomenon was also found in protein expression, IL-1β (*P* < 0.001), IL-6 (*P* < 0.05), TNF-α (*P* < 0.001) (n = 6) Fig(4) E. Moreover, minocycline administration dramatically decreased the infiltration of monocytes (CD45highCD11b+Ly-6C+) (*P* < 0.05) and macrophages (CD45highCD11b+F4/80+) (P<0.01) in the cerebellum (n = 4) Fig(4) F. Our results verified the previous finding, the cerebellar microglia played a pro-inflammatory role after CH. When we specifically inhibited microglia activation, the relative acute neuroinflammation was relieved.



Fig(4) [50]. Inhibition of microglia activation alleviates acute neuroinflammation in CH mice. A. diagrammatic drawing. C57BL/6 mice received 50 mg/kg/day 3 days after CH induction by intraperitoneal administration (i.p.), then the CH mice in both Minocycline treatment and vehicle group were sacrificed for inflammation assessment. B. Iba-1 Immunofluorescence staining. Iba-1 (green fluorescence), DAPI (blue fluorescence), Scale bar = 25 μm. C. Flow cytometry analysis for percentage of TNF-α positive microglia in the cerebellum, with gating strategy and summarized data. D. mRNA expression of typical pro-inflammatory cytokines: IL-1β, IL-6, TNF-α. E. Protein expression of IL-1β, IL-6 TNF-α, were detected by ELISA. F. Flow cytometry analysis for the number of monocyte

(CD45highCD11b+Ly 6C+) and macrophage (CD45highCD11b+F4/80+) infiltrated into the cerebellum. The values are expressed as mean ± SEM. Compared with Sham group, n.s. represents no significance. *P < 0.05, **P < 0.01, ***P < 0.001. Others' contribution: I assisted Shutao Xie to finish Fig(4) A. Shutao Xie contributed to Fig(4) B.

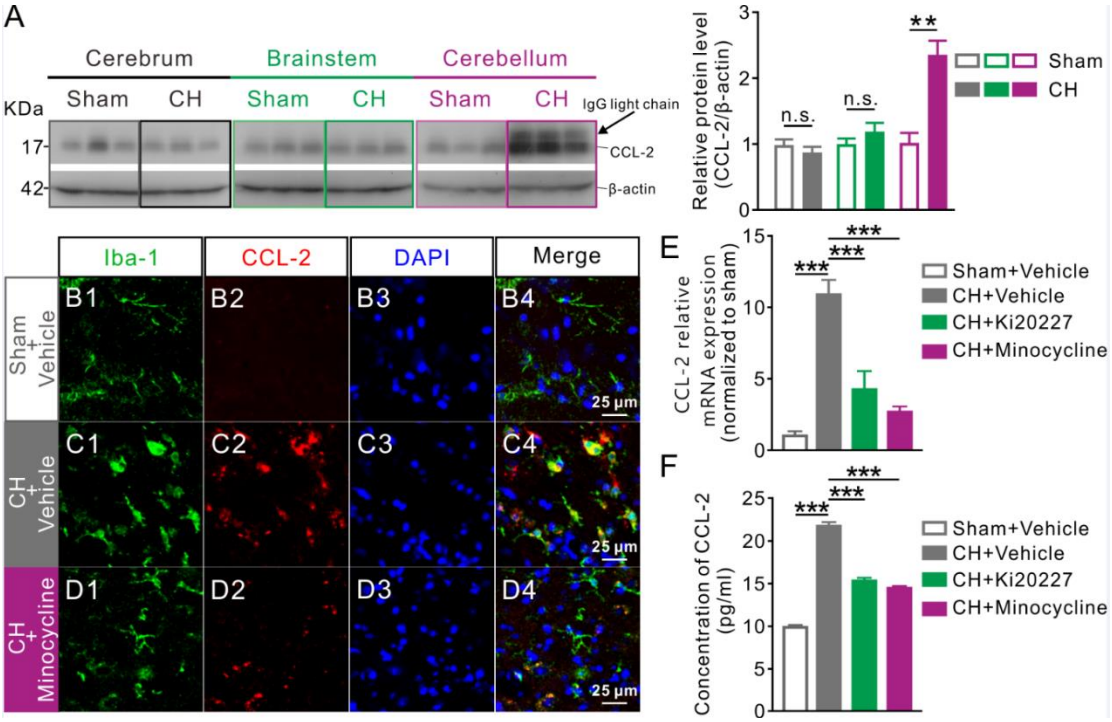
3.6. CEREBELLAR REACTIVE MICROGLIA-DERIVED CHEMOKINE CCL2 WAS INCREASED AFTER THE OCCURRENCE OF CH

C-C Motif Chemokine Ligand 2 (CCL2), has been proved to be a detrimental chemokine involved in acute neuroinflammation [78]. In both mouse and human brains, CCL2 is abundantly expressed by reactive microglia [79]. Besides, a higher circulating concentration of CCL2 was related to a greater risk of stroke [80]. Intracerebral hemorrhage patients with elevated CCL2 had worse prognosis [81]. Thus, we tested the CCL2 expression in the brain tissues, after cerebellar hematoma was induced. The results of western blot demonstrated CCL2 highly expressed in the cerebellum ($P < 0.01$), not cerebrum or brainstem ($P > 0.05$) ($n = 6$) Fig(5) A. The enhanced cerebellar CCL2 signal was mainly co-labeled with Iba-1 positive cells in CH mice. Inhibition of microglial activation by minocycline treatment reduced CCL2 positive staining and co-expression with microglia Fig(5) B-D. Following CH, CCL2 was mainly released by cerebellar reactive microglia. Compared with CH mice, the mRNA ($P< 0.001$) and protein ($P< 0.001$) expression levels of CCL2 in the cerebellum of CH mice administered with drugs: Ki20227 or minocycline, were significantly lower ($n = 6$) Fig(5) E-F. These results suggested cerebellar reactive microglia-derived chemokine CCL2 was elevated after CH.

3.7. MICROGLIA-DERIVED CHEMOKINE CCL2 AGGRAVATED ACUTE NEUROINFLAMMATION FOLLOWING CH

Bindarit has prominent anti-inflammatory function, and this characteristic is associated with inhibiting the monocyte chemotactic protein, mainly includes CCL2, thereby selectively interfering with recruitment of monocytes [82]. We explored the role of CCL2 in CH induced acute immune response by intragastric administration of Bindarit Fig(6) A. As Fig(6) B suggested, the mRNA expression of CCL2 in the cerebellum was significantly reduced in Sham and CH group with drug treatment ($n = 6$, Sham: Vehicle group versus Bindarit group: $P<0.01$;

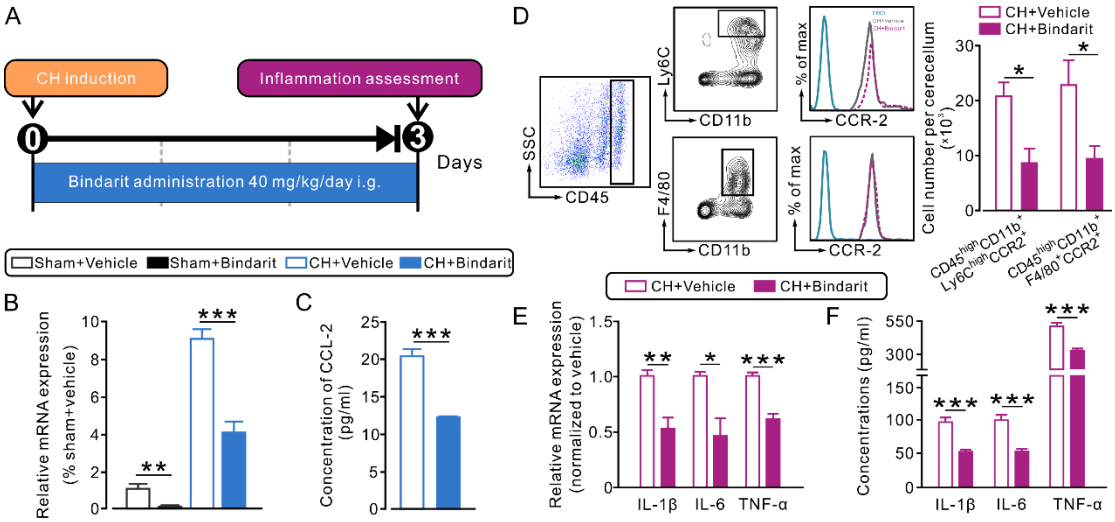
CH: Vehicle group versus Bindarit group: $P < 0.001$). The data of ELISA indicated the protein expression of CCL2 was also decreased ($n = 6$, $P < 0.001$) Fig(6) C. Thus, the synthesis of CCL2 in the cerebellum of CH mice was effectively inhibited by Bindarit. In view of the known function of CCL2, and the secretion of this chemokine at injured site, which is vital for the recruitment of circulating inflammatory C-C Motif Chemokine Receptor 2 (CCR2) positive monocytes and macrophages to central nervous system [83]. We assessed above immune cells infiltration by flow cytometry.



Fig(5) [50]. Increased level of microglia-derived chemokine CCL-2 was found in the cerebellum after CH. A. Western blot analysis for protein expression of CCL-2 in cerebrum, brainstem and cerebellum. The direction of arrow: light chain of Immunoglobulin G (IgG). B-D. Immunofluorescence staining in blue color (DAPI), green color (Iba-1) and red color (CCL-2), scale bar = 25 μm, in Sham + Vehicle, CH + Vehicle and CH + Minocycline groups. Administration of minocycline decreased the microglial activation together with CCL-2 expression (D). E-F. The mRNA (E) and protein (F) level of CCL-2 were detected in the cerebellum of Sham + Vehicle, and CH mice treated with saline, Ki20227 and minocycline, respectively. The values are expressed as mean ± SEM. Compared with Sham group, n.s. represents no significance. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Others' contribution: Shutao Xie contributed to Fig(5) B-D.

The results demonstrated infiltration of cerebellar monocytes (CD45highCD11b+Ly-6ChighCCR2+, $P < 0.05$) and macrophages (CD45highCD11b+F4/80+CCR2+, $P < 0.05$) were dramatically decreased, when CH mice received Bindarit ($n = 4$) Fig(6) D. In addition, both the

mRNA (n =6) and protein (n =9) expression of IL-1β (mRNA: $P < 0.01$, protein: $P < 0.001$), IL-6 (mRNA: $P < 0.05$, protein: $P < 0.001$) and TNF-α (mRNA: $P < 0.001$, protein: $P < 0.001$) were declined in CH + Bindarit group Fig(6) E-F. Our data indicated that microglia-derived CCL-2 aggravated acute neuroinflammation in CH mice, with infiltration of cerebellar pro-inflammatory monocytes and macrophages and increased expression of pro-inflammatory cytokines.

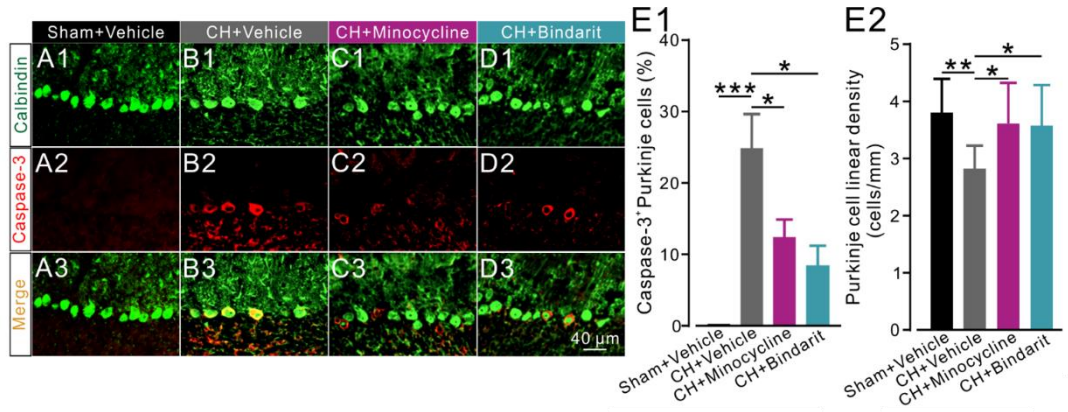


Fig(6) [50]. Blocking the synthesis of CCL-2 reduces monocyte and macrophage infiltrated into cerebellum after CH. A. diagrammatic drawing. C57BL/6 mice received 40 mg/kg/day 3 days after CH induction, then the CH mice in both Bindarit treatment and vehicle group were sacrificed for inflammation assessment. B. mRNA expression of CCL-2 in the cerebellum of CH mice and Sham group with or without Bindarit. C. Protein expression of CCL-2 in the cerebellum of CH mice. D. Flow cytometry analysis for the number of CCR2 positive monocytes and macrophages infiltrated into the cerebellum, with gating strategy and summarized data. E. mRNA expression of typical pro-inflammatory cytokines: IL-1β, IL-6, TNF-α. F. Protein expression of IL-1β, IL-6 TNF-α. The values are expressed as mean ± SEM. Compared with Sham group, n.s. represents no significance. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Others' contribution: Shutao Xie contributed Fig(6) A.

3.8. SUPPRESSION OF MICROGLIA ACTIVATION AND BLOCKAGE OF CCL2 PRODUCTION DECREASED PURKINJE CELLS APOPTOSIS

It is noted that Purkinje cells are the only neurons in the cerebellar cortex [84], which means all the information within the cortex is transmitted by these neurons [85]. Ataxia-like symptoms are associated with loss of Purkinje neurons in the cerebellum [86, 87]. Besides, neuronal apoptosis is widely observed in patients with hemorrhagic stroke and ultimately contributes to

acute brain injury [88, 89]. Unnecessary triggered inflammatory responses can exacerbate neuronal degeneration [90]. Thus, we next explored the apoptosis of Purkinje cells following CH and when the neuroinflammation was reduced by drugs treatments. Purkinje cells were stained with calbindin. The immunofluorescence results suggested caspase-3 labeled cerebellar apoptotic Purkinje cells expressed highly in CH mice Fig(7) A-B. As shown in Fig(7) C-E, administration of minocycline and bindarit decreased the percentage of cerebellar apoptotic Purkinje cells (Sham: Vehicle group: n = 7, CH : Vehicle group: n = 6; Minocycline group: n = 7; Bindarit group: n = 8, $P < 0.05$), while increasing the Purkinje cell linear density to the normal level (Sham: Vehicle group: n = 7, CH: Vehicle group: n = 6; Minocycline group: n = 7; Bindarit group: n = 8, $P < 0.05$). Hence, inhibition of microglial activation and CCL2 synthesis alleviated apoptosis and loss of Purkinje neurons.

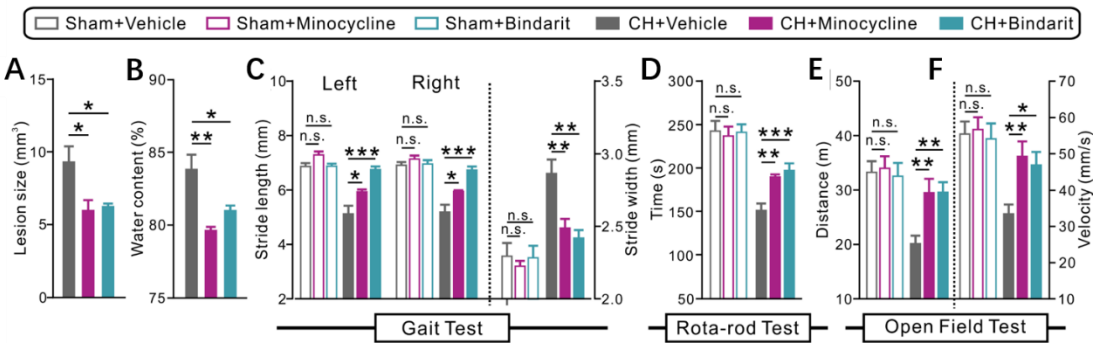


Fig(7) [50]. Inhibition of microglial activation and blockage of CCL-2 production reduce Purkinje cell apoptosis after CH. A-D. Immunofluorescence staining for calbindin (green color) and caspase-3 (red color) in Sham + Vehicle, CH + Vehicle, CH + Minocycline, CH + Bindarit group. Scale bar = 40 μm. E1-E2. The percentage of caspase-3 positive Purkinje cells and Purkinje cell linear density were measured. The values are expressed as mean ± SEM. Compared with Sham group, n.s. represents no significance. *P < 0.05, **P < 0.01, ***P < 0.001. Others' contribution: I assisted Shutao Xie to finish Fig(7) A-D. Shutao Xie contributed to Fig(7) E.

3.9. SUPPRESSION OF MICROGLIA ACTIVATION AND CCL2 SYNTHESIS ALLEVIATED BRAIN INJURY AND ATAXIA-LIKE SYMPTOMS AFTER CH

According to the previous results, the acute neuroinflammation and neuronal apoptosis following CH can be significantly reduced by treatments that targeting microglial activation and chemokine CCL2 synthesis. Hence, we finally evaluated the influence of minocycline and

bindarit on brain injury and motor deficiency in CH mice. Lesion size of treated groups showed a smaller level (CH: Vehicle group: n=9; Minocycline group: n=9; Bindarit group: n =10, $P < 0.05$) Fig(8) A. The similar declined phenomenon was also found in cerebellar water content (n=6, CH: Minocycline group versus Vehicle group: $P < 0.01$; CH: Bindarit group versus Vehicle group: $P < 0.05$) Fig(8) B. Drug administration did not affect locomotor performances in Sham groups (Sham: Vehicle group: n=10; Minocycline group: n=10; Bindarit group: n=10) Fig(8) C-F. The spatial stability of footprints was improved (CH: Vehicle group: n = 11; Minocycline group: n = 13; Bindarit group: n = 12). Stride lengths were extended (CH: both left and right: Minocycline group versus Vehicle group: $P < 0.05$; Bindarit group versus Vehicle group: $P < 0.001$), and stride width was shortened ($P < 0.01$) Fig(8) C. The endurance time on the accelerated rota-rod machine was greatly enhanced (n = 12, CH: Minocycline group versus Vehicle group: $P < 0.01$; Bindarit group versus Vehicle group: $P < 0.001$) Fig(8) D. The improvement was also found in total locomotor distance ($P < 0.01$) and velocity (CH: Minocycline group versus Vehicle group: $P < 0.01$, Bindarit group versus Vehicle group: $P < 0.05$) (CH: Vehicle group: n = 12; Minocycline group: n = 12; Bindarit group: n = 10) Fig(8) E-F.



Fig(8) [50]. Inhibition of microglial activation and blockage of CCL-2 production relieve brain injury and ataxia-like motor symptoms after CH. Relative behavioral tests were performed in Sham + Vehicle, Sham + Minocycline, Sham + Bindarit, CH + Vehicle, CH + Minocycline, CH + Bindarit. A. Lesion size of CH groups. B. Water content of CH groups. C. Gait test. To evaluate spatial distribution of footprints: left and right stride length, stride width. D. Rota-rod test. To detect the endurance time that mice walked on the rotating beam. E-F. Open field test. To measure the total locomotor distance and velocity. The values are expressed as mean ± SEM. Compared with Sham group, n.s. represents no significance. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Others' contribution: Shutao Xie and Bo Song contributed to Fig(8) A. Shutao Xie, Bo Song and I finished Fig(8) B-F together.

In general, reactive microglia together with its derived chemokine CCL2 play pro-inflammatory roles in acute neuroinflammation following CH. Inhibition of pro-inflammatory microglial activation and blockage of microglia-derived CCL2 production alleviated inflammatory responses and relevant apoptosis of Purkinje neurons, and significantly improving cerebellar injury and motor deficits.

4. DISCUSSION

4.1. REACTIVE MICROGLIA IN ACUTE NEUROINFLAMMATION FOLLOWING CH

Our study first showed reactive cerebellar microglia had a detrimental influence on acute neuroinflammation and caused a series of inflammatory responses, further aggravating brain injury and disability after CH.

Microglia are widely viewed as innate immune cells in the CNS, which colonize there as early as the embryonic phase [91]. The conventional perspective considers them to be a group of macrophage-like cells. When the branched and ramified resting microglia with weak immune reactivity turn into an amoeboid morphological phenotype, they become activated with intense immune reactivity [92]. They are also regarded as the primary defensive responders in CNS, thus the enabled response occurs rapidly, within hours, following hemorrhagic stroke, and subsequent inflammatory responses reach the peak on the third day[93]. Increasing evidence indicated reactive microglia exert diverse functions when exposed to different types of stimuli. They sometimes mainly play a neuroprotective role, such as clearing cell debris by phagocytosis, secreting anti-inflammatory mediators such as IL-4, IL-10, and TGF-β, suppressing inflammatory responses and promoting neurogenesis [94]. Activated microglia attenuated neurological deficits and promoted hematoma clearance after ICH by secreting Interleukin-27 to regulate neutrophil maturation and increasing production of lactoferrin [95]. Whereas microglia exhibit adverse effects under some circumstances, releasing cytotoxic substances such as oxygen free radicals, overproducing pro-inflammatory cytokines and recruiting peripheral leukocytes [96]. Infiltration of leukocytes occurs after microglial activation in hematoma induced brain injury [97]. Further observing our disease interest, the dual roles of microglia were also found in the majority of hemorrhagic stroke [38]. However, the exact influence of microglia in CH is unclear. Aside from the external environment, microglia themselves display spatial differences in the brain. Cerebellar microglia have the lowest density in the brain, less than half of the other brain regions, but higher immunologic alert to the surroundings [39, 40, 98]. Considering the differences, it becomes worth investigating cerebellar microglia in the acute neuroinflammation following CH.

We used unilateral microinjection of collagenase into the cerebellar paramedian white matter in our study to induce a CH model, simulating clinical pathological characteristics and ataxic symptoms of CH patients. Hematoma formation induced acute neuroinflammation with activated TNF-α-positive microglia and overproduction of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. Of these cytokines, IL-1β and TNF-α have been proven to be vital for the development of edema [93]. Microglial activation and relative expression of inflammatory mediators of CH mice were restricted in the cerebellum, not involving cerebrum and brain stem. Depleting microglia by CSF1R inhibitor dramatically reduced monocyte and macrophage infiltration and expression levels of pro-inflammatory cytokines, but did not affect anti-inflammatory cytokines including IL-2, IL-4, IL-10, IL-12 (p35), IL-12 (p40), G-CSF in the brain. Moreover, elimination of microglia alleviated cerebellar lesion size, brain edema and promoted motor functional recovery after CH. Then when we selectively suppressed the activation of microglia, not polarizing into pro-inflammatory phenotype by minocycline, the relative acute neuroinflammation was relieved. Therefore, cerebellar microglia play a pro-inflammatory role in CH-induced acute neuroinflammation.

4.2. REACTIVE MICROGLIA DERIVED CHEMOKINE CCL2

After brain injury, the activated microglia also release chemokines to trigger subsequent chain reactions. One of the most potent microglia derived chemokines is CCL2 [99]. CCL2 has been proved to be highly expressed by microglia [100]. Two clinical reports demonstrated that ICH patients with elevated serum CCL2 concentration had poor functional outcomes, and this chemokine was also confirmed as an independent variable for the correlation [81, 83]. In addition, in the experimental stroke model, CCL2 deficient mice significantly attenuated tissue damage and reduced the production of IL-1β, which may contribute to the protective effect. [101]. In the current study, we found CH mice had a significantly increased concentration of CCL2 in the cerebellum, and the chemokine CCL2 was co-expressed with reactive microglia. When the specific elimination or inhibition of microglial activation was performed, the increased expression levels of cerebellar CCL2 were significantly suppressed, suggesting that the chemokine CCL2 derived from microglia may play an important role in the acute

neuroinflammation of CH.

CCR2 is the receptor of chemokine CCL2, and they could work together to regulate blood brain barrier permeability, driving recruitment of peripheral monocytes into the lesion site of the brain [102]. Compared with normal mice, Ccr2$^{-/-}$ mice had less blood derived inflammatory monocytes but improved locomotor deficits after ICH [83], and the infiltration of fewer leukocytes was also found in another study when there was a lack of CCL2 or CCR2 [37]. Therefore, the CCL2-CCR2 chemotactic system may have a crucial effect on the cerebellar acute neuroinflammation. Blockage of CCL2 production in our study remarkably reduced the circulating inflammatory CCR-2^{+} monocytes and macrophages that migrate into the cerebellum after CH. It has been reported that monocytes contribute to the inflammation by differentiating into macrophage populations under different sorts of local conditions [103]. Thus, the enhanced CCR-2^{+} macrophages in the cerebellum after CH may be differentiated from CCL2 recruited circulating inflammatory CCR-2^{+} monocytes. In addition to the changes of typical leukocytes, the release of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, lesion size and brain edema were also significantly alleviated by blocking CCL2 synthesis. Taken together, we believe that CCL2, a chemokine derived from cerebellar reactive microglia plays a crucial role in aggravating acute neuroinflammation, mainly through driving recruitment of monocytes and macrophages to the cerebellar area of injury.

4.3. APOPTOSIS OF PURKINJE CELLS

The cerebellum is the brain region, which mainly takes responsibility for motor coordination. Amongst its complex structures, the Purkinje cells are known as the only efferent in the cerebellar cortex, sending information to the deep cerebellar nuclei. In other words, the loss of these neurons will lead to functional deficits of the cerebellum [104]. Besides, neuronal apoptosis has been reported to be a vital contributor to many neurological disorders, including stroke [105]. In six cerebellar infarcted monkeys, the decreased number of Purkinje cells had a great association with ataxia symptoms [106]. Neuronal degeneration can be induced by improper inflammatory responses [90]. The activity of microglia contributed to the apoptosis of Purkinje neurons in the developing mouse cerebellum [107]. In our study, the

immunofluorescence results suggested caspase-3 stained cerebellar apoptotic Purkinje cells expressed highly in CH mice, and with lower liner density. To reduce the inflammation degree by inhibition of microglial activation and CCL2 synthesis can significantly alleviate apoptosis and loss of Purkinje neurons. The receptors of cytokines are proven to be expressed on the Purkinje cells, such as IL-1 receptor including two types of receptor proteins: p80 type I and p68 type II, IL-6Rα, TNFR1 and TNFR2 [108-110]. Hypoxia induced death of Purkinje cells was regulated by reactive microglia derived IL-1β and TNF-α. In addition to the enhanced levels of pro-inflammatory cytokines in the cerebellum, their receptor proteins TNF-R1 and IL-1R1 were also highly expressed in the Purkinje neurons. When the researchers treated the cultured Purkinje cells with a reactive-microglia medium, which specifically neutralized IL-1β and TNF-α therein, the apoptotic condition of the neurons was effectively improved [111]. Hence, we speculate that CH-induced microglial-mediated acute neuroinflammation probably cause the degeneration of Purkinje neurons. Suppression of microglial activation or relative chemokine CCL-2 synthesis, with decline of pro-inflammatory cytokines, attenuated the loss of Purkinje neurons following CH.

4.4. POTENTIAL TREATMENT STRATEGY FOR CH TARGETING ACUTE NEUROINFLAMMATION

CH is a life-threatening neurological disease, associated with long-term disability such as ataxia [18, 112]. The hemorrhage occurs in the narrow posterior fossa and presents a high rate of incidence [113]. Surgical intervention has long been considered as the principal approach for managing cerebellar hematoma. However, increasing evidence suggested in comparison to conservative treatment, there was no significant beneficial effect on the functional outcomes when CH patients received hematoma evacuation [17, 114]. Considering the dismal prognosis following cerebellar bleeding, it becomes necessary to seek an alternative therapeutic strategy for this devastating type of stroke, and targeting the CH induced acute neuroinflammation could be a possibility. The relationship between neuroinflammation and ataxia-like symptoms has been established. Locomotor deficits can be triggered by lipopolysaccharide (LPS) that is microinjected into the mouse cerebellum, because of the induced neuroinflammation, with over

release of pro-inflammatory cytokines following microglial activation [115]. In the Spinocerebellar Ataxia Type 1 (SCA1) model, a type of inherited cerebellar neurodegenerative disease, researchers found that before the occurrence of Purkinje neurons death and ataxic symptoms, microglia were activated much earlier [116]. When eliminating microglia in the transgenic mouse model of SCA1, their degree of neuroinflammation was attenuated, while motor function was improved significantly [117]. Ibuprofen is a non-steroidal anti-inflammatory drug. It has a positive influence on the LPS induced motor incoordination in ataxia telangiectasia mutated-deficient mice [118]. We treated the operated sick mice with Ki20227, minocycline and bindarit. Ki20227 is a kind of colony stimulating factor-1 receptor, which is responsible for the survival of microglia [119]. Minocycline is a tetracycline antibiotic that specifically inhibits microglia polarizing into pro-inflammatory phenotype and it has been tested for efficacy among depression patients in some clinical trials [120]. Bindarit is known as a potential neuroprotective drug by inhibiting CCL2 synthesis [121]. In the current study, lesion size became smaller and the significantly declined trend was also investigated in cerebellar water content. The gait stability was improved, with longer stride lengths and shortened stride width. Other motor performances such as the duration time on the rotating beam, movement distance and velocity in the open field were greatly enhanced. Moreover, each drug treatment did not affect behavioral conditions in the Sham group. Hence, modulating the CH induced acute neuroinflammation by suppression of reactive microglia and CCL2 production will probably become an alternative treatment strategy for cerebellar bleeding, effectively improving motor deficiency such as ataxia.

4.5. CONCLUSION

In summary, our study suggested activated microglia played a detrimental role in the acute neuroinflammation following CH. It initiated a series of inflammatory reactions, including overproduction of typical pro-inflammatory cytokines and chemokine CCL2, subsequently driving recruitment of peripheral circulating monocytes and macrophages. Inhibition of microglial activation and blockage of CCL2 synthesis dramatically alleviated neuronal death, and improved brain injury, motor deficits in CH mice. We first reveal the mechanism of CH

induced acute neuroinflammation and provide an important reference for the development of a novel therapeutic strategy for cerebellar hemorrhagic stroke.

Part 2. Neuroinflammation in chronic brain injury: Alzheimer's disease

5. INTRODUCTION

5.1. ALZHEIMER'S DISEASE

Past:

Alzheimer's disease was first reported by a German psychiatrist and neuropathologist, Alois Alzheimer, in 1906. He noticed many unusual clumps and neurofibrillary tangles in brain autopsy of his 50-year old patient Auguste D., who suffered from progressive memory loss and personality changes [122]. Nevertheless, the finding did not achieve much attention. Until 1910, the term 'Alzheimer's disease' was introduced in the 8th edition of the textbook 'Psychiatry' by Emil Kraepelin, a renowned German psychiatrist and Alois Alzheimer's superior. Since then, it has been extensively accepted and diagnosed gradually around the world [123, 124]. However, it took almost a century for the understanding of the molecular biology underlying the clumps which spread over AD brains when the disease progresses to evolve; these pathologic hallmarks include the extracellular deposition of insoluble amyloid β (Aβ) that contribute to senile plaques and tau mediated intracellular aggregation of neurofibrillary tangles [125, 126].

Present:

AD now is the most common form of dementia and the most prevalent chronic neurodegenerative disorder manifesting with progressive cognitive impairment, spatio-temporal disorientation and executive dysfunction. It is considered to be associated with neurotransmitter decrease, plaques and tangles-induced irreversible neuronal injury and synaptic dysfunction that usually starts from the entorhinal cortex and spreads across the neocortex [127]. There are many etiological hypotheses, and in almost all cases, Aβ, tau and Apolipoprotein (APOE) and other genes are deemed to play important roles [128]. However, the exact mechanism driving the onset of AD are not fully clarified up to now.

Diagnostic criteria for AD were revised four times from the first version that was proposed in 1984 to the guidelines in 2018, which were issued by National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders

Association and International Working Group, or National Institute of Aging-Alzheimer's Association working group [129-133], in order to standardize diagnosis and improve accuracy. Even though autopsy is the gold standard for AD diagnosis, this is impractical in clinical routine settings where patients have to be identified in vivo. Thus, diagnosis is based on clinical symptoms, cognitive examinations and biological assessments such as brain imaging. With the discoveries of fluid biomarkers and development of neuroimaging techniques, the accuracy of AD clinical diagnoses has increased. However, the sensitivity and specificity still need to be further investigated with more clinical applications. The average duration of patients living with AD symptoms is 8-10 years, but there is a long preclinical stage during which plaques and tangles slowly accumulate in the brain over several years without causing symptoms [134].

Although billions of dollars have been spent both by governments and industry to develop effective disease-modifying AD drugs, no effective method has been developed to stop or retard this devastating degeneration so far. Hence, some researchers instead try to concentrate on preventative strategies, targeting preclinical, prodromal periods and mild cognitive impairment related to AD, to discover the optimal therapeutic time window [135]. Interventions to reduce chronic inflammation prior to apparent clinical manifestation might protect against the onset of AD [136, 137], however, clear evidence about therapeutic effectiveness has yet to be shown [138, 139].

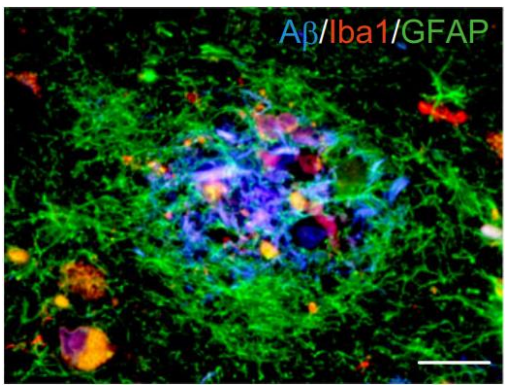
Future:

As estimated, the population aged over 65 worldwide will rise to approximately 1 billion in 2030 [140]. With the coming of aging society, AD has been regarded as one of the biggest health challenges in the 21st century [141]. It is not only a great physical and mental burden, but also increases the economic pressure on patients, their families and society. Currently, there are more than 44 million people suffering from AD globally, and the prevalence will double by 2050 [142]. The annual total cost people spend on healthcare for AD is estimated to increase to about $500 billion in the United States by 2040 [143, 144]. In Germany, caregivers have to spend nearly 97 hours every week to take care of a sick family member with severe AD [145]. In other words, AD will affect more people, far beyond our statistical evaluation.

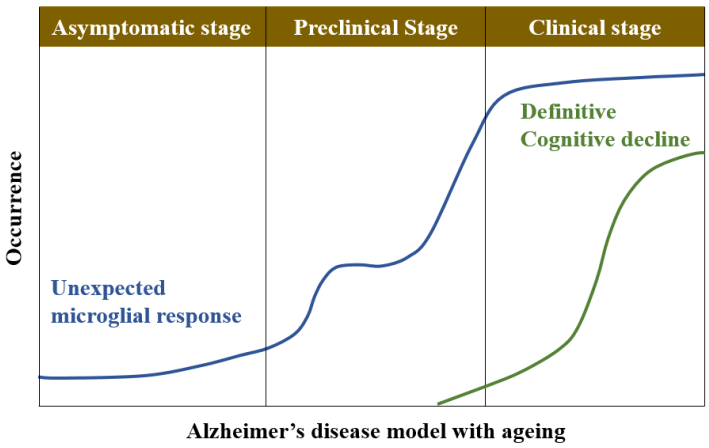
As a result, to clarify the possible pathogenesis of AD and explore feasible therapeutic strategies become urgent issues in the medical field.

5.2. CHRONIC NEUROINFLAMMATION AND AD

Over the years, a host of studies focused on the mechanisms of AD and proposed some hypotheses, among which chronic neuroinflammation is a well-accepted causative factor, and glia cells are meant to protect the microenvironment in the brain, including microglia and astrocytes, take part in the process. In the preclinical stage of AD, inflammation arises with mild stimuli, presenting with more glia cells activated and gathered around the Aβ plaques Fig(9) [146]. Together with tau oligomers aggregation, microglia fail to play their defensive and clearing roles, hence the debris and plaques accumulate between neurons [147]. Moreover, defective microglia with TREM2 mutations, which lose their receptor to clear Aβ, result in impaired phagocytosis [148]. In addition, the abnormally activated cells secreting pro-inflammatory cytokines, further accelerate neuronal damage. Compared with controls, the pro-inflammatory cytokines, such as TNF-α and IL-6 were found elevated in AD brains [149]. Some of these cytokines could promote the formation of plaques by modulating γ secretase, resulting in more amyloid precursor protein being cleaved into Aβ [150, 151]. It evolves to be a vicious circle in late stage AD, contributing to the persistent inflammation in AD models [152] Fig(10).



Fig(9) [146]. Dense-cored Aβ plaques are in blue. Ionized calcium binding adaptor molecule 1 (Iba1): the marker of microglia, microgliosis stained in red; Glial fibrillary acidic protein (GFAP): the marker of astrocyte, astrocytosis stained in green.



Fig(10). The chronic neuroinflammatory AD model with ageing: unexpected microglial activation occurs earlier than the patients have typical cognitive impairment. The defective microglia chronically cause injury of the neuronal networks during the whole stages of AD [152].

The innate immune response in the brain has been proven to influence neurodegeneration. Meanwhile, some reports suggested that the peripheral inflammation, to a certain extent, may aggravate the response in the CNS [153]. A clinical study based on the Framingham Heart Study offspring cohort evaluated the relationship between serum C-reactive protein (CRP), a biomarker of mild chronic systematic inflammation, and AD together with brain volume. In comparison with non-carriers of the *APOE* ε4 AD risk allele, ε4 carriers with higher CRP levels had an increased risk of developing AD and related hippocampus atrophy [154].

The chronic neuroinflammation continually driven by some unknown stimuli, not only the simple toxic protein deposition, might act as a trigger or synergy factor for the onset of Alzheimer's disease.

5.3. INFECTION COULD BE THE CONTRIBUTOR

With years of efforts, people gradually understand that the microbe *Helicobacter pylori* (*Hp*) causes peptic ulcers, as well as gastric cancers, and long-term Hepatitis B infection leads to hepatic cell carcinoma [155]. There, we are tempted to ask: is the chronic neuroinflammation in AD triggered or accelerated by infection?

Actually, the infection hypothesis of AD was proposed 30 years ago. In the early 1990s, Ruth F. Itzhaki and her colleagues first identified the evidence of Herpes Simplex Virus Type

1 (HSV-1), which is responsible for the common sores surrounding the mouth and lips, in AD patients' brains [156]. Later in 1998, Alan P. Hudson detected the positive genetic presence of *Chlamydia pneumoniae* (*Cpn*) in the typical neuropathology among the majority of tested AD patients (17/19), which was rarely seen in control samples (1/19) [157]. Then this medical direction attracted more and more physicians' and researchers' work. Subsequent studies further suggested although HSV-1 infected over half of the people worldwide, and always occurs in childhood, resulting in lifelong infection [158], there is no obvious positive signal in the brain tissues of young adults, which is in contrast to the high frequency of HSV-1 DNA in elderly people, indicating the virus may hide somewhere in the body and enter into the brain with age as the immune system weakens [159]. It was shown that in HSV-1 infected populations carrying the *APOE* ε4 allele e AD risk is increased [160]. Multiscale analysis based on new bioinformatics technologies reveals that human herpesviruses 6A and 7 are abundant in post mortem entorhinal cortex and hippocampus from individuals with AD, and the viral activity is closely connected with AD susceptibility genes, for instance *PSEN1* and *BACE1* [161]. Moreover, some other pathogens, such as Cytomegalovirus (CMV) [162] and *Hp* [163], were also possibly relevant to the progression of AD, but the clinical conclusions remain controversial because of inconsistent findings, with some teams failing to confirm the association [164].

On the other hand, the link between infectious agents and neuroinflammation with Aβ amyloidosis was established by basic research. In human neural cell culture and transgenic AD mice, the soluble Aβ oligomer, acting as an antimicrobial peptide, which fights against viral and bacterial infections, was found to have a protective role in the CNS immune response [165]. But conversely, the protective behavior promotes seeding and Aβ peptide deposition in the brain [166].

The infectious etiology of AD has been debated and deliberately ignored for decades, including some authoritative organizations [167]. However, the evidence we have generated so far pushes us to reevaluate this possibility.

It is time to carefully summarize clinical evidences and investigate whether it could be the

strong support.

5.4. A SYSTEMATIC REVIEW AND META-ANALYSIS

Every year, thousands of medical studies are published in different journals all over the world. It is impractical for either physicians or researchers to evaluate all the new information or findings, even within the field that they are interested in. Besides, individual studies are often one-sided. Reading narrative reviews is a good choice for understanding researchers' ideas about a problem, but still not sufficient to reach an all-round understanding, because the author's subjective preference is inevitable. It is also unreliable to make clinical decisions with possible false positive results and small sample size[168].

A systematic review and meta-analysis is a scientific, quantitative and objective solution to the issue mentioned above, whereby it is possible to integrate a large number of research results. Combining various clinical results from different sources is not a simple task and was first tried by K. Pearson in 1904 [169], then gradually establishing standardization of outcomes that could be evaluated on the same scale and calculated together [170, 171]. The optimization of methodologies led to systematic review and meta-analysis becoming widely applied in an increasing number of medical fields. In 1993, the Cochrane Collaboration was established as a global collaborative network that is determined to help to produce high quality systematic reviews, setting standards and guidelines for the synthesis of researches [172].

The procedure when performing a systematic review and meta-analysis has been well established, including literature review, brief and detailed study screening, valid data extraction and statistical analysis by software, such as Review Manager 5, Stata and Comprehensive Meta Analysis.

The general introduction and comparison among these three frequently-used softwares are presented below.

1) Review Manager 5 (RevMan 5)

Review Manager is an official free software of the Cochrane Collaboration that was developed for preparing not only Cochrane reviews but also reviews from other sources. We could use it

to write intervention review, diagnostic test accuracy review, methodology review, overview of reviews and flexible review depending on different requirements. The operation interface is new users friendly, we were able to find where to enter our data and generate images quickly [173].

2) Stata

Stata is a powerful software with statistical packages for distinct purposes, including meta-analysis associated macros, such as meta-regression, publication bias and more, which can be conveniently downloaded and incorporated into Stata. We can encode in a dialog box, run entered program, and view the sent results in a DOS-like window. This command-driven software allows us to manage our data and create tables and graphs [174].

3) Comprehensive Meta-Analysis (CMA)

CMA is a commercial software for meta-analysis. It provides an interactive menu-driven program, and accepts various formats of data involving the three most common ones: events and sample size, mean and standard deviations, point estimate and confidence interval (CI). We can enter different data types in the same analysis and produce pooled effect size without any other calculational actions [175].

4) Comparison

Table(4). Comparisons among three softwares [176, 177].

Software	Advantages	Shortages
RevMan 5	1. Free of charge 2. Easy to follow	1. Limited functions for meta-analysis, such as meta-regression, Egger's test, L'Abbe plot et al. cannot be conducted 2. Requires the same format of entered data
Stata	1. Comprehensive functions for meta-analysis	1. Requires the same format of entered data 2. Forest plot provides limited options(study name, the point estimate and CI) for customization
CMA	1. Accepts more than 100 formats of entered data 2. Copy and paste data are much simpler	1. Not ideal for diagnostic test accuracy review

The fixed-effect model and the random-effects model are the two statistical models in meta-

analysis. For the purpose of estimating correct general effect size, it is vital to select the appropriate model. Unlike the fixed one which only considers study internal variation, the random one assesses both internal and external variance among studies, on account of the assumptions of these two models [178]. Participants in different trials may be exposed to diverse environments or experience multi-purpose interventions, exhibiting substantial diversity. Thus when we extract data, we are unable to assume all the studies shared one true effect size characterized by the fixed effect model. As such, we need to choose random effect model [179].

At the mention of meta-analysis, we have to realize and deal with the presence of heterogeneity and bias. Subgroup analysis could be used to explore and exclude the potential source of heterogeneity and emphasize clinicians' interested elements [180]. Whereas excessive use of subgroups should be avoided as it breaks randomness of the original studies to some extent. Sensitivity test, performed by leaving out studies one by one, could also be performed to evaluate heterogeneity, finding the influence of each included study [181]. As the meta-analysis always summarizes published papers, but the journals prefer to accept manuscripts with significant results, publication bias occurs more frequently than we thought [182]. The egger's regression test, trim and fill method were performed to check this bias, assisting us to understand the pooled effect size correctly.

Systematic review and meta-analysis is a high-efficiency bridge, linking previous and subsequent researches. Before we clarify the relationship between infection and Alzheimer's disease. Three previous meta-analyses [183-185] that already explored this topic should be mentioned, however, all three were characterized by a focus on a single type of infection and limited subgroup analyses. In addition, with the development of new detection methods and more standardized phenotyping of clinical cohorts, additional studies with large sample sizes have emerged. We therefore undertook a new meta-analysis to synthesize the current evidence relating to the most common sources of infection, viruses and bacteria, and their association with risk of AD aiming to provide a more comprehensive and detailed overview.

6. MATERIALS AND METHODS

6.1. STUDY PROTOCOL

To obtain consistent and unbiased results, standardized protocols must be followed while preparing a systematic review and meta-analysis, with a particular focus on extracting the relevant data from primary studies. Therefore, the thesis was performed following the guidelines proposed by the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) Statement [186].

6.1.1. What are PROSPERO and PRISMA-P

PROSPERO: *International Prospective Register of Systematic Reviews*

This is an online registry, where researchers can directly register the planned systematic reviews of human studies or animal research. It is supported by the US National Institute for Health Research. One purpose of the website is to reduce unwanted duplication. When conducting a systematic review, we could start with searching relevant keywords on the website, to determine whether there are no associated systematic reviews or if an update of existing reviews is required. Moreover, authors are required to provide documentation of methods, containing inclusion and exclusion criteria, outcomes, progress and other key characteristics during the registration, which helps to increase transparency in the process of completing a review [187].

6.1.2. PRISMA-P: *Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols*

A 17-items (26 sub-items) checklist, PRISMA-P, was published to help investigators prepare documentation and improve the quality of every registration in PROSPERO that is mentioned above. The official protocol consists of three main parts: administrative information, introduction, and methods. For new users, the protocol could help them get familiar with the rationale of the review and preplanned approaches about how to carry out the project, avoiding amendments that would affect results. For experienced users, it prevents arbitrary decision-making and may even offer the opportunity to find available collaboration [188].

6.2. SEARCH STRATEGY

The literature review included a combination of search terms: ‘Alzheimer*’ AND ‘infection’ OR ‘viruses’ OR ‘bacteria’. Studies were identified through searching Pubmed, a free search engine comprising the MEDLINE database, life science journals and some online books. All searches were performed before June 4th, 2020 with no time span specified. ‘Alzheimer*’ AND ‘infection’ : n=4214; ‘Alzheimer*’ AND ‘viruses’ : n=1752; ‘Alzheimer*’ AND ‘bacteria’ : n=2316. (n: number of papers)

6.3. STUDY SELECTION

All articles yielded with the keywords were imported to EndNote X9. Duplicate references were first removed. Then the titles and abstracts were scanned to evaluate topic relativity according to the inclusion and exclusion criteria described in Part 6.4. For eligible articles check, we read the full text for further assessment. The doubt of article recruitment was resolved via discussion with my supervisor.

6.4. INCLUSION AND EXCLUSION CRITERIA

The eligible studies included in this systematic review and meta-analysis should meet the following criteria:

a) Papers were published in a peer-reviewed journal with original data;

b) Case-control studies;

c) Study subjects were humans with AD and control groups; AD patients were diagnosed by clinical presentations and/or confirmed histologically. Control subjects were free of any form of neurodegeneration.

d) The precise number of positive and total individuals in each group was presented, so that OR and 95% CI could be calculated.

e) Full text in English.

The exclusion criteria are as follows:

a) The patients have other unspecified dementias;

b) Other types of text, such as review, book chapter, conference abstract;

c) Other studies, such as case reports, case series;

d) Basic research, such as animal or cellular studies, not human tissues studies.

6.5. DATA EXTRACTION

The pertinent papers were entirely read and evaluated for the following data extraction. Extracted data include author's names, year of publication, institute, cohort wherever applicable, country, study population, pathogens involved in each study, number of positive cases in AD, AD cases, number of positive cases in control, control cases, detection method, materials tested, AD patients with *APOE* ε4 allele evidence.

6.6. STATISTICAL METHODS

Stata (version 14; Stata Corp, College Station, TX, USA) was used in our study to investigate the relationship between infectious risk and the development of AD statistically. The fixed effect model was initially selected if minimal variation was found, otherwise, a random-effect model was chosen instead [189]. Under appropriate effects models, the corresponding OR and 95% CI of respective study and pooled effect size were calculated.

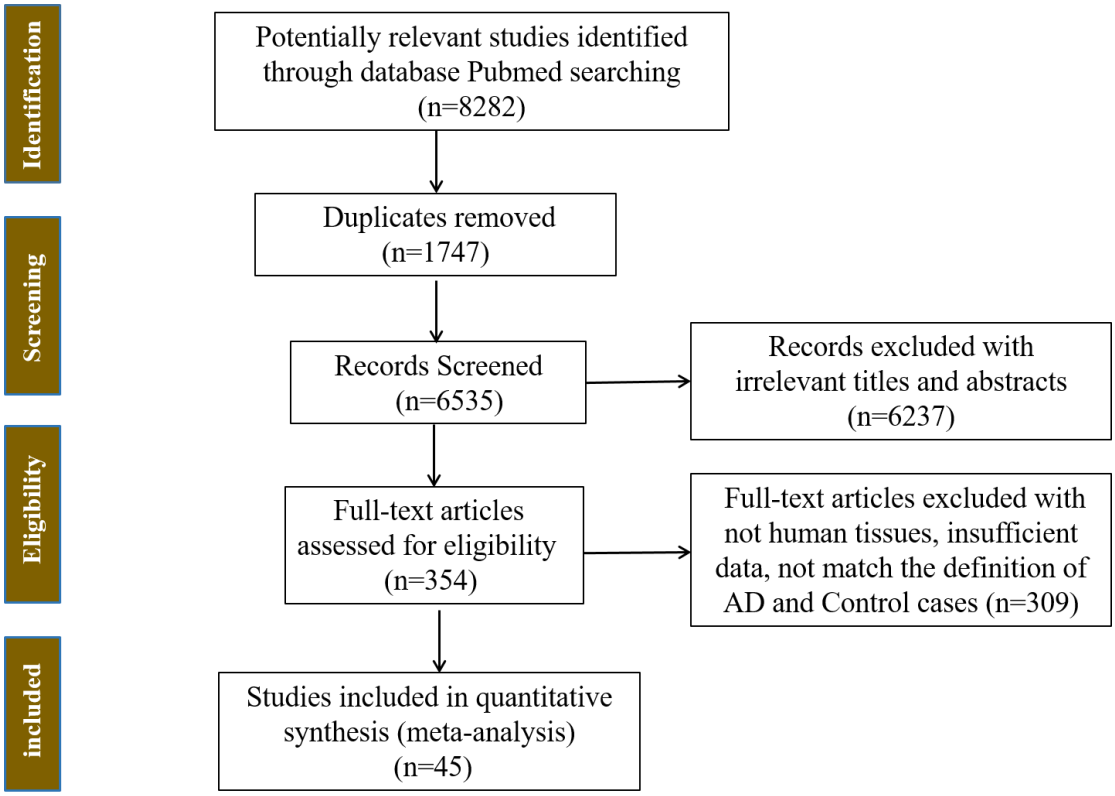
Heterogeneity of initial results among studies was evaluated by Cochran's Q ($\chi 2$) and I^2 statistics. Q test with P-value indicates the existence and inexistence of heterogeneity. Besides, to avoid the insufficient test power due to few studies included in the analysis, we performed another corrected method for compensation [190]. I^2 statistics report the extent of heterogeneity [191]. Different I^2 values, 25, 50, and 75%, represent low, moderate and high degrees of heterogeneity, respectively [192]. A p-value less than 0.10 or an I^2 index of>50% is considered to indicate substantial heterogeneity [193].

Funnel plot, visualized scatterplots without evaluated value, is a qualitative tool for publication bias analysis, and the egger test is designed for quantifying funnel plot asymmetry [194]. It is recommended for the work with more than 10 included studies, because if the data size is small, the test may not have enough statistical power to find the bias [195]. We also used the Trim and Fill method to assess publication bias, and recompute an adjusted summary effect size [196]. The first step is to exclude studies with small sample size, and then add the assumptive missing

studies that could make the funnel plot symmetric [197]. The assumption of this method only imputes funnel plot asymmetry to publication bias, which is a one-sided view. Hence, it should be regarded as a tool of sensitivity analysis. Another sensitivity analysis method, leave-one-out, was also performed to identify influential studies in this meta-analysis. During the process, meta-analyses were conducted circularly by excluding each included study at each analysis, in order to investigate the effect of every single study on the final pooled effect size [198]. For subgroup analysis, the data are divided into several groups defined by a categorical criterion, such as geographical region, methods of examination, materials tested and AD patients with or without *APOE* ε4 allele. The comparison of estimated effect value among subgroups helps us to further assess the variance in each group and explore the source of heterogeneity [199].

7. RESULTS

7.1. CHARACTERISTICS OF STUDIES



Fig(11). Flow chart of study selection process.

As outlined in Fig(11), in total, there were 8282 potentially relevant records identified through literature review, of which 1747 were directly excluded because of duplication. The titles and abstracts of the remaining 6535 results were carefully screened. Of them, 6237 studies with different topics were removed. Then we read the full text of the selected 354 articles for eligibility checking, 45 of which met all inclusion and exclusion criteria for quantitative synthesis. The detailed information of each study was presented in Table(5), including publication year, the corresponding institute and country where the researchers work, the cohort from which they obtained human samples, pathogens involved in each study, the respective number of AD patients and control cases, tissues or body fluid tested, detection method, and evidence that reveal the potential role of *APOE* ε4 together with pathogens in the development

of AD.

Table(5). Characteristics of included studies.

Reference, Year	Institute/Cohort	Country	Pathogen	Materials tested	Detection method	AD patients with *APOE* ε4 allele evidence
[200], 1983	University of Manchester	United Kingdom	HSV	Brain tissue	Immunohistochemistry (HSV antigen VA)	No
[201], 1989	Montrose VA Hospital	United States	Spirochetosis: Treponema; Borrelia	CSF	Fluorescent Antibody Test	No
[202], 1990	Centre Hospitalier Universitaire	France	HSV-1, CMV, VZV, EBV	Blood	ELISA (anti-HSV-1 ab, anti-CMV ab, anti-VZV ab); Anticomplement IF test (Ab to Epstein-Barr nuclear antigen)	No
[203], 1992	University of Manchester / Bristol Brain Bank; Edinburgh Brain Bank	United Kingdom	HSV-1	Brain tissue	PCR (DNA, viral TK gene)	No
[204], 1993	University of Lausanne	Switzerland	Spirochetosis	Blood, CSF, Brain tissue	Dark field microscopy	No
[205], 1996	University of Manchester / by G. Wilcock	United Kingdom	HSV-1, VZV	Brain tissue	PCR (HSV-1: TK, ICP0 gene; VZV: origin of replication (OR); TK gene)	Yes
[206], 1997	University of Manchester	United Kingdom	HSV-1, VZV	Brain tissue	PCR (HSV-1: TK gene; VZV: OR; TK gene)	No
[207], 1997	University of Manchester	United Kingdom	HSV-1	Brain tissue	PCR (HSV1: TK gene)	Yes
[208], 1997	Tohoku University School of Medicine	Japan	HSV	Brain tissue	PCR (HSV glycoprotein D DNA (type I and II))	Yes
[209], 1998	McGill University	Canada	HSV-1	Brain tissue	PCR (HSV-1 glycoprotein D gene)	Yes
[157], 1998	Allegheny University of the Health Sciences / Harvard Brain Tissue Resource Center (Boston, Mass., USA) and the MCP-Hahnemann School of Medicine Department of Pathology (Philadelphia, Pa., USA).	United States	*Cpn*	Brain tissue	PCR	No
[210], 1999	McGill University / Patients: a double-blind controlled clinical drug trial; Controls: a World Health Organization sponsored longitudinal study	Canada	Spirochetosis	Blood	Dark field microscopy	No
[211], 2000	Beckman Research Institute at the City of Hope / the Alzheimer's Disease Research Center Neuropathology Core, USC School of Medicine.	United States	*Cpn*	Brain tissue	PCR (*Cpn* DNA)	No
[212], 2000	National Institutes of Health / Johns Hopkins University Alzheimer's Disease Research Center (Baltimore; National Institutes of Health grant AG 05146)	United States	Spirochetosis: Borrelia	Brian tissue	PCR	No
[213], 2001	National Institutes of Health / by Dr Juan Troncoso from the Johns Hopkins University Alzheimer's Disease Research Center	United States	HSV-1	Brain tissue	PCR	No
[214], 2002	University of Manchester	United Kingdom	HSV-1, HSV2, CMV, HHV-6	Brain tissue	PCR	No
[215], 2002	University of Bristol, Frenchay Hospital	United Kingdom	*Cpn*	Brain tissue	PCR and IHC	No
[216], 2002	Oregon Health and Sciences University / Oregon Brain Bank (Oregon Health and Sciences University, Portland)	United States	Spirochetosis: Treponema	Brian tissue	IHC; PCR	No
[217], 2003	University of Turku	Finland	HSV-1, VZV, HHV-6	Brain tissue	PCR	Yes
[218], 2003	University Hospital of Linköping / the Clinic of Geriatric Medicine at the	Sweden	*Hp*	Blood	ELISA (anti *Hp* ab)	No

	University Hospital, Linköping, Sweden. The time period for patient inclusion was January 1995 to December 1997.					
[219], 2003	University of Manchester	United Kingdom	*Cpn*	Brain tissue	PCR (*Cpn* gene for rRNA)	No
[220], 2004	Aichi Medical University School of Medicine	Japan	HSV-1	Brian tissue	PCR (HSV-1 glycoprotein D gene)	No
[221], 2005	The University of Manchester / (a) the Oxford Project To Investigate Memory and Ageing (OPTIMA); (b) South West dementia brain bank (University of Bristol); (c) University Hospitals Alzheimer Center, Fairhill Center for Aging, Cleveland, Ohio, USA; (d) Oxford Radcliffe Hospitals NHS Trust.	United Kingdom	HSV-1, HHV-6	Blood, CSF	ELISA (anti HSV-1 IgG)	Yes
[222], 2005	Showa University School of Medicine / Showa University Karasuyama Hospital	Japan	*Cpn*	Blood	ELISA (*Cpn* specific IgG and IgA)	No
[223], 2006	Aristotle University of Thessaloniki, Ippokration Hospital	Greece	*Hp*	Blood, Gastric mucosa biopsy	ELISA (anti *Hp* ab)	No
[224], 2006	Wayne State University School of Medicine / the Michigan Alzheimer's Disease Research Center (Ann Arbor, MI), the Canadian Brain Tissue Bank (Toronto, Ontario, Canada), the UCLA Brain Tissue Bank (Los Angeles CA, USA), and the Alzheimer's Research Center of the Health Partners Research Foundation at Regions Hospital (St Paul, MN, USA).	United States	*Cpn*	Brain tissue	PCR	Yes
[225], 2007	Medical University, Traugutta	Poland	*Cpn*	CSF	PCR	No
[226], 2008	S. Gerardo Hospital / Dementia Outpatient Unit	Italy	Spirochetosis: Borrelia	Blood	*Borrelia burgdorferi* IgG/IgM antibodies by Enzyme Linked Fluorescent Assay	No
[227], 2010	Philadelphia College of Osteopathic Medicine / Dr. William Hill of the Medical College of Georgia (Augusta, GA), from the MCP-Hahnemann School of Medicine Department of Pathology, currently Drexel University College of Medicine (Philadelphia, PA), and the Alzheimer's Research Center of the Health Partners Research Foundation at Regions Hospital (St Paul, MN)	United States	*Cpn*	Brain tissue	IHC (anti Chlamydia ab)	No
[228], 2011	Oita University Faculty of Medicine / Memory and Dementia Outpatient Clinic at the Department of General Medicine in Oita University Hospital from August 2002 to March 2009	Japan	*Hp*	Urine	A rapid urine test (RAPIRUN *H. pylori* antibody, Otsuka Pharmaceutical Co., Tokyo, Japan)	No
[229], 2013	The Jikei University School of Medicine / The Jikei University Hospital (Tokyo) or The Jikei University Kashiwa Hospital (Kashiwa city, Chiba prefecture).	Japan	HSV-1	Blood	ELISA (anti HSV-1 IgG)	No
[162], 2013	University of Bologna / Blood: the longitudinal "Conselice study" (Ravaglia et al., 2001); Brain tissue: Brain Bank of the Department of Neurosciences and Pathology, University of CaliforniaeSan Diego.	Italy	CMV, EBV, HHV-6	Blood (peripheral blood leukocytes); Brain tissue	PCR, ELISA (anti CMV IgG, EBV Epstein Barr nuclear antigen IgG, EBV viral capsid antigen IgG, and HHV-6 IgG).	Yes
[230], 2013	Uppsala University / Memory Clinic at the Department of Geriatrics in Uppsala University Hospital	Sweden	CMV	Blood	ELISA (anti CMV IgG)	No

[231], 2014	Third Military Medical University / Chongqing Daping Hospital and Chongqing Mental Health Center from January 2012 to June 2013	China	HSV-1, CMV, *Hp*, *Cpn*	Blood	ELISA (anti *Cpn*, *Hp*, CMV and HSV-1 IgG)	No
[232], 2014	Umea University / Medical Biobank in Umea (The Northern Sweden Health and Disease Study Cohort	Sweden	HSV	Blood	ELISA (anti HSV IgG, IgM), but the reactive antigen was acquired by growth of HSV-1 virus	No
[233], 2014	University of Milano / Fondazione Don Gnocchi, IRCCS in Milano	Italy	HSV-1	Blood	ELISA (anti HSV-1 IgG)	No
[234], 2014	University of Milano / dementia clinic of the Fondazione Don C. Gnocchi (Milano, Italy)	Italy	HSV-1, CMV	Blood	ELISA (anti HSV-1, CMV IgG)	No
[235], 2015	Hippokrateion General Hospital	Greece	*Hp*	Gastroscopy and histological examination(tissue specimens), Blood (serum)	Cresyl-Violet staining, *Hp* IgG antibody	No
[236], 2016	University of Milano / Fondazione Don Gnocchi, IRCCS in Milano	Italy	HSV-1, CMV, HHV-6	Blood	ELISA (anti HSV-1, CMV, HHV6 IgG)	No
[237], 2016	University of California at Davis / Alzheimer's Disease Center at the University of California Davis	United States	*E. coli*	Brian tissue	Western Blot	No
[238], 2017	Umea University, Uppsala University / Medical Biobank in Umea (Northern Sweden Health and Disease Study)	Sweden	HSV-1, HSV-2, CMV	Blood	ELISA (anti HSV-1, HSV-2 IgG, CMV IgM IgG)	No
[239], 2018	University of Milan / Rehabilitative Neurology Unit of the IRCCS Santa Maria Nascente, Don C. Gnocchi Foundation – ONLUS, Milan, Italy	Italy	HSV-1	Blood	ELISA (anti HSV-1 IgG)	No
[240], 2019	Umea University / Medical Biobank in Ume a (Northern Sweden Health and Disease Study)	Sweden	HSV-1, HSV-2, CMV, *Cpn*	Blood	ELISA (anti HSV-1, HSV-2, Cpn IgG, CMV IgM IgG)	No
[241], 2020	Uppsala University	Sweden	CMV	Blood	ELISA (anti CMV IgG)	No
[242], 2020	National Institutes of Health / Mount Sinai Brain Bank; Religious Orders Study, Memory and Aging Project; Johns Hopkins Brain Resource Center	United States	HHV-6, EBV, CMV	Brain tissue	RNA-Seq; ddPCR	No

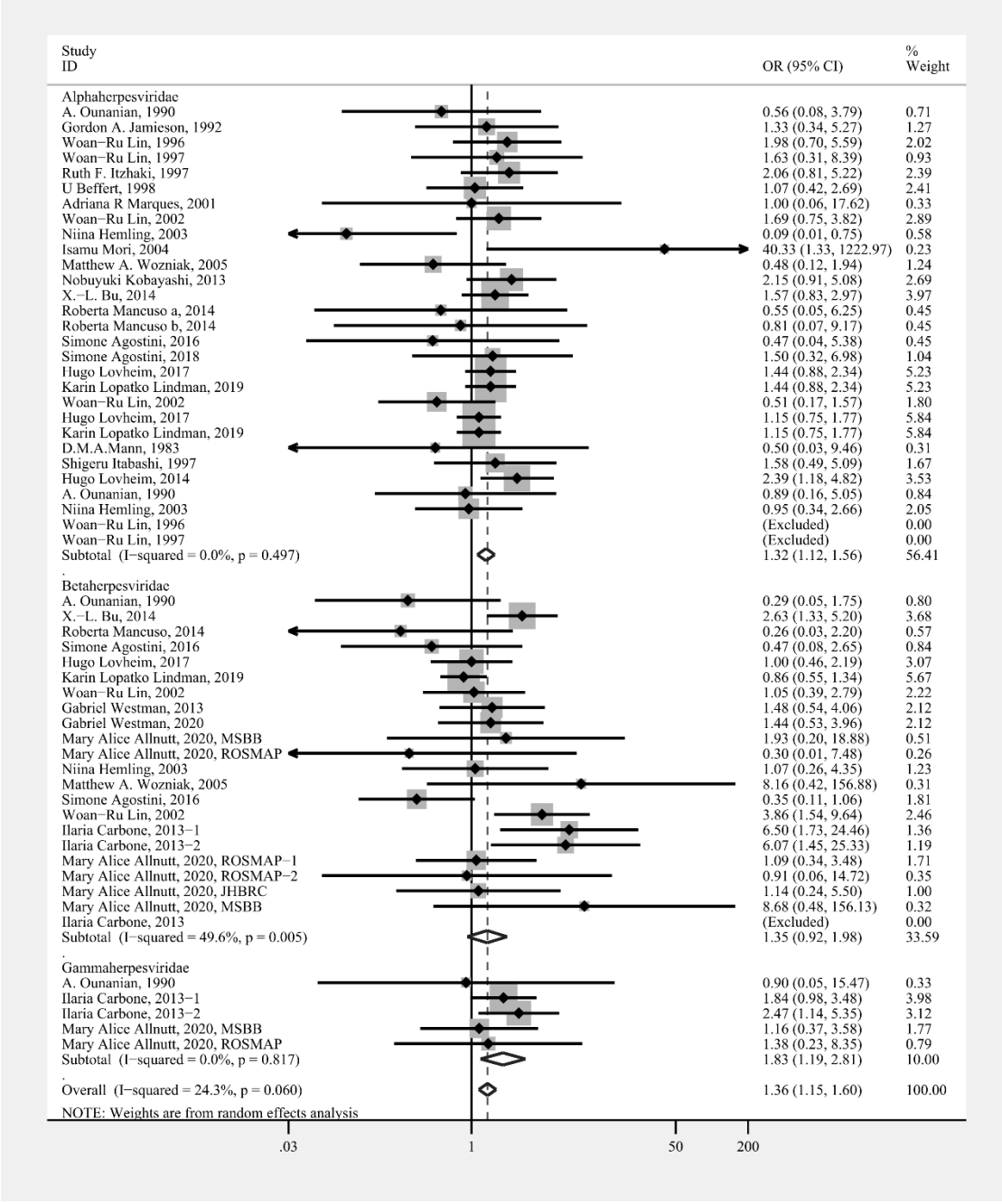
Herpes Simplex Virus (HSV), Herpes Simplex Virus Type 1 (HSV-1), Herpes Simplex Virus Type 2 (HSV-2), varicella zoster virus (VZV), Epstein–Barr virus (EBV), Cytomegalovirus (CMV), Human Herpes Virus Type 6 (HHV-6), *Chlamydia pneumoniae* (*Cpn*), *Helicobacter pylori* (*Hp*), *Escherichia coli* (*E. coli*), Cerebrospinal Fluid (CSF), antibody (ab), Immunohistochemistry (IHC), Immunofluorescence (IF), thymidine kinase (TK), Enzyme Linked Fluorescent Assay (ELFA), Polymerase chain reaction (PCR), RNA sequencing (RNA-seq), droplet digital PCR (ddPCR).

7.2. VIRUS

7.2.1. Herpesviridae increase the risk of AD

Herpesviridae is a group of enveloped, double-stranded DNA viruses. Currently, there are at least 9 types of herpes viruses known to infect human beings, of which 7 types were included in our meta-analysis: HSV-1, HSV-2, varicella zoster virus (VZV), Epstein–Barr virus (EBV), CMV, and Human Herpes Virus 6 (HHV-6 A and B). According to their physicochemical properties, these herpes viruses can be divided into three subfamilies: α (HSV-1, HSV-2, VZV),

β (CMV, HHV-6), and γ (EBV) subfamily.



Fig(12). Forest plot of the association between three subfamilies of herpesviridae (α, β, and γ subfamily), or overall herpes viruses and AD. Johns Hopkins Brain Resource Center (JHBRC), Mount Sinai Brain Bank (MSBB), Religious Orders Study, Memory and Aging Project (ROSMAP).

In total, there are twenty-six papers included in the analysis, comprising 3602 AD patients and 3141 control individuals. To be noted, besides a common case-control study, Ilaria Carbone et al. [162] also focused on a follow up study among 150 elderly people. Some of them developed

AD, and the others remained in good cognitive health at the end of the 5-year follow-up. So we used their EBV and HHV-6 DNA test data in this cohort as well. In Allnutt's et al.'s work [242], they simultaneously detected the presence of HHV-6 in three independent brain banks: Mount Sinai Brain Bank, Religious Orders Study, Memory and Aging Project and Johns Hopkins Brain Resource Center, including 1228 subjects. The studies conducted by Mann et al. in 1983 [200], Itabashi et al. in 1997 [208], and Lovheim et al. in 2014 [232] detected HSV in human tissues, and did not further distinguish the virus into type 1 or type 2. Hence, we classified them into α subfamily, but did not analyze them as any certain type of herpes virus.

Under random effects model, the overall odds ratio (OR) for the herpesviridae family was 1.36, and 95%CI was 1.15-1.60, with low degree of heterogeneity. There was an increased risk of AD with detectable evidence of α and γ subfamily herpes viruses infection, with an OR of 1.32 (95%CI = 1.12-1.56), 1.83 (95%CI = 1.19-2.81), respectively, and no significant heterogeneity was found in both groups. While β subfamily infection has the tendency to increase disease incidence, but did not reach statistical significance. Then we noticed two included papers, by Lovheim et al. and Lopatko Lindman et al.. They worked in the same group and used the same cohort to perform their researches. Thus if there was overlap, we reserved their data of recent work, and generated the new pooled OR, 1.36 (95%CI = 1.13-1.64); I-squared = 26.5%, p=0.05. The general findings were not changed, and the relative outcomes are shown in Table(6). When focusing on specific viruses, HSV-1 and EBV were the two most stable contributors to the onset of AD.

Table(6) New meta-analysis outcomes of herpesviridae related studies, without the same data.

OR(95%CI), *I-squared, p-value*		
Herpesviridae	**Subfamily**	**Herpes Virus**
1.36(1.13-1.64) *I-squared=26.5%, p=0.05*	α subfamily: 1.34(1.09-1.64) *I-squared=3.5%, p=0.41*	HSV-1, 1.41(1.08-1.83), *I-squared=3.9%, p=0.41*
		HSV-2, 0.91(0.45-1.87), *I-squared=42.7%, p=0.19*
		VZV, 0.93(0.38-2.26), *I-squared=0.0%, p=0.95*
	β subfamily, 1.35(0.92-1.98) *I-squared=49.6%, p=0.005*	CMV, 1.09(0.75-1.59), *I-squared=28.0%, p=0.18*
		HHV-6, 2.04(0.98-4.24), *I-squared=57.6%, p=0.01*
	γ subfamily, 1.83(1.19-2.81) *I-squared=0.0%, p=0.82*	EBV, 1.83(1.19-2.81), *I-squared=0.0%, p=0.82*

To identify the relationship between overall, or three subfamilies of herpesviridae (α, β, and γ subfamily), or respective herpes viruses, and AD.

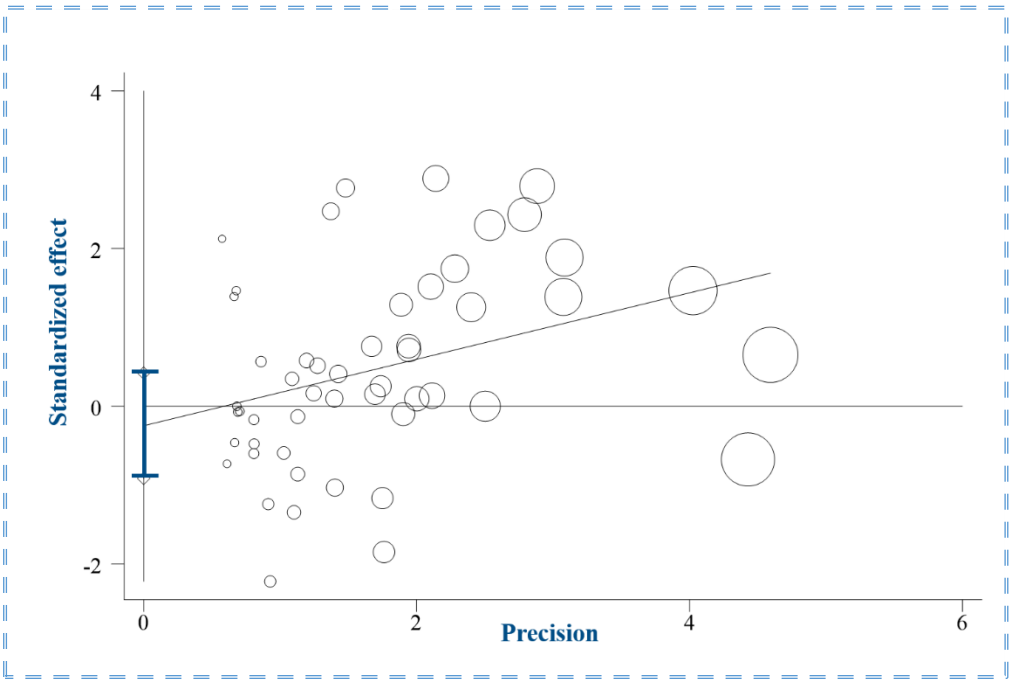
7.2.2. Egger's regression test

Egger's regression test is an effective method to explore publication bias. The precision of effect estimates was regression analyzed by using the standard normal deviation (SND), with the formula: SND = a + b × precision. For binary variable, the OR divided by its standard error (SE) was regarded as a standardized effect, and the reciprocal of the SE was defined as precision [190].

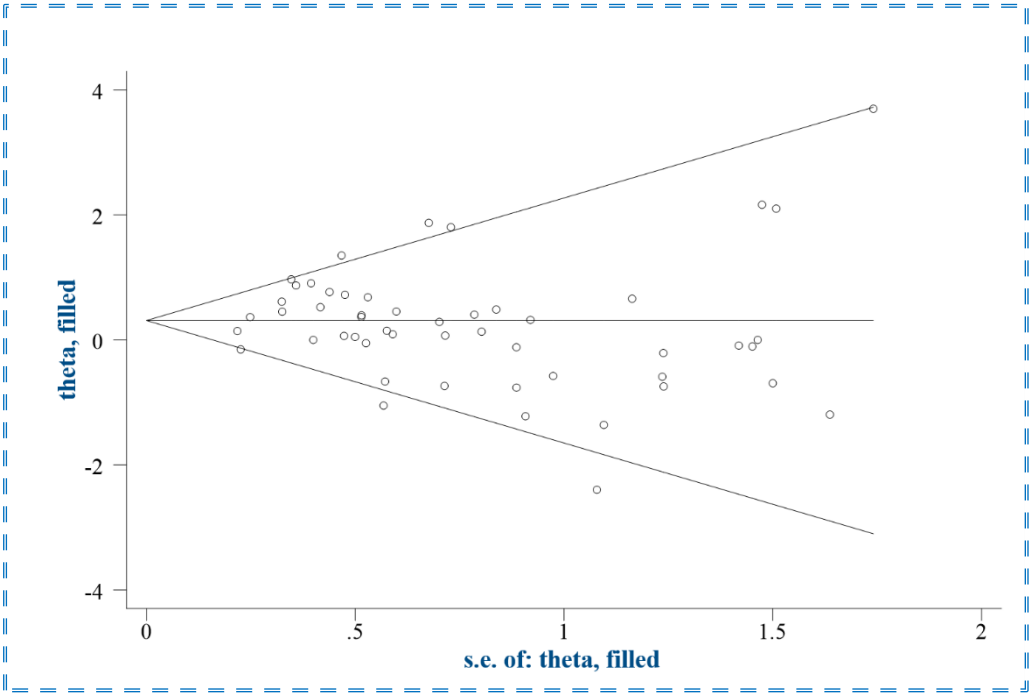
Studies with different sample sizes will produce an extensive range of relative values, such as the larger SE of small sample size trials, so they will be close to both axes, whereas large trials will have a more precise assessment. If there is asymmetry, the regression line will not cross the zero point. So intercept in the graph represents the degree of asymmetry. In our analysis Fig(13), the intercept 'a' was -0.25, and bias p-value was 0.46 (more than 0.1). Egger's regression test suggested that no evidence of publication bias was found in herpesviridae related studies.

7.2.3. Sensitivity analysis

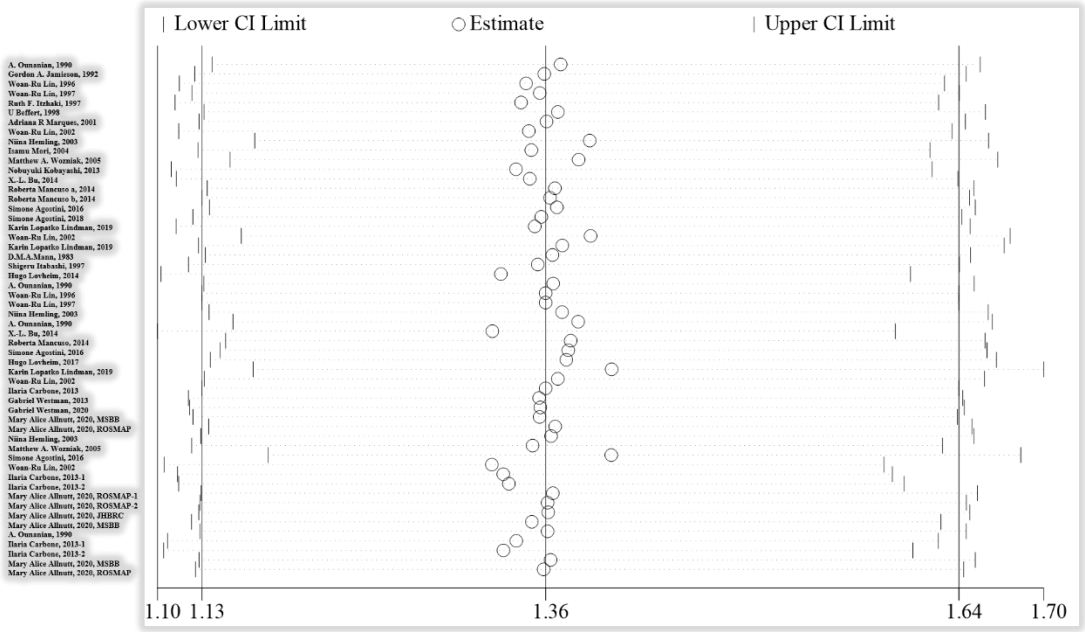
Trim and fill method allows us to reduce the bias in pooled estimates and reexamine previous findings with adjusted OR, if publication bias leads to between-study heterogeneity. The program was performed to look for missing studies. Under fixed and random effect models, the imputed point estimates were 1.34 (95%CI = 1.13-1.58), 1.32 (95%CI = 1.06-1.64), which were the same statistical significance as our initial pooled effect size. Hence, no trimming or filling studies was implemented in the analysis Fig(14). Trim and fill method indicated that there was no missing study in our herpesviridae meta-analysis.



Fig(13). Evaluation of publication bias for herpesviridae relevant studies using Egger's regression test. Each circle represents an independent included study, and the size was determined by weights.



Fig(14). Correction for asymmetric funnel plot attributing to publication bias using trim and filled method. Filled funnel plot with pseudo 95% confidence limits. Each circle represents an independent included study.



Fig(15). Leave-one-out analysis for herpesviridae related studies. Each circle and two vertical lines in the same row represent pooled estimate and 95% confidence interval (CI) without one study, which is listed on the left side.

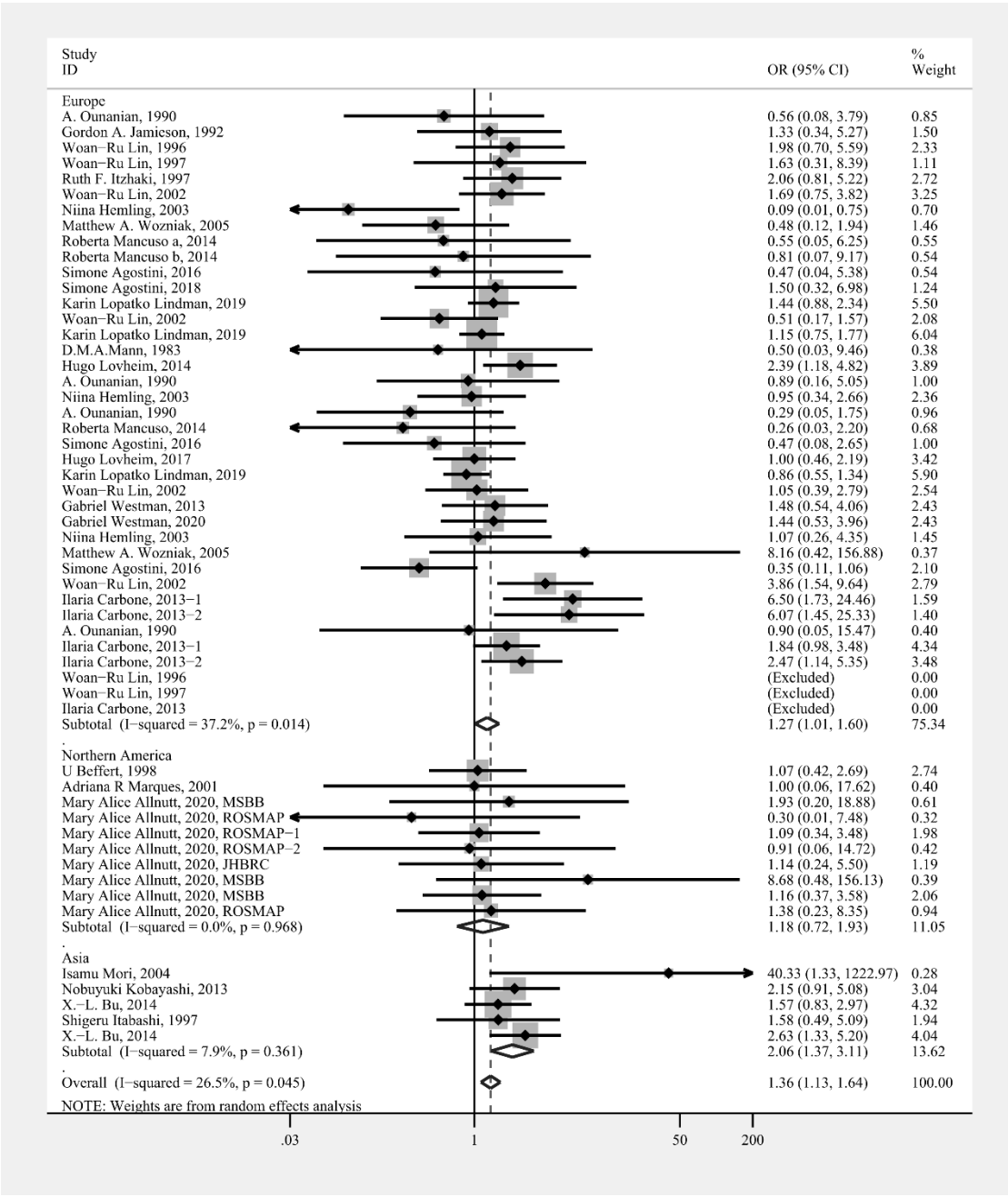
Leave-one-out was used to enhance the robustness of the pooled estimates. In our analysis, no matter which study was removed, the pooled OR and 95%CI were more than 1.10 from the beginning to the end Fig(15). Therefore, although a low-moderate degree of heterogeneity was reported, no influential study that contributed to the heterogeneity was found.

7.2.4. Subgroup analysis

A. Geographical region subgroup

Sorting by geographical regions where the study was performed Fig(16), subgroup analysis showed more explorations between infection and AD were conducted in Europe, with pooled risk estimate 1.27, 95%CI = 1.01-1.60, I-squared = 37.2%, p = 0.01. Ten independent investigations were conducted in Northern America, OR = 1.18, 95%CI = 0.72-1.93, without significant heterogeneity. Four included studies were performed in Asia, OR = 2.06, 95%CI = 1.37-3.11, I-squared = 7.9%, p = 0.36. Results about the pooled outcomes of herpesviridae, its subfamilies and specific herpes virus were shown in Table(7), when there were greater than or equal 5 assessed studies. The presence of α subfamily increased the risk of AD in Asia, OR = 1.88 (95%CI = 1.09-3.23), while EBV infection contributed to the development of AD in

Europe, OR = 2.03 (95%CI = 1.25-3.29).



Fig(16). Forest plot of geographical region subgroup analysis for herpesviridae related studies.

Table(7). Meta-analysis outcomes of geographical region subgroup for herpesviridae related studies.

Geographical Region	OR(95% CI), *I-squared, p-value*						
	Total	**α subfamily**	**β subfamily**	**HSV-1**	**CMV**	**HHV-6**	**EBV**
Europe	1.27(1.01-1.60), *37.2%, 0.01*	1.26(0.99-1.60), *5.6%, 0.39*	1.26(0.79-2.03), *59.4%, 0.002*	1.30(0.94 1.80), *3.5%, 0.41*	0.92(0.67-1.25), *0.0%, 0.62*	2.46(0.86-7.08), *72.5%, 0.003*	2.03(1.25-3.29), *0.0%, 0.72*
Northern America	1.18(0.72-1.93), *0.0%, 0.97*	1.06(0.44-2.56), *0.0%, 0.97*	1.25(0.58-2.70), *0.0%, 0.73*	1.06(0.44-2.56), *0.0%, 0.97*	1.04(0.16-6.65), *0.0%, 0.36*	1.30(0.56-3.03), *0.0%, 0.58*	1.22(0.47-3.16), *0.0%, 0.87*
Asia	2.06(1.37-3.11), *7.9%, 0.36*	1.88(1.09-3.23), *16.5%, 0.31*	2.63(1.33-5.20)	2.12(0.95-4.73), *43.2%, 0.17*	2.63(1.33-5.20)		

Pooled risk estimates, 95% CI, I-squared and p-value of herpesviridae, subfamilies and specific herpes viruses (included studies: n⩾5). Lack of I-squared and p-value meant there was only one study in the analysis.

B. Materials tested subgroup

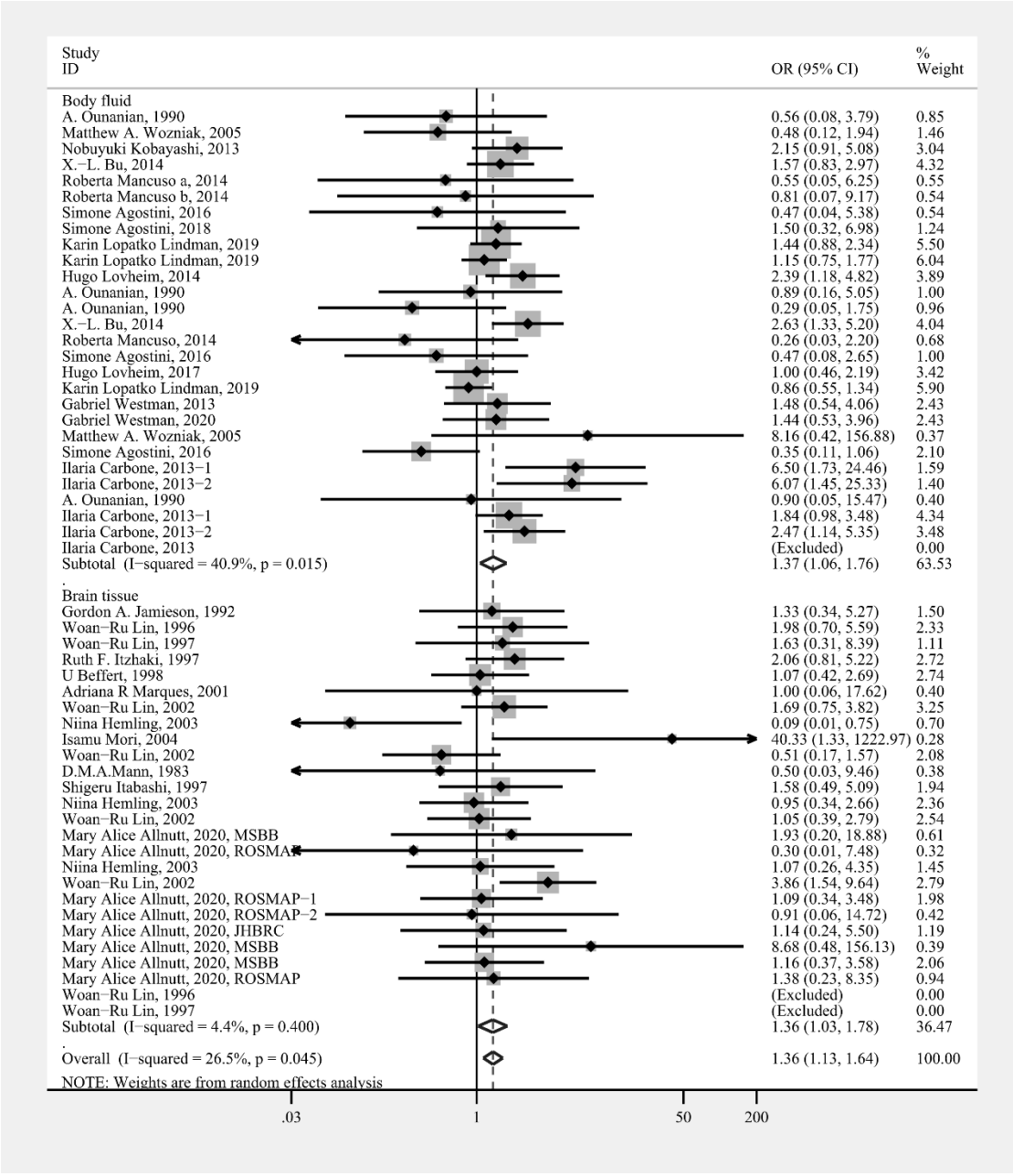
If assuming tested materials could be the confounder for the final results Fig(17), we found when researchers detected the presence of herpesviridae in body fluid, including blood, peripheral blood leukocytes, cerebrospinal fluid, the pooled OR was 1.37 (95%CI = 1.06-1.76) with low-moderate heterogeneity. On the other hand, when tested materials were brain tissues, the OR was 1.36 (95%CI = 1.03-1.78), without obvious heterogeneity. The rest of other analyses were listed in Table(8). The risk estimates and 95% CI of α subfamily and EBV were 1.37 (95%CI = 1.08-1.74), 2.03 (95%CI = 1.25-3.29), respectively with remarkable homogeneity, when researchers applied fluid samples to find the virus.

C. Detection method subgroup

The approach of verifying the presence and absence of herpesviridae could be the variable, which contributed to the outcome heterogeneity Fig(18). When researchers detected antibodies, including IgG, and one study [238] tested IgM by ELISA, the overall point estimate was 1.28 (95%CI = 1.02-1.60) with low-moderate heterogeneity. Then if choosing nucleic acid to find herpes viruses by Polymerase Chain Reaction or RNA sequencing, OR was 1.52 (95%CI = 1.11-2.08), with low-moderate heterogeneity. The risk estimates and 95% CI of α subfamily and EBV were 1.36 (95%CI = 1.07-1.73) and 2.03 (95%CI = 1.25-3.29), respectively, when the anti-virus antibodies were used as detective standard. At nucleic acid level, our analysis demonstrated the evidence of β subfamily and HHV6 infection increase the occurrence of AD, more than two fold, accompanied with I-squared = 28.2%, p = 0.18, and I-squared = 29.1%, p = 0.20, respectively Table(9).

D. *APOE* ε4 subgroup

APOE ε4 is widely considered as an AD susceptibility gene. We found, compared with *APOE* ε4 non-carriers, herpesviridae infection occurred more frequently in *APOE* ε4 carriers among AD patients, 2.07 (95%CI = 1.09-3.95), I-squared = 33.2%, p = 0.13. Subgroup analysis revealed that AD cases had greater possibilities to be infected with HSV-1 if simultaneously carried *APOE* ε4 gene, with a pooled OR of 2.55 (95%CI = 0.73-8.88), but showed obvious heterogeneity Fig(19).

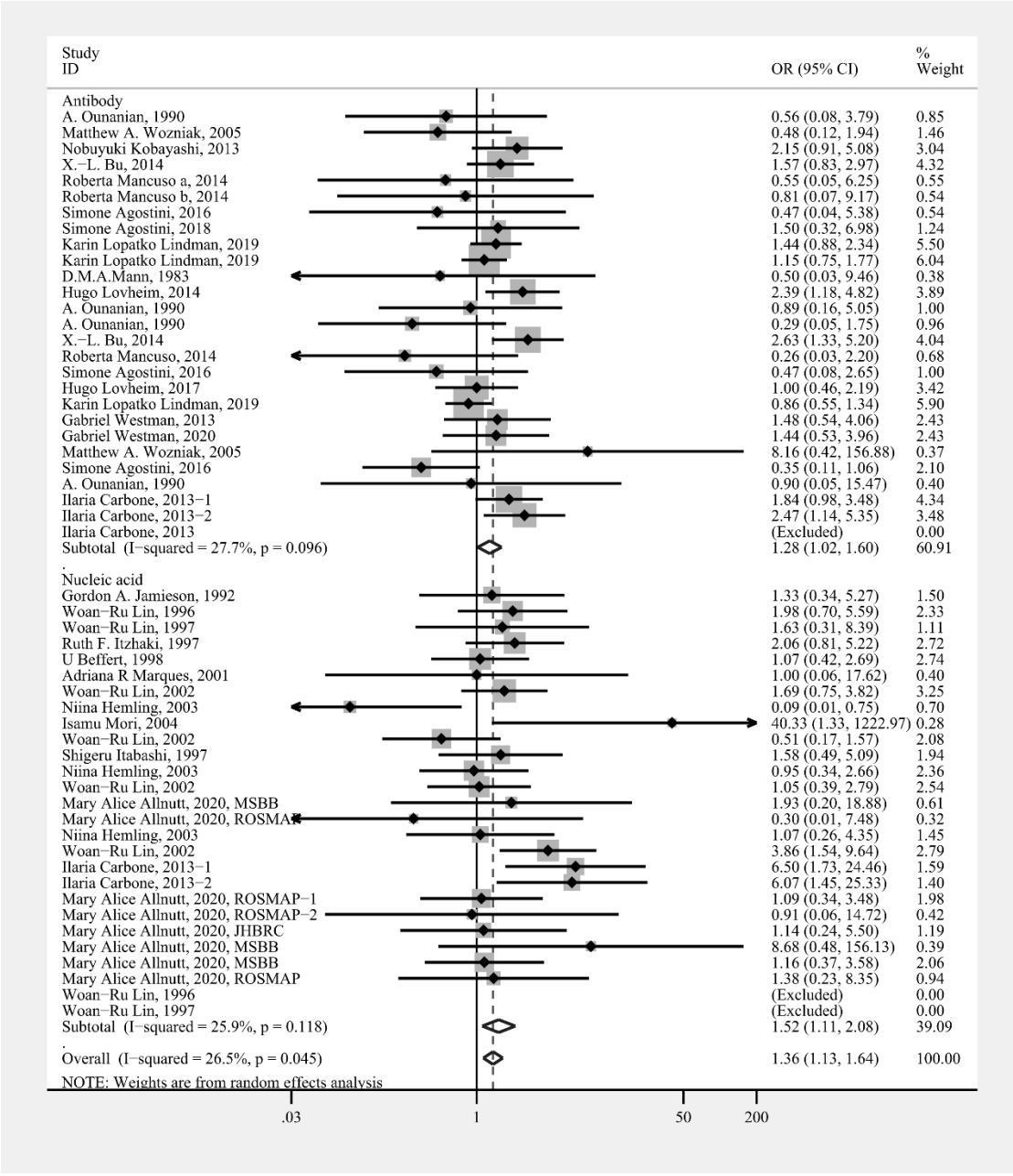


Fig(17). Forest plot of materials tested subgroup analysis for herpesviridae related studies.

Table(8). Meta-analysis outcomes of materials tested subgroup for herpesviridae related studies.

Materials tested	**OR(95% CI), *I-squared, p-value***						
	Total	**α subfamily**	**β subfamily**	**HSV-1**	**CMV**	**HHV-6**	**EBV**
Body fluid	1.37(1.06-1.76), *40.9%, 0.02*	1.37(1.08-1.74), *0.0%, 0.62*	1.27(0.75-2.15), *64.4%, 0.001*	1.37(0.99-1.88), *0.0%, 0.68*	1.07(0.68-1.71), *46.3%, 0.07*	1.86(1.00-3.45), *10.3%, 0.35*	2.03(1.25-3.29), *0.0%, 0.72*
Brain tissue	1.36(1.03-1.78), *4.4%, 0.40*	1.25(0.83-1.88), *23.8%, 0.20*	1.60(0.99-2.57), *0.0%, 0.46*	1.45(0.85-2.47), *32.7%, 0.16*	1.05(0.44-2.49), *0.0%, 0.65*	2.85(0.50-16.32), *80.8%, 0.001*	1.22(0.47-3.16), *0.0%, 0.87*

Pooled risk estimates, 95% CI, I-squared and p-value of herpesviridae, subfamilies and specific herpes viruses (included studies: n≥5).

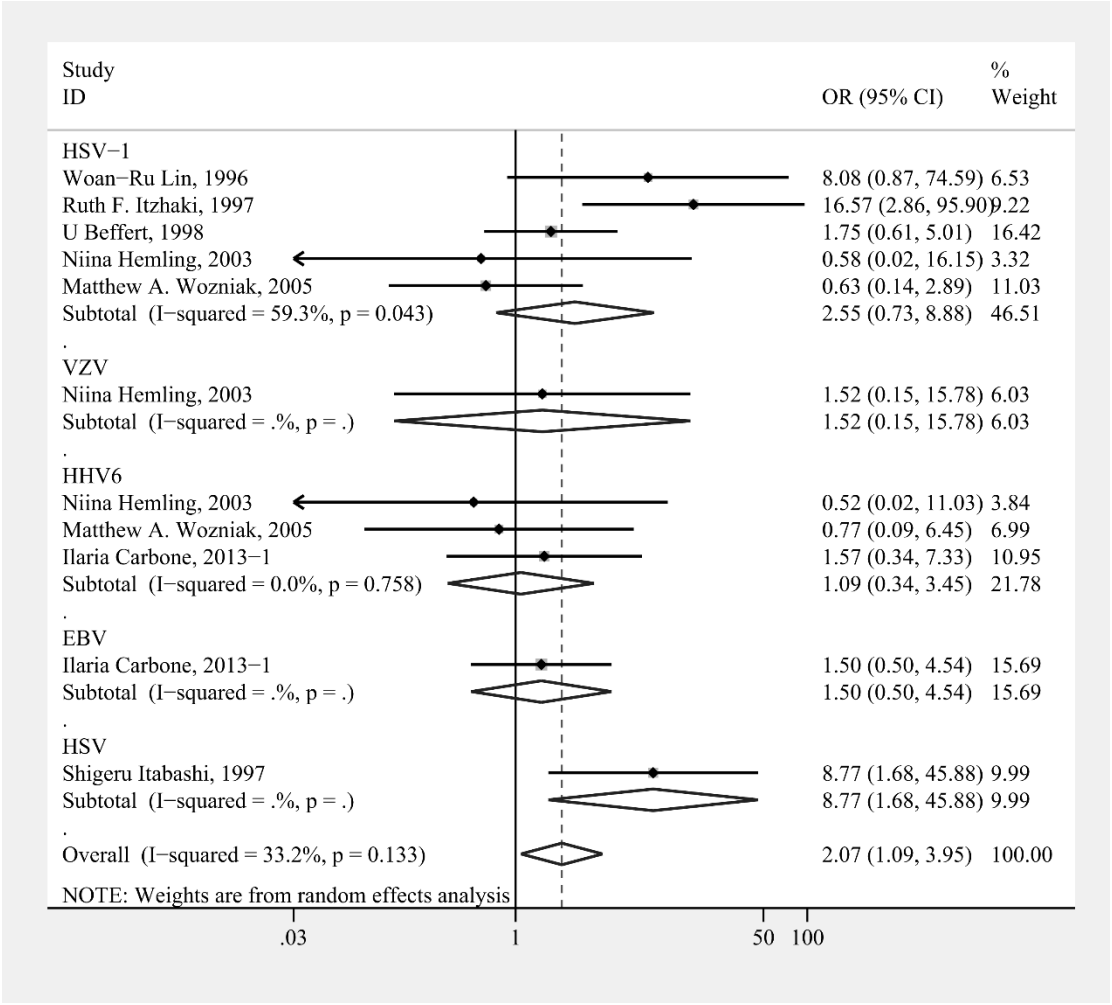


Fig(18). Forest plot of detection method subgroup analysis for herpesviridae related studies.

Table(9). Meta-analysis outcomes of detection method subgroup for herpesviridae related studies.

Detection method	OR (95% CI), *I-squared, p-value*						
	Total	**α subfamily**	**β subfamily**	**HSV-1**	**CMV**	**HHV-6**	**EBV**
Antibody	1.28(1.02-1.60), *27.7%, 0.10*	1.36(1.07-1.73), *0.0%, 0.66*	0.98(0.61-1.59), *51.9%, 0.028*	1.37(0.99-1.88), *0.0%, 0.68*	1.07(0.68-1.71), *46.3%, 0.07*	1.27(0.05-29.62), *75.7%, 0.04*	2.03(1.25-3.29), 0.0%, 0.72
Nucleic acid	1.52(1.11-2.08), *25.9%, 0.12*	1.26(0.83-1.93), *28.4%, 0.17*	2.06(1.20-3.51), *28.2%, 0.18*	1.45(0.85-2.47), *32.7%, 0.16*	1.05(0.44-2.49), *0.0%, 0.65*	2.53(1.37-4.67), *29.1%, 0.20*	1.22(0.47-3.16), 0.0%, 0.87

Pooled risk estimates, 95% CI, I-squared and p-value of herpesviridae, subfamilies and specific herpes viruses (included studies: n≥5).



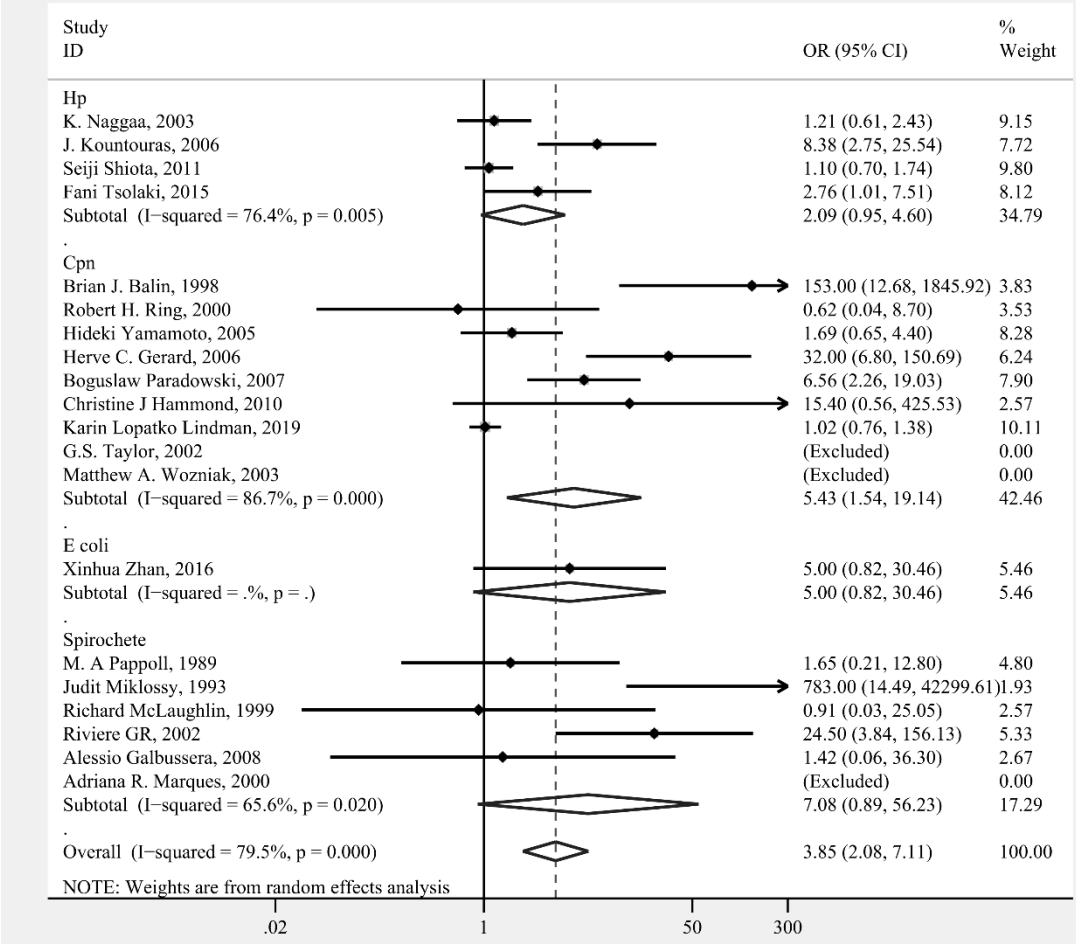
Fig(19). In AD subgroup, meta-analysis of the risk of herpesviridae and specific herpes viruses infection according to the presence and absence of *APOE* ε4.

7.3. BACTERIA

7.3.1. Gram negative bacteria could be the contributor to the onset of AD

There were four types of bacteria included in our analysis, *Hp*, *Cpn*, *Escherichia coli* (*E. coli*) and Spirochete, consisting of 1222 AD cases and 887 control subjects. According to their gram

stain reactions, they are generally assigned to gram negative bacteria. The pooled outcomes suggested gram negative bacteria positive subjects had a significantly increased occurrence of AD than negative ones, OR = 3.85 (95%CI = 2.08-7.11) with high heterogeneity. Of which, the point estimate of *Hp* was 2.09, *Cpn* was 5.43 and Spirochete was 7.08, and all of them showed different degrees of heterogeneity. Only one paper mentioned *E. coli*, and its OR was 5. In the *Hp* analysis, J. Kountouras's work tested *Hp* positivity in blood and gastric mucosa biopsy in the same population. Because gastric mucosal examination for the presence of *Hp* is known as the gold standard for diagnosis of *Hp* infection, we excluded their blood data [223].

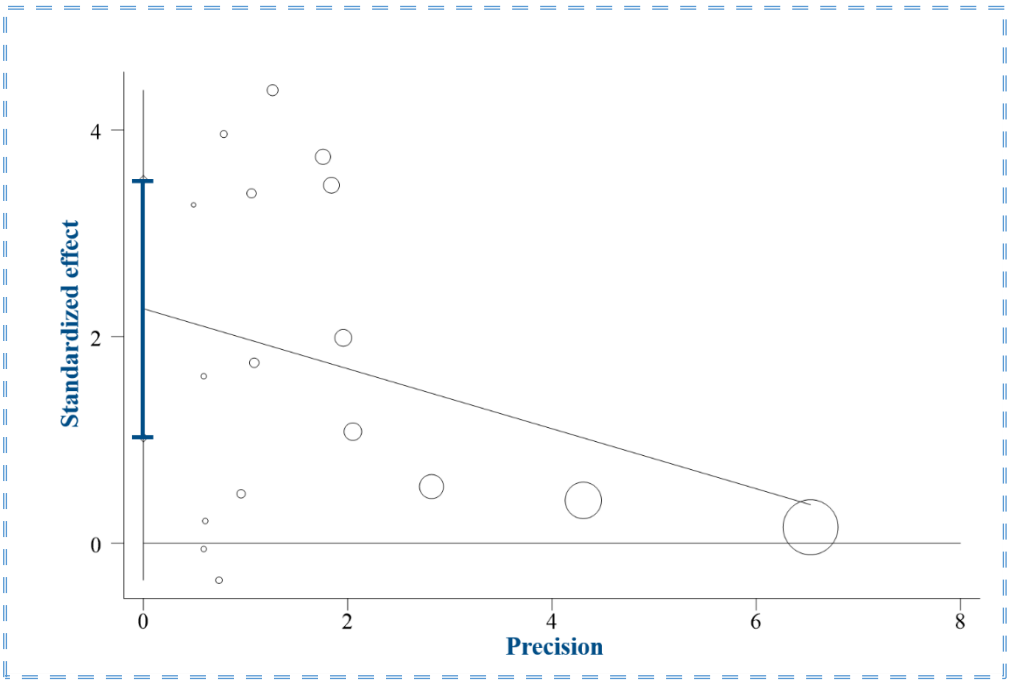


Fig(20). Forest plot of the association between four types of gram negative bacteria and AD.

7.3.2. Egger's regression test

In our analysis Fig(21), the intercept 'a' was 2.27, 95%CI = 1.02-3.51, and bias p-value was

0.001 (less than 0.1). Egger's regression test suggested that significant publication bias was found in gram negative bacteria related studies. On the other hand, bias p-value of *Cpn* was 0.05, indicating the presence of publication bias. Spirochete was 0.89, and *Hp* was 0.11, which revealed no evidence of publication bias was found in them.

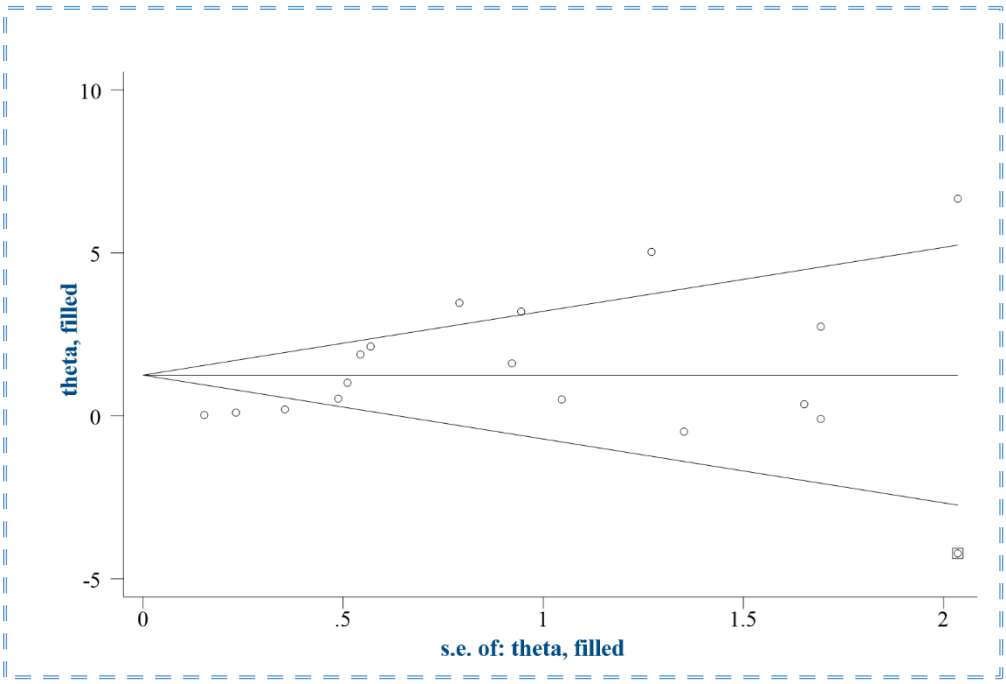


Fig(21). Evaluation of publication bias for gram negative bacteria relevant studies using Egger's regression test. Each circle represents an independent included study, and the size was determined by weights.

7.3.3. Sensitivity analysis

Under the fixed effect model, our initial outcome was 1.77 (95%CI = 1.46-2.14), and the adjusted pooled estimate and 95%CI were 1.54, 1.25-1.89. Likewise, the new produced result was 3.48 (95%CI = 1.88-6.44) under the random effect model Fig(22). The statistical significance did not change after Trim and fill program, which means the publication bias had little influence on the combined effect size. Even though the included studies in each type of bacteria were less than 10, it made little sense to evaluate the asymmetry of funnel plot, we still performed this method and listed results in Table(10) for references. All of the adjusted ones did not change their previous estimates' trend.

Another sensitivity analysis, leave-one-out, suggested no study could break the statistical significance of pooled OR Fig(23). However, to exclude studies of Seiji Shiota in 2011 [228] or Karin Lopatko Lindman in 2019 [240] increased the risk estimate to 4.62 and 4.64, and when removing works of Brian J. Balin [157], Herve C. Gerard [224] decreased this value to 3.21 and 3.19, respectively. For specific bacteria, Spirochete, leave-out-analysis indicated Richard McLaughlin's research in 1999 [210] influenced the final estimate. When excluding it, the pooled estimate changed into 11.15 (95%CI = 1.04-119.45). By reason of the limited included studies in the *Hp* section and a wide range of pooled estimates in the *Cpn* section, we cannot achieve similar outcomes for Spirochete, so no further results for them were shown in this part.

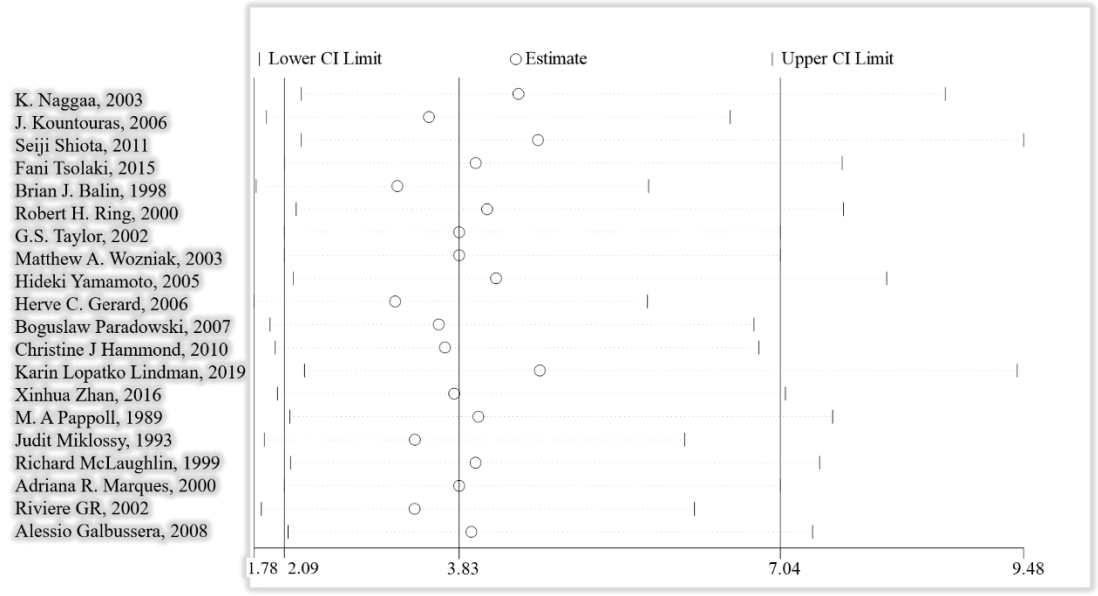


Fig(22). Correction for asymmetric funnel plot attributing to publication bias using trim and filled method. Filled funnel plot with pseudo 95% confidence limits. Each circle represents an independent included study. The square represents a filled study.

Table(10). The previous and adjusted pooled estimates after trim and filled method for *Hp, Cpn* and Spirochete.

Bacteria	OR(95% CI)		Adjusted OR(95% CI)	
	Fixed	Random	Fixed	Random
Hp	1.54(1.11-2.14)	2.09(0.95-4.60)	1.51(1.08-2.12)	2.09(0.95-4.60)
Cpn	1.62(1.27-2.09)	5.43(1.54-19.14)	1.43(1.09-1.87)	5.41(1.55-18.85)
Spirochete	8.88(3.37-23.44)	7.09(0.89-56.23)	6.82(2.20-21.20)	7.07(0.91-55.10)

Under fixed and random effects models.



Fig(23). Leave-one-out analysis for gram negative bacteria related studies. Each circle and two vertical lines in the same row represent a pooled estimate and 95% CI without one study, which is listed on the left side.

7.3.4. Subgroup analysis

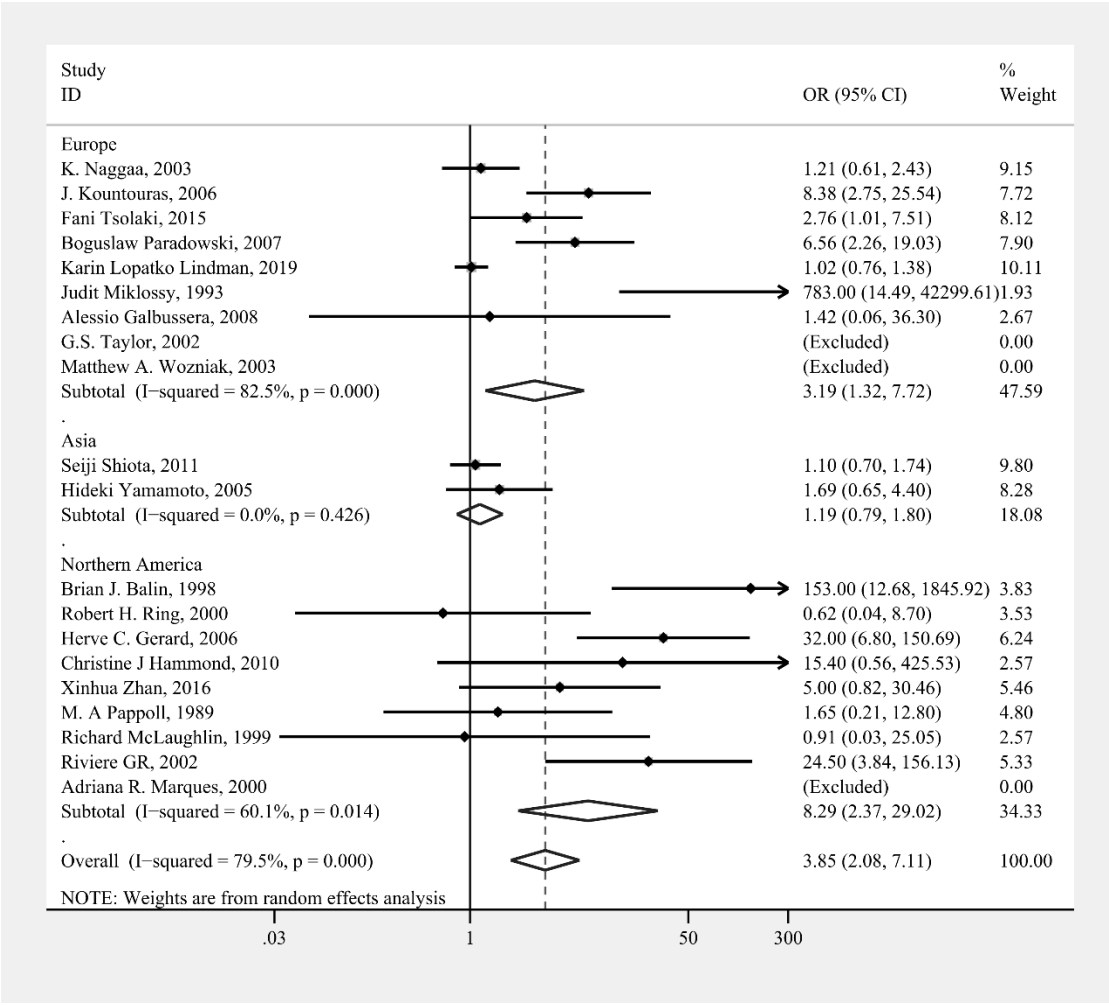
A. Geographical region subgroup

The first area subgroup analysis suggested an increased occurrence of AD in Europe and Northern America, when the evidence of gram negative bacterial infection was found. The respective OR was 3.19 (95%CI = 1.32-7.72), 8.29 (95%CI = 2.37-29.02), and both of them have remarkable heterogeneity Fig(24). The results of *Cpn* infection revealed a significantly higher possibility of Alzheimer's disease in Northern America, OR (95%CI) = 15.90 (1.83-137.98), I-squared = 68.8%, p = 0.02 Table(11).

B. Materials tested subgroup

When exploring the role of tested materials in risk estimates, we found that body fluid was less sensitive to detect bacteria. No matter what the human tissues are, the outcomes of whole the bacteria showed a growing trend with statistical significance Fig(25). Of them, two papers [223, 235] focused on *Hp* used non brain tissue, gastric mucosa, as their examined samples, and another Japanese study applied a rapid urine test among included subjects [228]. Compared with the control group, the presence of *Cpn* was more likely to be detected in brain tissue, a pooled OR was 15.90 (95%CI = 1.83-137.98), but notably with considerable heterogeneity

Table(12).

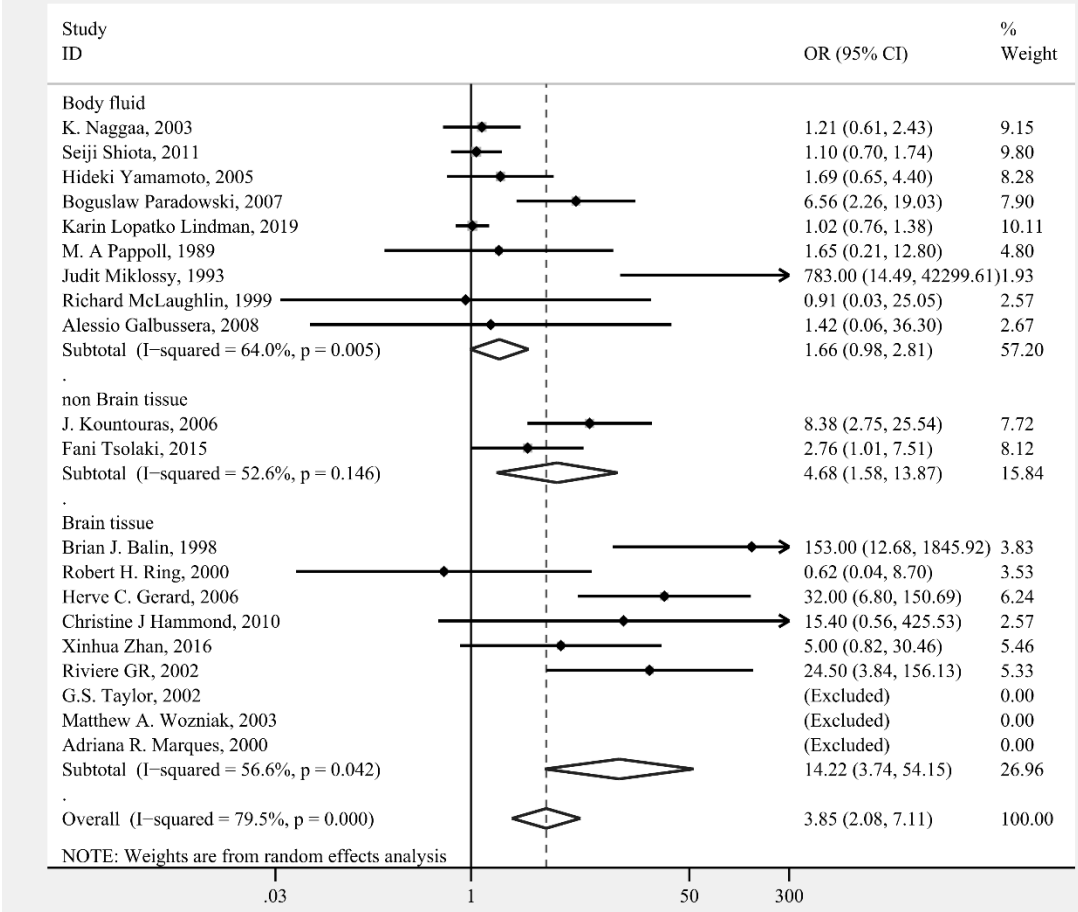


Fig(24). Forest plot of geographical region subgroup analysis for gram negative bacteria related studies.

Table(11). Meta-analysis outcomes of geographical region subgroup for gram negative bacteria related studies.

Geographical Region	**OR (95% CI), I-squared, p-value**			
	Total	***Hp***	***Cpn***	**Spirochete**
Europe	3.19(1.32-7.72), *82.5%, 0.00*	2.84(0.94-8.60), *76.6%, 0.01*	2.41(0.39-14.88), *90.8%, 0.001*	29.87(0.06-14605.31), *82.8%, 0.02*
Northern America	8.29(2.37-29.02), *60.1%, 0.01*		15.90(1.83-137.98), *68.8%, 0.02*	4.15(0.05-34.20), *59.7%, 0.08*
Asia	1.19(0.79-1.80), *0.0%, 0.43*	1.10(0.70-1.74),	1.69(0.65-4.40)	

Pooled risk estimates, 95% CI, I-squared and p-value of total and specific bacteria. Lack of I-squared and p-value meant there was only one study in the analysis.

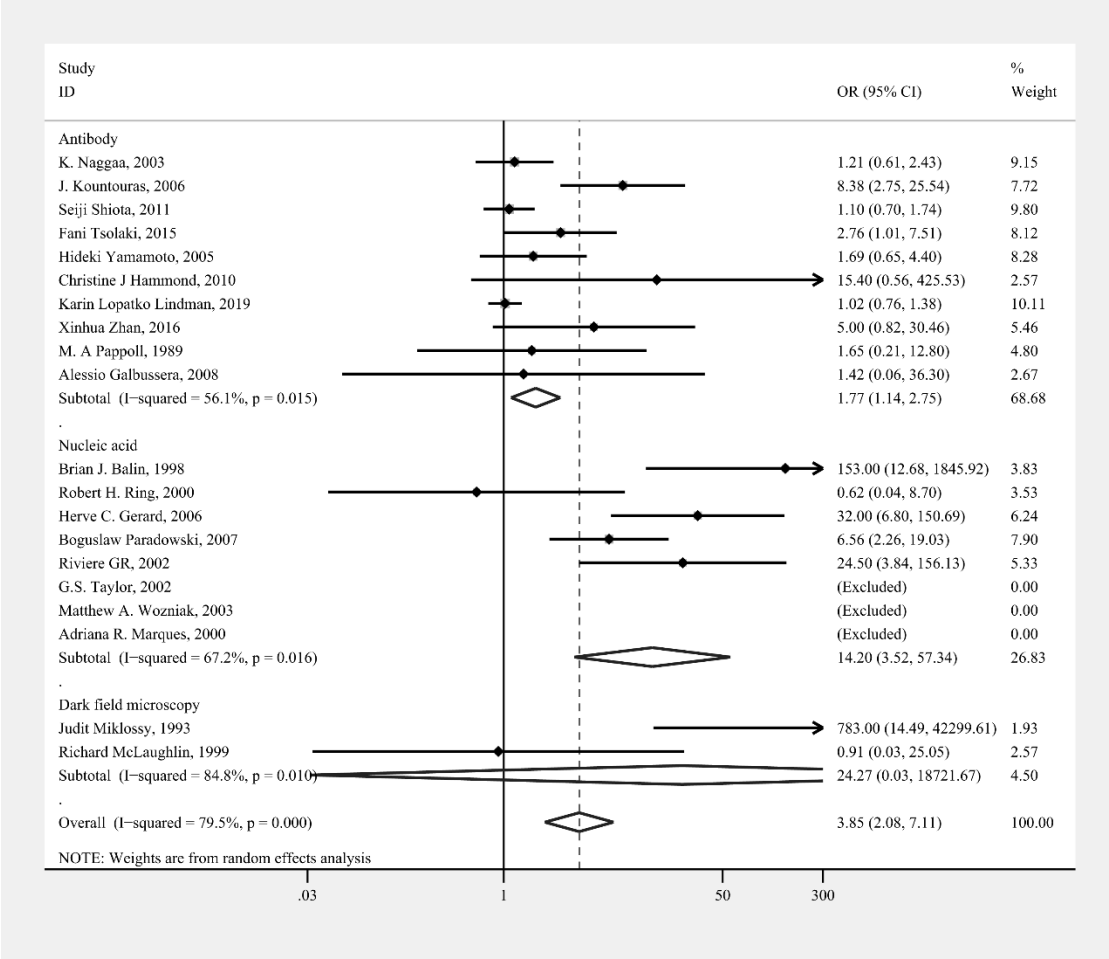


Fig(25). Forest plot of materials tested subgroup analysis for gram negative bacteria related studies.

Table(12). Meta-analysis outcomes of materials tested subgroup for gram negative bacteria related studies.

Materials tested	**OR (95% CI), I-squared, p-value**			
	Total	***Hp***	***Cpn***	**Spirochete**
Body fluid	1.66(0.98-2.81), *64.0%, 0.005*	1.13(0.77-1.66), *0.0%, 0.82*	2.05(0.72-5.89), *82.5%, 0.003*	4.93(0.33-73.56), *67.9%, 0.03*
Brain tissue	14.22(3.74-54.15), *56.6%, 0.04*		15.90(1.83-137.98), *68.8%, 0.02*	24.50(3.84-156.13)
non Brain tissue	4.68(1.58-13.87), *52.6%, 0.15*	4.68(1.58-13.87), *52.6%, 0.15*		

Pooled risk estimates of materials tested subgroup meta-analysis, 95% CI, I-squared and p-value of total and specific bacteria. Lack of I-squared and p-value meant there was only one study in the analysis.



Fig(26). Forest plot of detection method subgroup analysis for gram negative bacteria related studies.

Table(13). Meta-analysis outcomes of detection method subgroup for gram negative bacteria related studies.

Detection method	**OR (95% CI), I-squared, p-value**			
	Total	***Hp***	***Cpn***	**Spirochete**
Antibody	1.77(1.14-2.75), *56.1%, 0.02*	2.09(0.95-4.60), *76.4%, 0.01*	1.34(0.67-2.70), *41.8%, 0.18*	*1.58(0.28-8.93), 0.0%, 0.94*
Nucleic acid	14.20(3.52-57.34), *67.2%, 0.02*		12.33(2.11-72.01), *74.1%, 0.01*	*24.50(3.84-156.13),*
Dark field microscopy	24.27(0.03-18721.67), *84.8%, 0.01*			24.27(0.03-18721.67), *84.8%, 0.01*

Pooled risk estimates, 95% CI, I-squared and p-value of total and specific bacteria. Lack of I-squared and p-value meant there was only one study in the analysis.

C. Detection method subgroup

The third subgroup analysis refers to the detection method. Two papers used dark field microscopy to observe spirochete, 30ul CSF was dropwise added on a slide [204, 210]. The

criteria of C.pneumoniae seropositivity was determined by both indexes of its specific IgG and IgA antibodies in Hideki Yamamoto's paper [222]. The results demonstrated a significantly increased incidence of AD if detecting gram negative bacteria by examining antibody or nucleic acid, with an OR of 1.77 (95%CI = 1.14-2.75) or 14.20 (95%CI = 3.52-57.34) Fig(26). In addition, when the presence of *Cpn* was identified by nucleic acid examination, we found an increased incidence of AD with a nearly high degree of heterogeneity Table(13).

D. *APOE* ε4 subgroup

The last subgroup was our interested topic, the relationship between *APOE* ε4 and gram negative bacterial infection among AD patients. However, no further meta-analysis could be performed. Because there was only one included paper that mentioned *APOE* ε4 [224], in the AD subgroup 11 *Cpn* positive individuals were detected in 14 *APOE* ε4 carriers, whereas 2 negative subjects were found in cases without *APOE* ε4.

8. DISCUSSION

8.1. HERPESVIRIDAE CONTRIBUTE TO THE DEVELOPMENT OF AD

8.1.1. Herpesviridae and alpha subfamily

Herpesviridae is a group of enveloped viruses with genetic materials, i.e. DNA. It is the etiological agent for various diseases, and those who are infected will carry the virus for life because of the latent characteristic. Until now, nine herpesviruses have been known to infect human beings. Except Human herpes virus type 7 and Kaposi sarcoma herpesvirus [243], the rest of the herpes viruses were included in our meta-analysis study. According to their biologic nature and structural properties, the herpes viruses are classified into three subfamilies: alphaherpesvirus, betaherpesvirus and gammaherpesvirus [244]. The alphaherpesvirus can be further divided into two genera: simplexvirus (HSV-1, HSV-2) and VZV [245]. CMV and HHV6 belong to betaherpesvirus, while EBV is involved in gammaherpesvirus. In the current study, herpesviridae (OR; 95% CI: 1.36; 1.13-1.64; I-squared=26.5%; p=0.05), and its alpha subfamily (OR; 95% CI: 1.34; 1.09-1.64; I-squared=3.5%; p=0.41) were associated with the occurrence of AD. More researches are required to explain the relationships, and microbial characteristics of herpes viruses should be taken into consideration. In contrast to the beta subfamily and gamma subfamily, which prefer to establish latent infection in immune cells, monocytes and B cells respectively, alpha subfamily are inclined to live in sensory neurons, which may provide some clues for future investigation [246].

8.1.2. Specific herpes virus

In regard to the individual virus, HSV-1 (OR; 95%CI: 1.41; 1.08-1.83; I-squared=3.9%; p=0.41) and EBV (OR; 95%CI: 1.83; 1.19-2.81; I-squared=0.0%, p=0.82) made the greatest contribution to the onset of AD without obvious heterogeneity. Compared with other herpes viruses, such as the diagnosis of VZV reactivation usually occurs once. The mathematical models suggested HSV-1 and EBV reactivate more frequently during the entire life but with fewer actually activated cells [246]. Hence, the relative latent infection with these two herpes viruses is a repeatedly triggered and chronic process.

I. Human Simplex Virus-1

The presence of HSV-1 was firstly identified in AD patients' brain sections by Ruth F. Itzhaki and her colleagues in 1991 [156]. A sizeable epidemiological trial in the US indicated the HSV-1 infection was connected with cognitive impairment to varying degrees across all ages. Its seropositivity had a strong association with impaired visuospatial processing in children, damaged coding speed in middle-aged adults, and declines in immediate memory were found in HSV-1 positive older adults [247]. The cognition of HSV-1 infected hosts may be influenced earlier in their lifetime. With global scientists' efforts [229, 240], its importance is being gradually uncovered in the development of AD. HSV-1 replication was also detected in normal aging brains [221]. In contrast to the normal elderly populations, only 24% of HSV1 DNA are plaque-related, the viral DNA of HSV-1 was mainly expressed within plaque in AD patients' brain sections, and 72% of them are plaque-related [159]. In addition, HSV-1 affected tau phosphorylation associated enzymes and further made tangles deposition in cell culture [248]. If the HSV-1 infected cells received antiviral treatments, the levels of abnormal proteins: Aβ and P-tau were significantly reduced [249]. Therefore, the clinical and experimental results greatly revealed the potential relationship between HSV-1 and senile plaque/neurofibrillary tangles formation. Aβ deposition has now been proved as a neuroprotective immune response in 5XFAD mouse, by entrapping viruses, because HSV-1 surface glycoproteins and oligomers would bind to each other [166]. Recurrent HSV-1 infected mice exhibit AD like symptoms such as accumulation of typical pathological features, pro-inflammatory condition and cognitive impairment [250]. The previous findings and our results support a stable link between HSV-1 and risk of AD.

II. Epstein-Barr Virus

EBV is another herpes virus that reached statistical significance in our analysis. Virus infected B lymphocytes by salivary transfer is considered to be the main pathway of transmission [251]. People usually obtain primary infection without symptoms at an early age, around 3 years old, especially in some Asian countries [252]. An Italian group tested the presence of EBV in peripheral blood leukocytes, 30 from 66 AD individuals whereas 33 from 106 controls were EBV-positive. In a further follow-up of 150 elderly persons with normal cognitive performance

at the baseline for 5 years, the presence of virus was significantly enhanced in subjects who progressed into clinical AD when the trial ended [162]. Moreover, another study used different cohorts and peptide screening, and they found the adaptive immunity, mainly the functions of CD8+ T effector memory CD45RA+ cells, can be dramatically triggered by EBV antigens among AD patients [253]. Even though the evidence we have had so far is favorable, only three studies were included in the meta-analysis. More clinical trials with larger sample size are needed in the future.

III. Human Herpes Virus 6

HHV6 in the current analysis did not reach statistical significance (OR; 95%CI: 2.04; 0.98-4.24; I-squared=57.6%; p=0.01). Considering the high-profile multiscale analysis, HHV6 should be considered as a potential pathogen. The virus has a high infection rate and prefers to infect $CD4^+$ T-lymphocytes [254]. Based on new bioinformatics technologies, the increased HHV-6 viral abundance was found in AD subjects in comparison to controls, and the findings were further confirmed in two additional cohorts [255]. The neuroprotective Aβ seeding and deposition that defend against HHV6 infection have also been reported in neuronal cell culture and AD mouse model [166]. In addition, the overlaps of herpes viruses in brain samples were detected by regression analysis, of which the HSV-1 positive AD patients were significantly more likely to carry HHV-6 [214]. This means that HHV-6 may not only simply be regarded as the potential contributor for AD development, but also have a synergistic effect on aggravating the injury induced by HSV-1 in AD.

8.1.3. *APOE* ε4

APOE ε4 is known as the susceptibility gene of Alzheimer's disease [256]. Accumulating evidence indicated carriage of *APOE* ε4 together with herpes virus infection probably takes part in increasing the risk of AD [207, 257, 258]. A large Swedish cohort study that enrolled 3,413 individuals, revealed HSV carriers couple with *APOE* ε4 had a significant decline in episodic memory, which is the typical early symptom of AD [259]. In current study, we found that compared with non *APOE* ε4 carriers, herpesviridae infection occurred more in *APOE* ε4 carriers among AD patients (OR; 95%CI: 2.07; 1.09-3.95; I-squared=32.2%; p=0.13). In

addition, AD cases had an increased risk of being infected with HSV-1 if they simultaneously carried *APOE* allele 4 gene (OR; 95%CI: 2.55; 0.73-8.88; I-squared=59.3%; p=0.04). The results support the hypothesis that a combination of exposure to herpes virus infection and carriage of *APOE* ε4 increase the risk of AD.

8.2. GRAM NEGATIVE BACTERIA COULD BE THE RISK FACTOR FOR THE ONSET OF AD

8.2.1. Gram negative bacteria

Gram negative bacteria can be effectively distinguished from Gram positive bacteria by staining with crystal violet-iodine and safranin, and present in pink after counterstaining. The outer membrane bound with LPS is known as their specific toxic structure [260]. In our meta-analysis, gram negative bacteria could be one of the potential etiological factors (OR; 95%CI: 3.85; 2.08-7.11; I-squared=79.5%; p=0.00), but with considerable heterogeneity. Prenatal administration of LPS induced AD-like features: a decline in recognition and spatial memory and a rise in Aβ42 and p-tau levels when the mice were 12 month old [261]. An increasing number of studies demonstrated that LPS plays an indispensable role in the occurrence of AD. Compared with healthy controls, the higher LPS concentration was detected in blood of AD cases [262]. Extracellular amyloid deposition can be triggered and increased by chronic bacterial infections [263].

8.2.2. Specific bacteria

I. *Chlamydia pneumoniae*

Of these included bacteria, *Cpn* (OR; 95%CI: 5.43; 1.54-19.14; I-squared=86.7%; p=0.00) had the strongest association with AD. It is a common infectious agent for pneumonia. Yet in 1998, the presence of *Cpn* was detected in the postmortem brains sections of 17 AD patients among the total 19 tested cases [157]. Then people started to discover and establish the link between *Cpn* and AD. Some clinical trials also showed a positive outcome, exposure to *Cpn* infection increased the risk of Alzheimer's disease [224, 225], but the phenomenon was inconsistent [211]. In addition, when the BALB/c mice received intranasal inoculation with *Cpn*, AD related

pathological hallmarks, amyloid plaque depositions were developed and observed in brains [264]. According to the findings and our data, it is worthwhile to keep investigating the relationship between *Cpn* and AD.

II. *Helicobacter pylori*

Hp is known as an independent risk factor for stomach ulcers. The presence of *Hp* was more frequent in patients with AD dementia [163, 235]. Impaired cognitive performance was in particular correlated with *Hp* infection in the epidemiological study [265]. So far, clinical studies that refer to the connection between *Hp* and AD are limited, and the association did not reach statistical significance in our meta-analysis (OR; 95%CI: 2.09; 0.95-4.60; I-squared=76.4%; p=0.005). However, if we choose gastric mucosa as detected tissue, there was a significant association (OR; 95%CI: 4.68; 1.58-13.87), with lower heterogeneity. A host of studies suggested the *Hp* could enter the CNS and work by oral-nasal-olfactory pathway or circulating monocytes that carried bacteria [266]. Therefore, *Hp* probably becomes another bacteria candidate.

8.3. CHRONIC NEUROINFLAMMATION WITH INFECTION IN AD

Chronic neuroinflammation is now accepted as the causative element for pathogenesis of AD, including activated glia cells, over response and persistent inflammatory state, which exacerbate relative neurological deficits [267, 268]. Elderly people with higher cytokines, IL-1 and TNFα, in extracted peripheral blood mononuclear cells had an increased risk of AD [269]. According to the results of several epidemiological studies, non-steroidal anti-inflammatory drugs (NSAIDs) could show a preventative effect on AD, because a lower incidence of disease was usually found among drug users [270, 271]. These findings strengthen the evidence for a pathophysiologic role of inflammatory state for the onset of AD. On the other hand, most prospective drug trials reported no promising results in terms of the treatment effect of NSAIDs on cognitive symptoms [272], which might indicate the complex mechanism with multiple targets underlying the long-term neuroinflammation, other than merely involving cyclooxygenase activity. Therefore, it is worthwhile to explore in more detail this inflammation topic. As we questioned at the beginning, what kind of substances drive these extended immune

reactions. One reasonable hypothesis is chronic infection. A nested case-control enrolled 10,106 subjects revealed that older populations with more infections were more likely to develop dementia [273]. Our meta-analysis also supports the assumption that the presence of herpesviridae (HSV-1 and EBV) and some of gram negative bacteria (*Cpn*) significantly cause AD development. Beta-amyloid depositions are an important part of the innate immune system for fighting against infection in CNS [274]. In addition, *Cpn* infection stimulated microglia overproducing pro-inflammatory cytokines TNFα and IL-6 [263]. Reactivation of HSV-1 in the trigeminal ganglia or cerebral cortex induced enhanced expressions of toll-like receptor-4, interferons that are regarded as neuroinflammation associated markers, and specific neurodegenerative proteins, such as p-tau [275]. The data suggested an attack by a recurrent HSV-1 infection probably promotes neurodegeneration with chronic neuroinflammation. Depending on the published reports and our findings, chronic infections with different virus and bacteria pathogens may result in neuroinflammation which further contributes to the progression of AD.

8.4. LIMITATIONS

First, the included studies were selected in a specific database and all written in English, so we analyzed the publication bias by egger's regression test, trim and fill, leave one out in Stata software to avoid ignoring. Second, regarding the type of studies, the statistical efficacy in case-control study is lower than randomized controlled trial (RCT). However, in reality it would be impossible to conduct RCT to seek the relationship between pathogens and AD. Third, the studies related to this field were still limited, especially for every single virus or bacteria. Thus more validations, referring to whether specific pathogen or a cocktail of infectious candidates, in different cohorts with large sample size are worthwhile and required in the future. Fourth, heterogeneity was found in our meta-analysis. Region areas, materials tested and detection method could be the contributors. Accordingly, we analyze them in subgroup tests to maximum adjusting. The final outcomes of gram negative bacteria were highly heterogeneous. In the subgroup analysis, in contrast with body fluid (OR; 95%CI: 2.05; 0.72-5.89), to apply brain tissue as tested *Cpn* materials showed a significantly increased incidence of AD (OR; 95%CI:

15.90; 1.83-137.98) with lower heterogeneity. While detection with *Cpn* antibody effectively reduced the heterogeneity, but it also decreased the significance of pooled estimate (OR; 95%CI: 1.34; 0.67-2.70; I-squared=41.8%; p=0.18). Besides, although virus associated results had high homogeneity, subgroup analysis still indicated region areas, materials tested and detection method could be the variants for final outcomes. Testing antibodies (OR; 95%CI: 1.27; 0.05-29.62) or nucleic acid (OR; 95%CI: 2.53; 1.37-4.67) changed significance of risk to develop HHV-6 related AD. From another point of view, the data we have reveal that researchers need to make a standard when carrying out relevant clinical trials, adjusting influence factors in advance. More accurate and consistent data would be easier to obtain, and then getting a more credible pooled estimate is undoubtedly logical. Finally, the majority of reports focused on the association between the presence of virus or bacteria with Alzheimer's disease, not fully identifying whether they are really active. It will be meaningful to further clarify the viral and bacterial condition by emerging technologies.

8.5. FUTURE OUTLOOKS

Alzheimer's disease is a chronic process of neurological injury and the patients will gradually lose memory. Until now scientists have not found any effective treatments to reverse it, which mainly attribute to the unclear etiology [263]. The results in our meta-analysis support chronic neuroinflammation with infection could be the causative factor for AD development. Recurrent infection without obvious symptoms leads to continuous inflammatory responses in CNS, consequently inducing or promoting AD pathology. Other recent systematic reviews and meta-analysis on this topic support the general hypotheses, including a publication with relatively liberal inclusion criteria [276], and the work targeting a specific pathogen (HSV-1) [277]. Other publications provide evidence on *Toxopla*sma gondii [278, 279] being a risk factor for the development of AD. Due to the complexity of the topic and the constantly growing evidence base, regularly updated reviews and standardized investigations are required.

Moreover, the acknowledged answer for this kind of query should be direct evidence, such as when clearing the virus or bacteria, patients are free from the disease or it has stopped its progression. A large retrospective cohort study in 2018 found anti-herpetic intervention

dramatically decreased dementia occurrence among HSV infected hosts [280]. If the conclusion can stand up to scrutiny, it would be an available treatment. Hence, further works on examining whether anti-infection drugs can control or prevent dementia progression, especially prospective clinical trials are expected in the future.

8.6 CONCLUSION

Our meta-analysis suggests the presence of herpesviridae and alpha subfamily increase the risk of AD, and in particular HSV-1 and EBV have the strongest association. When the herpes virus infection occurred together with carriage of *APOE* ε4 there would be a further enhanced AD risk. Another common pathogen gram negative bacteria, especially *Cpn* could also be potential etiological factors. It becomes good evidence to support the assumption that AD related chronic neuroinflammation is driven by viral or bacterial infection, and subsequently promoting AD pathology. Additional studies are required to strengthen this relationship and determine whether preventative treatments with anti-herpetic and anti-bacterial drug are deserved to expect.

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To be a good doctor, cheer up!

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I hereby declare, that the submitted thesis entitled:

Neuroinflammation in acute and chronic brain injury: Cerebellar hemorrhage and Alzheimer's disease

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Place, Date
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Signature doctoral candidate
Chen Aoxue