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Ricardo De Miguel Moral

Advances in the field of aluminum-based adjuvants in sheep: clinicopathological and immunological studies and interaction with small ruminant lentiviruses

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Tesis Doctoral

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Autor

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PhD Thesis

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Autor

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Facultad de Veterinaria

2022

A mis abuelos, mis padres y mis hermanos. En especial a mi abuelo Jesús. Porque venimos del campo y al campo volveremos. Gracias por enseñarme que la vida consiste en eso: en sembrar con ilusión, en entender la tierra, en predecir las nubes, en confiar en el sol… y en mantener la paciencia y la esperanza hasta el día de la recolección.

“Todos llevamos una espina y un clavel, luces y cruces que habitan bajo la piel, usamos nuestro pincel sobre un destino de papel, sabiendo que la muerte es una cifra cruel”

Ignacio Fornés Olmo (Nach), 2021

“Todo hombre puede ser, si se lo propone, escultor de su propio cerebro, y que aun el peor dotado es susceptible, al modo de las tierras pobres, pero bien cultivadas y abonadas, de rendir copiosa mies”

Santiago Ramón y Cajal, 1912

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Esta tesis doctoral tiene tantos autores como personas han influido en ella. Unas lo han hecho de forma directa, aportando ideas, conocimiento o simplemente ilusión al proyecto. Otras muchas de forma indirecta, con su alegría en el día a día, su comprensión en los tragos amargos y su serenidad en los momentos de euforia. Esta tesis es el fruto de una oportunidad. La oportunidad de formarme como patólogo, investigador y persona, en Zaragoza y Pamplona.

En esta etapa ha habido dos personas que han brillado de una manera especial, y a quienes dedico todos los méritos conseguidos, pues son más suyos que míos. Espero haberos inspirado durante estos años, aunque sea una décima parte de lo que me habéis inspirado a mí. A Lluís Luján, nombre escrito con dos "eles" y pronunciado solo con una por la mayoría de los castellanoparlantes. Eres y siempre serás mi sensei en el mundo de la patología. Una persona paciente, confiada y optimista. Capaz de delegar, obligándote a estar preparado. Gracias a ti soy diplomado por el ECVP y se abre ante mí un camino profesional lleno de oportunidades. Porque escribo los artículos científicos a tu imagen y semejanza. Y porque también pienso que esa última "frase resumen" al final de la introducción de cada artículo, simplemente sobra. Gracias. A Ramsés Reina, quien siempre escribe esa última frase de la introducción. Me has enseñado todo lo que sé sobre inmunología, virología y Navarrería. Eres mi sensei en el campo de la biología molecular. Cada discusión científica contigo me ha frustrado y enseñado a partes iguales, y qué necesario es lo primero y qué bonito que es lo segundo. Siempre sabes ver más allá, tanto en la vida como en la ciencia. Transmites calma, a pesar de ser puro nervio. Eres una mente brillante y admiro tu forma de ver la vida. Ojalá haber compartido la época universitaria juntos. Porque el rap es mejor que el rock. Y porque no hay nada como un poco de queso y vino en mitad de una ruta en bici. Gracias.

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PREFACE

The present PhD Thesis has been arranged in five independent Chapters, which correspond to the scientific articles that have been -or are expected to be- published in indexed international scientific journals. Each chapter has been written so that it could be understood independently of the remaining parts of the PhD Thesis. In this sense, each Chapter contains its own: Abstract, Introduction, Material and methods, Results, Discussion and Conclusions. Moreover, this PhD Thesis also includes: General Introduction, which contains a broad state-of-the-art; Global material and methods, which collects and amplify the methodological information that is shared by several Chapters; Global discussion and future perspectives, which evaluate the five chapters altogether and reflect on the scientific opportunities opened consequence of the results herein collected; Conclusions, which summarizes the conclusions of each individual Chapter and References, which contain all the publications cited along the manuscript.

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SCIENTIFIC PUBLICATIONS

de Miguel R*, Asín J*, Rodríguez-Largo A, *et al*. Growth performance and clinicopathological analyses in lambs repetitively inoculated with aluminum-hydroxide containing vaccines or aluminum-hydroxide only. *Animals*. 2021;11:1–18.

* Ricardo de Miguel and Javier Asín contributed equally to this work.

de Miguel R*, Asín J*, Rodríguez-Largo A, *et al*. Detection of aluminum in lumbar spinal cord of sheep subcutaneously inoculated with aluminum-hydroxide containing products. *J Inorg Biochem*. 2020;204:110871.

*Ricardo de Miguel and Javier Asín contributed equally to this work.

Echeverría I*, de Miguel R*, Asín J, *et al.* Replication of small ruminant lentiviruses in aluminum hydroxide-induced granulomas in sheep: a potential new factor for viral dissemination. *J Virol*. 2020;95.

*Irache Echeverría and Ricardo de Miguel contributed equally to this work.

de Miguel R*, Arrieta M*, Rodríguez-largo A, *et al.* Worldwide prevalence of small ruminant lentiviruses in sheep: A systematic review and meta-analysis. *Animals*. 2021;11:1–21.

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TABLE OF ABBREVIATIONS

ABAs	Aluminum-based adjuvants
Al	Aluminum
ADG	Average daily gain
AGID	Agar gel immunodiffusion assay
APC	Antigen-presenting cells
ASC	Apoptosis-associated speck-like protein containing a CARD
ASIA syndrome	Autoimmune/Inflammatory Syndrome Induced by Adjuvants
BAL	Bronchoalveolar lavage
BBB	Blood-brain barrier
BCG	Bacilli Calmette-Guerin
BIV	Bovine immunodeficiency virus
BSCB	Blood-spinal cord barrier
BTV	Bluetongue virus
BVDV	Bovine viral diarrhea virus
C	Cell-associated deposits
Ca	Capsid
cDNA	Complementary DNA
CAEV	Caprine arthritis encephalitis virus
CARD	Caspase activation and recruitment domain
CNS	Central nervous system
COVID-19	Coronavirus disease emerged in 2019
DAMPs	Danger-associated molecular patters
DEPC H_20	Dietilpirocarbonate-treated water
dpi	Days after the first inoculation
EIAV	Equine infectious anemia virus
ELISA	Enzyme-linked immunosorbent assay
ENV	Outer envelope
FIV	Feline immunodeficiency virus

GaHV-2	Gallid herpesvirus 2
GM	Gray matter
HE	Hematoxylin-eosin
HIV	Human immunodeficiency virus
IBD	Infectious bronchitis disease
ICP-MS	Inductively coupled mass spectrometry
IFN-γ	Interferon gamma
In	Integrase
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
ILT	Infectious laryngotracheitis
IQR	Interquartile range
ISCOMs	Immuno-stimulating complexes
ISG	Interferon stimulated genes
Ma	Matrix
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response 88
Nc	Nucleocapsid
NC	Non cell-associated deposits
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NLR family pyrin domain containing 3
NLR	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIPES-buffer	1,4-Piperazine-diethanesulfonic acid buffer

PNS	Peripheral nervous system
Pr	Protease
PRR	Pattern recognition receptors
PRRS	Porcine reproductive and respiratory syndrome
qPCR	Quantitative PCR
RT-qPCR	Quantitative reverse transcription PCR
RT	Retrotranscriptase enzyme
SD	Standard deviation
SIV	Simian immunodeficiency virus
SRLV	Small ruminant lentiviruses
T	Total deposits
T_h cells	T helper cells / CD4+ cells
TIR	Toll-interleukin-1 receptor
TLR	Toll like receptors
TH-GFAAS	Transversely heated graphite atomic absorption spectroscopy
TRIF	TIR-domain-containing adapter-inducing interferon-β
UK	United Kingdom
USA	United States of America
VMV	Visna/maedi virus
VPLs	Virus-like particles
WHO	World health organization
WM	White matter

SUMMARY

Vaccines are key elements in human and veterinary medicine and one of the greatest public and animal health achievements of the last centuries. Vaccines try to mimic the immunogenic aspects elicited by natural infections in order to provide long-term protection against them. Veterinary vaccines have a positive impact on animal welfare and economics achieving a drastic reduction in the morbidity and mortality induced by spontaneous infections.

Intensification in domestic animal production has drastically increased during the last decades favoring animal overcrowding and infection spread. Good management practices and appropriate vaccination strategies to prevent diseases are cornerstone of any successful health plan. In sheep husbandry, vaccination protocols differ depending on a variety of factors such as production system, geographical location, climate, and/or disease prevalence. These protocols can be further modified by compulsory vaccination campaigns to fight against emerging or re-emerging epizootics. A recent example was the compulsory vaccination campaign against bluetongue virus (BTV) that took place in most European countries during the first decade of the 21st century. The mass vaccination successfully controlled the infection in ruminants but raised several sanitary concerns in different European countries. In Spain, a wasting syndrome associated with neurological signs was reported and classified under the umbrella of the autoimmune/autoinflammatory syndrome induced by adjuvants (ASIA syndrome).

Immune activation after vaccination highly depends on the type of vaccine employed. Most ovine vaccines are based on inactivated pathogens or recombinant proteins that are often poorly immunogenic as they need adjuvants to strengthen the immune response. Aluminum-based adjuvants (ABAs) have been used in human and veterinary vaccines for more than 70 years. However, precise mechanisms of action of these adjuvants are just partially elucidated. Inoculation of ABAs induce the recruitment of inflammatory cells to the injection site and evolves towards the formation of a chronic inflammatory nodule, the so-called injection-site granuloma as it is mainly composed of activated macrophages. Aggregates of Al-laden macrophages can reach and accumulate in the regional lymph node.

Small ruminant lentiviruses (SRLV) cause highly prevalent chronic infections in sheep and goats worldwide. SRLV are highly heterogenic single-stranded RNA

retroviruses. Indeed, four main genotypes (A, B, C and E) and more than 35 subgroups have been already characterized. SRLV have tropism for the mononuclear-phagocyte system and induce slow, chronic and persistent inflammation in four main target organs: lung, joints, nervous system and mammary gland. The occurrence of each clinical form and the severity of the lesions depend on viral factors as well as the host immune response. In the articular form, the main clinical sign is unilateral or bilateral carpal arthritis caused by genotype B2 strains in sheep.

The aim of this PhD Thesis is to study the effects of the repeated inoculation of aluminum (Al)-oxyhydroxide in sheep and its relationship with the SRLV pathogenesis. Experiment 1 included 84 lambs that were divided into four flocks (Flocks 1-4) of 21 animals each. In each Flock, lambs were divided into three treatment groups of 7 animals each: Vaccine group, inoculated with commercial vaccines; Adjuvant-only group, which received the equivalent dose of Al-oxyhydroxide, and Control group, injected with phosphate-buffered saline. The experiment lasted 15 months and lambs received 19 subcutaneous inoculations, mimicking the amount of Al that animals can receive during their productive lifespan. Experiment 2 was based on 15 adult (>4 year-old) female commercial sheep naturally infected by SRLV and showing bilateral arthritis. Similarly to Experiment 1, sheep were divided into 3 treatment groups (Vaccine, Adjuvant-only and Control). Six animals were excluded after the quarantine period for animal welfare reasons and the final number of animals in each group was: Vaccine group (n=4), Adjuvant-only group (n=3) and Control group (n=2). The experiment lasted 75 days and animals received a total of eight subcutaneous inoculations. To complement molecular and serologic results of Experiment 2, an additional group (Vaccine-extra group; n=2) was inoculated with 4 doses of commercial vaccines along 40 days.

Chapter 1 is based of animals of Flocks 1-4 of the Experiment 1 and provides an analysis of the **growth performance and clinicopathological analyses in lambs repetitively inoculated with Al-oxyhydroxide containing vaccines or Al-oxyhydroxide alone**. Mild differences in average daily gain and fattening index were observed, indicating a reduced growth performance in Vaccine groups likely related to short-term episodes of pyrexia and anorexia. Clinical and hematological parameters remained within normal limits. Histology showed no significant differences between groups,

although there was a tendency to present higher frequency of hyperchromatic, shrunken neurons in the lumbar spinal cord in the Adjuvant-only group. Although Al-oxyhydroxide was previously linked to granulomas at the injection site and with the production of ethological changes in sheep, results of Chapter 1 indicate that injected Al-oxyhydroxide is not enough to fully reproduce the wasting presentation of the ovine ASIA syndrome. Other factors such as sex, breed, age, production system, genetic background, diet or climate conditions could play a role.

Chapter 2 is based on animals of Flock 1 of the Experiment 1 and report the **detection of Al in lumbar spinal cord of lambs repetitively inoculated with Al-oxyhydroxide containing vaccines or Al-oxyhydroxide alone**. Deposits were significantly more abundant in the lumbar spinal cord than in the parietal lobe in the Adjuvant-only group and they showed a marked statistic trend in the Vaccine group. In the lumbar spinal cord, Al deposits were higher in both the Vaccine and Adjuvant-only groups compared with the Control group. In the parietal lobe, Al deposits were higher in the Vaccine group compared with Control group and Adjuvant-only group. In the lumbar spinal cord, lumogallion reactive Al deposits were more abundant in the gray matter than in the white matter in both Vaccine and Adjuvant-only groups and Al deposits were mostly associated with glial-like cells. In the parietal lobe, few Al deposits, which were sometimes related to vessels, were found. Results of Chapter 2 indicate that in sheep, Al-oxyhydroxide adjuvants inoculated in the subcutaneous tissue selectively accumulates in the lumbar spinal cord.

Chapter 3 is based on animals of Flock 1 of the Experiment 1 and provides a deep analysis of the **the inflammatory and immune signaling pathways at injection-site granulomas, regional lymph node and spleen of lambs repetitively inoculated with Al-oxyhydroxide containing vaccines or Al-oxyhydroxide alone.** Immunological activation was more evident in secondary lymphoid organs (i.e., lymph node and spleen) and involved the overexpression of toll-like receptors, pro-inflammatory cytokines, costimulatory molecules and antiviral proteins. Few differences were found in granulomas likely due to the unavailability of proper control tissue. However, reduction in IL-10 expression and the increase of IL-6 may indicate a constant immune activation in granulomas vaccinated animals. Results of Chapter 3 indicate that in sheep, pro-

inflammatory and immune signaling pathways are upregulated after the inoculation of Al-oxyhydroxide, being this upregulation higher when Al-oxyhydroxide is part of a vaccine.

Chapter 4 is based on animals of Flocks 2 and 4 of the Experiment 1 and animals of the Experiment 2. This chapter addresses the **impact of Al-induced granulomas on serological response and virus kinetics in SRLV infected animals**. In Flock 2 and 4 of Experiment 1, new SRLV-seropositive animals were more abundant in lambs from Vaccine and Adjuvant-only groups compared to Control group. Accordingly, arthritic sheep reported an increase in antibody titers along the experiment in Vaccine, Vaccine-extra and Adjuvant-only groups but not in Control group. SRLV viral DNA copies in blood slightly increased in Control animals, whereas Vaccine, Vaccine-extra and Adjuvant-only groups showed a marked decrease. Moreover, radiographic and thermographic analyses showed an accelerated, significant progression of articular lesions in Vaccine and Adjuvant-only groups. These animals presented injection-site granulomas that evinced granular, intracytoplasmic SRLV immunolabelling in macrophages. These SRLV-containing granulomas are likely responsible of the increased antibody titers in Vaccine, Vaccine-extra and Adjuvant-only groups along the experiment. Results of Chapter 4 indicate that serological response against SRLV as well as virological and pathological features may be affected in animals inoculated with ABAs, underscoring the importance of Al-induced granulomas in the pathogenesis of macrophage-tropic viruses.

Chapter 5 develops **a systematic review and meta-analysis** of the scientific publications of the last 40 years (1981-2020) in the **SRLV** field owing to address **worldwide prevalence in sheep.** Most articles used a single diagnostic test to estimate SRLV prevalence whereas articles using 3 or more test were scarce. ELISA has progressively replaced AGID over the last decades. SRLV infection in sheep is widespread across the world, with Europe showing the highest individual prevalence (40.9%). Europe is also the geographical area in which most studies have been performed. Africa, Asia and North America showed values ranging from 16.7% to 21.8% at the individual level. South and Central America showed the lowest individual SRLV prevalence (1.7%). There was a strong positive correlation between individual and flock prevalence. Results of Chapter 5 evinced that in spite of the global importance of small ruminants, the

knowledge on SRLV prevalence is patchy and inconsistent. There is lack of a gold standard method and a defined sampling strategy among countries and continents.

This PhD Thesis is a multidisciplinary approach to a complex scientific issue and provides a substantial advance in the understanding of ABAs' mechanism of action and their interaction with SRLV. The integrated analyses of clinical, biochemical, histopathological, serological, molecular and epidemiologic data provide a strong evidence of the importance of both, ABAs and SRLV, and their interaction. Safety concerns related to the persistence of Al-induced granulomas, translocation of ABAs to the central nervous system and SRLV replication within Al-loaded macrophages demand the design of new adjuvants for safer vaccines in sheep.

RESUMEN

Las vacunas son elementos clave en medicina humana y veterinaria y suponen uno de los mayores logros en salud pública y animal de los últimos siglos. Las vacunas intentan reproducir los aspectos inmunológicos provocados por las infecciones naturales para proporcionar protección a largo plazo contra ellas. En veterinaria, las vacunas tienen un impacto positivo en el bienestar de los animales y el rendimiento económico de las explotaciones, ya que reducen drásticamente la morbilidad y la mortalidad debidas a infecciones espontáneas.

La intensificación de la producción animal ha aumentado drásticamente durante las últimas décadas, favoreciendo el contacto estrecho entre animales y el contagio de infecciones. Las buenas prácticas de manejo y una estrategia de vacunación adecuada son la piedra angular de cualquier plan sanitario de éxito. En ganadería ovina, los protocolos de vacunación difieren en función de múltiples factores, como el sistema de producción, la localización geográfica, el clima y/o la prevalencia de enfermedades. Además, los protocolos de vacunación pueden verse modificados por campañas de vacunación globales para luchar contra epizootias emergentes o reemergentes. Un ejemplo reciente fue la campaña de vacunación obligatoria contra el virus de la lengua azul (BTV) que tuvo lugar en la mayoría de los países europeos durante la primera década del siglo XXI. La vacunación masiva controló con éxito la infección en rumiantes, pero ocasionó diversos efectos adversos en varios países europeos. En España se describió un síndrome caquectizante asociado a signos neurológicos que fue englobado dentro del síndrome autoinmune/autoinflamatorio inducido por adyuvantes (síndrome ASIA).

El tipo de activación del sistema inmune tras la vacunación depende en gran medida del tipo de vacuna empleada. La mayoría de las vacunas ovinas se basan en patógenos inactivados o proteínas recombinantes que a menudo son poco inmunogénicas *per se*, por lo que necesitan adyuvantes para aumentar la respuesta inmunitaria. Los adyuvantes a base de Aluminio (ABAs) se han utilizado en vacunas humanas y animales durante más de 70 años. Sin embargo, se desconocen gran parte del mecanismo de acción de estos adyuvantes. La inoculación de ABAs induce el reclutamiento de células inflamatorias en el punto de inyección que evoluciona hacia la formación de un nódulo inflamatorio crónico, el llamado granuloma postvacunal, que

está compuesto principalmente por macrófagos activados. Estos agregados de macrófagos cargados con Al pueden migrar hacia el linfonodo regional y acumularse en él.

Los lentivirus de pequeños rumiantes (SRLV) causan infecciones crónicas en ovejas y cabras en todo el mundo con una alta prevalencia. Los SRLV son retrovirus de ARN monocatenario con una alta heterogeneidad genética. De hecho, se han caracterizado cuatro genotipos principales (A, B, C y E) y más de 35 subtipos. Los SRLV tienen tropismo por el sistema mononuclear-fagocítico e inducen una inflamación lenta, crónica y persistente en cuatro órganos diana principales: pulmón, articulaciones, sistema nervioso y glándula mamaria. El tropismo por cada uno de estos tejidos, así como la severidad de las lesiones, dependen de factores asociados a la estirpe del virus y a la respuesta inmune del hospedador. En la forma articular, el principal signo clínico es artritis en la articulación del carpo, la cual puede ser unilateral o bilateral y en ovinos es causada principalmente por estirpes del genotipo B2.

El objetivo de esta Tesis Doctoral es estudiar los efectos de la inoculación repetida de oxihidróxido de aluminio (Al) en ovinos y su relación con la patogenia del SRLV. El Experimento 1 incluyó 84 corderos repartidos en cuatro rebaños (Rebaños 1-4) de 21 animales cada uno. En cada Rebaño, los corderos se dividieron en tres grupos de tratamiento de 7 animales cada uno: Grupo *Vaccine*, inoculado con vacunas comerciales; grupo *Adjuvant-only*, que recibió la dosis equivalente de oxihidróxido de Al, y el grupo *Control*, al que se le inyectó solución salina tamponada con fosfato. El experimento duró 15 meses a lo largo de los cuales los corderos recibieron 19 inoculaciones subcutáneas, reproduciendo la cantidad de Al que pueden recibir estos animales a lo largo de su vida productiva. El Experimento 2 se basó en 15 ovejas hembras adultas (> 4 años) obtenidas de rebaños comerciales, infectadas de forma natural por SRLV y con signos clínicos de artritis bilateral. De manera similar al Experimento 1, los animales fueron dividieron en 3 grupos de tratamiento (*Vaccine*, *Adjuvant-only* y *Control*). Seis animales fueron excluidos tras el período de cuarentena por razones de bienestar animal y el número final de animales en cada grupo fue: grupo *Vaccine* (n=4), grupo de *Adjuvant-only* (n=3) y grupo *Control* (n=2). El experimento duró 75 días a lo largo de los cuales los animales recibieron un total de ocho inoculaciones subcutáneas.

Para complementar los resultados moleculares y serológicos del Experimento 2, un grupo adicional (grupo *Vaccine-extra*; n=2) fue inoculado con 4 dosis de vacunas comerciales a lo largo de 40 días.

El Capítulo 1 emplea animales de los Rebaños 1-4 del Experimento 1 y proporciona un **análisis de los índices productivos y parámetros clinicopatológicos asociados a inoculación repetida de oxihidróxido de Al en corderos, ya sea solo o como parte de una vacuna**. Se observaron ligeras diferencias en la ganancia media diaria y el índice de engrasamiento, lo que indica una reducción en la tasa de crecimiento en los animales vacunados, probablemente relacionado con breves episodios de pirexia y anorexia en los días posteriores a la inoculación. Los parámetros clínicos y hematológicos permanecieron dentro de los rangos normales. La histología no mostró diferencias significativas entre los grupos tratados, aunque se observó una tendencia a presentar una mayor frecuencia de neuronas hipercromáticas (i.e., *dark neurons*) en la médula espinal lumbar en el grupo *Adjuvant-only*. Aunque el oxihidróxido de Al se ha asociado con la aparición granulomas en el sitio de inyección y con cambios etológicos en ovejas, los resultados del Capítulo 1 indican que el oxihidróxido de Al inyectado por vía subcutánea no es suficiente para reproducir completamente la clínica caquectizante del síndrome ASIA ovino. Otros factores como el sexo, la raza, la edad, el acervo genético, el sistema de producción, la dieta o las condiciones climáticas podrían influir en el desarrollo de este síndrome.

El Capítulo 2 emplea animales del Rebaño 1 del Experimento 1 para la **detección de Al en la médula espinal lumbar tras la inoculación repetida de oxihidróxido de Al, ya sea solo o como parte de una vacuna**. Los depósitos de Al fueron significativamente más abundantes en la médula espinal lumbar en comparación con el lóbulo parietal en el grupo *Adjuvant-only* y mostraron una tendencia estadística en el grupo *Vaccine*. En la médula espinal lumbar, los depósitos de Al fueron más numerosos en los grupos *Vaccine* y *Adjuvant-only* en comparación con el grupo *Control*. En el lóbulo parietal, los depósitos de Al fueron mayores en el grupo de *Vaccine* en comparación con el grupo *Control* y el grupo *Adjuvant-only*. En la médula espinal lumbar, los depósitos de Al fueron más abundantes en la sustancia gris que en la sustancia blanca en los grupos *Vaccine* y *Adjuvant-only* y fundamentalmente se encontraban asociados a células de tipo glial. En

el lóbulo parietal se encontraron pocos depósitos de Al, en ocasiones relacionados con vasos saguíneos. Los resultados del Capítulo 2 indican que, en ovejas, el oxihidróxido de Al inoculado en el tejido subcutáneo se acumulan selectivamente en la médula espinal lumbar.

El Capítulo 3 emplea animales del Rebaño 1 del Experimento 1 y proporciona un **profundo análisis de las vías de señalización inflamatoria e inmunitaria en los granulomas postvacunales, los ganglios linfáticos regionales y el bazo de corderos tras la inoculación repetida de oxihidróxido de Al, ya sea solo o como parte de una vacuna**. La activación inmunológica fue más evidente en los órganos linfoides secundarios (linfonodo regional y bazo) e implicó la sobreexpresión de toll-like receptors, citoquinas pro-inflamatorias, moléculas co-estimuladoras y proteínas antivirales. Se encontraron pocas diferencias en los granulomas probablemente debido a la falta de un tejido control adecuado. Sin embargo, se observó una reducción en la expresión de interleucina 10 (IL-10) y un aumento de IL-6 que pueden indicar una activación inmune constante en los granulomas de animales vacunados. Los resultados del Capítulo 3 indican que, en ovejas, las vías de señalización pro-inflamatoria e inmunitarias se estimulan tras la inoculación de Al-oxihidróxido, siendo esta estimulación mayor cuando el Al-oxihidróxido forma parte de una vacuna.

El Capítulo 4 emplea animales de los Rebaños 2 y 4 del Experimento 1 y animales del Experimento 2. Este capítulo aborda el **impacto de los granulomas inducidos por oxihidróxido de Al en la respuesta de anticuerpos, la carga viral y la evolución de las lesiones de SRLV**. En los Rebaños 2 y 4 del Experimento 1, el número de nuevos animales seropositivos frente a SRLV fue mayor en los grupos *Vaccine* y *Adjuvant-only* que en el grupo *Control*. En consecuencia, las ovejas artríticas mostraron un aumento en el título de anticuerpos a lo largo del experimento en los grupos *Vaccine*, *Vaccine-extra* y *Adjuvant-only*, pero no en el grupo de control. La cantidad de ADN viral en sangre aumentó ligeramente en los animales del grupo *Control*, mientras que los grupos *Vaccine*, *Vaccine-extra* y *Adjuvant-only* mostraron una marcada disminución. Además, el análisis de radiografías y termografías mostró una progresión acelerada de la artritis en los grupos *Vaccine* y *Adjuvant-only*. Estos animales presentaban granulomas en punto de inoculación en los que se demostró la presencia de SRLV en el citoplasma de

los macrófagos. Estos granulomas infectados por SRLV son probablemente los responsables del aumento de los títulos de anticuerpos en los grupos *Vaccine*, *Vaccine-extra* y *Adjuvant-only*. Los resultados del Capítulo 4 indican que la respuesta serológica contra SRLV, así como la dinámica viral y la patología asociada pueden verse afectadas en animales inoculados con ABA, lo que subraya la importancia de los granulomas inducidos por Al en la patogenia de los virus con tropismo por macrófagos.

El Capítulo 5 desarrolla una **revisión sistemática y un meta-análisis** de las publicaciones científicas en el campo de los SRLV durante los últimos 40 años (1981-2020), para evidenciar su **prevalencia mundial en ovinos.** La mayoría de los artículos utilizaron una sola prueba diagnóstica para estimar la prevalencia de SRLV, mientras que los artículos que utilizaron 3 o más pruebas fueron escasos. La técnica ELISA ha reemplazado progresivamente a AGID a lo largo de las últimas décadas. La infección por SRLV en ovejas está muy extendida en todo el mundo, y Europa es el continente con mayor prevalencia individual (40,9 %). Europa es también el área geográfica en la que se han realizado más estudios. África, Asia y América del Norte muestran valores que oscilan entre el 16,7 % y el 21,8 % a nivel individual. América del Sur y Central muestran la prevalencia individual de SRLV más baja (1,7%). Hubo una fuerte correlación positiva entre la prevalencia individual y prevalencia colectiva. Los resultados del Capítulo 5 demostraron que, a pesar de la importancia mundial de la ganadería ovina, el conocimiento sobre la prevalencia de SRLV es irregular e inconsistente. Es necesario un método *gold standard* de diagnóstico y una estrategia de muestreo definida entre países y continentes.

Esta Tesis Doctoral proporciona un avance sustancial en la comprensión del mecanismo de acción de los ABAs y su interacción con los SRLV. El enfoque multidisciplinar que supone el análisis integrado de datos productivos, bioquímicos, histopatológicos, serológicos, moleculares y epidemiológicos permiten poner de manifiesto la importancia de los ABA, los SRLV y la interacción entre ellos. La persistencia de granulomas en el punto de inyección, la translocación de ABA al sistema nervioso central y la replicación de SRLV dentro de macrófagos cargados con Al exigen el diseño de nuevos adyuvantes vacunales que permitan el desarrollo de vacunas más seguras en ovejas.

INTRODUCTION

1 VACCINES

1.1 History of Vaccination

First attempts of active immunization in China and India, known as *variolation* (Plotkin and Plotkin, 2011), consisted in the inoculation of smallpox virus into the skin or nose of healthy children, preventing them from developing natural smallpox (Leung, 2011). *Variolation* rapidly extended to the Middle-East, where Lady Montagnu, British ambassador's wife in the Ottoman Empire, recognized its significance and imported the practice to the United Kingdom (UK) in 1721 (Parish, 1965; Plotkin and Plotkin, 2018).

The first vaccination dates from 1774, when an English cow breeder named Benjamin Jesty inoculated his wife and two children with cowpox collected from his infected herd to protect them against smallpox (Pead, 2003; Riedel, 2005). Based on this and other similar experiences, Edward Jenner perceived the efficacy of cowpox virus infection in the prevention of human smallpox. Moreover, he discovered that cowpox could be passed directly from one person to another, providing a large-scale model of immunization (Smith, 2011). Jenner referred to smallpox as *Variolae Vaccinae*, giving birth to the term "vaccine" (Baxby, 1999). Jenner studies were published in 1798 and they are considered the origin of live-attenuated vaccines (Jenner, 1798; Riedel, 2005). During the first decades of the 19th century, arm-to-arm transfer was the main method of human vaccination. This implied the attenuation of the virus, which originated the concept of "attenuation through passages" (Ballard, 1868; Plotkin and Plotkin, 2018).

The next major step in vaccine development took place in France. Louis Pasteur established the modern concept of vaccination, which is based on designing vaccines with the same agent that causes the disease. By developing several attenuation methods, he standardized the production of reproducible live attenuated vaccines. Pasteur initially worked in the attenuation of chicken cholera bacterium (currently *Pasteurella multocida*) and published part of his methods and results in 1880 (Pasteur, 1880; Bazin, 2011). At that moment, a ferocious competition started in France between Pasteur and the veterinarian Henry Toussaint to develop the vaccine against anthrax. It is widely accepted that Toussaint was first in achieving attenuation of the bacteria by

filtration and phenol acid extraction (Bazin, 2011; Plotkin and Plotkin, 2018). However, Toussaint came down with a neurologic disease and Pasteur announced his vaccine against anthrax in 1881 (Pasteur, Chamberland and Roux, 1881). Years later Pasteur developed the vaccine against rabies virus based on desiccated spinal cords of experimentally infected rabbits (Pasteur, 1885).

In 1886, Daniel Salmon and Theobald Smith achieved for the first time chemical inactivation of whole bacteria using *Salmonella*, a bacteria named to honor their scientific advances (Salmon, 1886; Salmon and Smith, 1886). They started the era of killed vaccines and allowed the development of vaccines against typhoid, plague, cholera and pertussis (Wright and Semple, 1897; Plotkin and Plotkin, 2018). The last years of the ninetieth century were cornerstone in the immunology field: Elie Metchnikoff at Institut Pasteur proposed the concept of cellular immunity and formulated the term "phagocyte" (Metchnikoff, 1905; Parish, 1965). Paul Ehrlich at Koch's Laboratory proposed the presence of "antibodies" based on the antitoxins found in the serum of animals previously inoculated with low doses of tetanus or diphtheria toxins (Lindenmann, 1984). Both, Metchnikoff and Ehrlich, shared the Nobel Prize in Medicine in 1908.

After the formulation of the fundamental concepts of vaccinology, during the first half of the twentieth century more crucial advances and understanding of some of the basic mechanisms in vaccinology were achieved. In the first decades, Albert Calmette and Camille Guerin developed the first vaccine against tuberculosis (Bacilli Calmette-Guerin; BCG) after 230 passages in culture of mycobacteria obtained from a bovine. In 1931, Goodpasture developed a method to culture viruses using chorioallantoic membranes of fertile hen eggs (Woodruff and Goodpasture, 1931). This advanced technique allowed Max Theiler to develop Yellow Fever vaccine, that was awarded with the Nobel Prize in 1951 (Plotkin and Plotkin, 2018). Later, Alexander Glenny, Barbara Hopkins and Gaston Ramon achieved to inactivate toxins with formalin to produce what is now called "toxoids" (Monod, 1964). This finding allowed the development of toxoids vaccines and the control of diphtheria and tetanus.

The latter half of the twentieth century is known as the golden age of vaccine development. After World War II, *in vitro* techniques for cell culture allowed virus

cultivation and viral vaccines refinement (Parish, 1965). “The Polio Wars” confronted multiple laboratories to be the first in developing a vaccine against this disease. Jonas Salk developed the trivalent formalin-inactivated polio vaccine, which was licensed in 1955 after a successful and historical “dual-control trial” in the United States of America (USA) (Salk *et al.*, 1954; Francis Jr *et al.*, 1955). Albert Sabin developed the live oral polio vaccine that was licensed in USA in 1960. Both vaccines have contributed to virtually eradicate polio from the western hemisphere, despite occasional cases of vaccine-associated polio disease (Plotkin and Plotkin, 2018). *In vitro* cell cultures also led to the development of vaccines against measles (Katz *et al.*, 1960), mumps (Hilleman *et al.*, 1968), rubella (Weller and Neva, 1962), adenovirus, varicella and rotavirus as well as the improvement of vaccines against rabies and Japanese encephalitis (Plotkin and Plotkin, 2018). Newer vaccine technologies were developed in the last decade of the 20th century, including re-assortment, reverse genetics, and cold adaptation. Cold-adapted influenza vaccines allow virus replication in the nostrils (32ºC), but not in warmer internal organs such as lungs (37ºC) (Abramson, 1999). Re-assortant technology has helped to develop influenza and rotavirus vaccines.

Regarding bacterial vaccines both, the discovery of capsular polysaccharides and the identification that protein-conjugated polysaccharides enhanced immunity, allowed the development of conjugated vaccines against *Haemophilus influenzae*, meningococci and pneumococci thus controlling the major causes of meningitis in children (Austrian *et al.*, 1976; Schneerson *et al.*, 1980; Pichichero, 2005; McNeil, 2006). Another important advance was the development of recombinant protein vaccines, first achieved by Valenzuela, Medina and Rutter at the University of California, where they cloned human hepatitis B surface antigen in yeast. Based on this technique, vaccines against hepatitis B, human papillomavirus and Lyme disease could be developed (McNeil, 2006).

More recently, with the establishment of whole-genome sequence techniques, the basics for reverse vaccinology (i.e. vaccines produced by genomics) were settled down (Rappuoli, 2000). This technology was pioneered by Rino Rappuoli and allowed the development of menigococcus serogroup B vaccine, which was licensed in USA in 2015 (Giuliani *et al.*, 2006; Plotkin and Plotkin, 2018).

Currently, vaccine development faces new challenges including COVID-19[1] pandemics, which has caused an important acceleration in the development of several vaccine platforms. The World Health Organization (WHO) publishes a regularly updated list of COVID-19 vaccines under development (*COVID-19 vaccine tracker and landscape*, 2021). By the end of August 2021, there were 112 vaccine prototypes in clinical phase and 185 in pre-clinical phase. Two new types of vaccine platforms have gained importance during COVID-19 pandemics: mRNA-based vaccines and viral vector-based vaccines (Ura *et al.*, 2021). Well-known examples of these vaccines are: *mRNA1273*, a lipid-encapsulated RNA vaccine manufactured by Moderna (Corbett *et al.*, 2020); *BNT162b1*, a lipid-encapsulated mRNA vaccine manufactured by BioNTech-Pfizer (Mulligan *et al.*, 2020); *ChAdOx1 nCoV-19*, a modified chimpanzee adenovirus manufactured by AstraZeneca-Oxford University (van Doremalen *et al.*, 2020) and *Ad26.COV2-S*, a modified human adenovirus manufactured by Janssen Pharmaceutical (Sadoff *et al.*, 2021).

1.2 Basic Principles of Vaccines

Vaccines are one of the greatest public health achievements of the last centuries (Vetter *et al.*, 2018). The main goal of vaccination is the active stimulation of the immune system by the administration of antigenic material (Kocourkova *et al.*, 2017). Vaccines try to mimic the immunogenic aspects elicited by natural infections in order to provide long-term protection against pathogens causing these infections (Canouï and Launay, 2019). Vaccination drastically reduces the morbidity and mortality induced by natural infections, which have a positive impact on public health, animal welfare and economic aspects. Moreover, vaccines protect both vaccinated and unvaccinated individuals by reducing inter-individual transmission and limiting the risk to be exposed. This indirect protection is called herd or community immunity and requires vaccination of a high proportion of the susceptible population (Fine, 1993; Kim, Johnstone and Loeb, 2011).

Immune activation after vaccination highly depends on the type of vaccine employed, since source and native properties of antigens highly determine the fate of

[1] COVID-19: Coronavirus disease emerged in 2019

the immune response (Montoya and Tchilian, 2021). This can be reduced to how the immune system process and presents the antigen delivered by a vaccine. Intracellular antigens are processed through the proteasome, linked to major histocompatibility complex (MHC) Class-I molecules and presented to CD8+ cytotoxic T cells, thereby exerting cytotoxic effector responses (Kumar, Abbas and Aster, 2021). Conversely, extracellular antigens are engulfed forming intracellular lysosomes in which protein digestion will render peptides arranged with MHC Class-II molecules and presented to CD4+ T helper (T_h) cells. Effector functions of these T_h cells include secretion of T_h1-like and T_h2-like cytokines and polarization of the immune response (Kumar, Abbas and Aster, 2021).

1.3 Vaccines in veterinary medicine

Infectious diseases of domestic animals have a major impact in animal husbandry. Consequences of infection in animal health and direct effects on animal production (i.e., poor productivity and condemnation of product) are the most relevant and immediate consequences. On top of that, the zoonotic potential of certain animal diseases and indirect effects as trade restrictions and are also of global concern.

Animal vaccines are becoming increasingly important as a prophylactic measure to prevent, manage and control diseases. This is especially evident nowadays due to the increase burden of antibiotic-resistant bacteria and antiparasitic-resistant parasites (Wellington *et al.*, 2013; Chambers, Graham and La Ragione, 2016). Moreover, the awareness of consumers about drug residues in meat and milk is leading to a marked decrease of such treatments and a compensatory increase in vaccine research and development (Roeder, Mariner and Kock, 2013; Mehdi *et al.*, 2018). However, vaccines should not be seen as the “silver bullet” against infections but just as a part of the solution. Indeed, vaccine efficacy depend on many others medical actions such as accurate disease diagnosis, implementation of surveillance programs, improvement of management conditions and identification and control of vectors and reservoirs control (Chambers, Graham and La Ragione, 2016).

Rinderpest (or cattle plague) is an excellent example of how combined control measures can dramatically improve animal health and human wellbeing (Roeder, Mariner and Kock, 2013). Rinderpest is a highly contagious viral disease of ruminants that was imported from India to Africa in 1889, causing the most catastrophic natural disaster ever to affect the whole African continent. The epidemics killed approximately 90% of the cattle in sub-Saharan Africa as well as many sheep and goats. This resulted in mass famine that eventually wipe out a third of the human population in Ethiopia and two-thirds of the Maasai people of Tanzania (Roth and Sandbulte, 2021). The Global Rinderpest Eradication Programme was a large-scale international collaboration involving vaccination that finished in 2011, when rinderpest infection was declared eradicated (*OIE Annual Report*, 2011).

As illustrated by rinderpest, vaccination strategies are usually directed towards the animal species of concern. Occasionally, vaccines are also designed for the animal reservoirs of certain pathogens, as the vaccination campaigns of European badgers in UK or wild boars in Spain against *Mycobacterium bovis*, that has reduced transmission to domestic ruminants and pigs (Gortazar *et al.*, 2014; Chambers *et al.*, 2017). Similarly, cats vaccinated against *Toxoplasma gondii* showed decreased rate of oocytes excretion after infection, thus reducing the likelihood of Toxoplasma-induced abortion in sheep and humans (Ramakrishnan *et al.*, 2019; Chu and Quan, 2021).

Another key application of animal vaccines is the protection of humans against zoonotic pathogens. In this sense, domestic dogs are vaccinated against rabies; poultry and pigs are vaccinated against zoonotic serovars of *Salmonella spp.* and cattle are vaccinated against EnteroHemorrhagic *Escherichia coli* O157:H7 (Varela, Dick and Wilson, 2013).

1.3.1 Health management in Small Ruminants

Professionalism in sheep and goat husbandry has drastically increased during last decades. Implementation of appropriate health management plans is crucial to guarantee farming sustainability by increasing production and animal welfare (Scott, Sargison and Wilson, 2007). Good husbandry practices and prophylactic measures to prevent diseases are cornerstone of any successful health management plan. These

measures include ectoparasite treatment and appropriate vaccination strategies (Scott, Sargison and Wilson, 2007).

Vaccination protocols in sheep farms differ depending on a variety of factors such as production system, geographical location, climate, and/or disease prevalence (Lacasta *et al.*, 2015). The main diseases that can be prevented or attenuated by vaccination in small ruminants can be grouped in:

- Diseases of the **digestive system** that can be prevented via vaccination are mainly caused by *Clostridium spp.*, *Escherichia coli*, and *Mycobacterium avium subspp. paratuberculosis*. Vaccination of pregnant ewes against colibacillosis and clostridial diseases is recommended 3-6 weeks before lambing to protect the newborn via passive immunity (Lewis, 2011; Lacasta *et al.*, 2015). Booster vaccines against *Clostridium spp.*can be applied twice a year, preferentially before entering new pastures (Ganter, 2008). Vaccination schedule against paratuberculosis includes a primovaccination of lambs at the age of 4-12 weeks followed by yearly booster vaccinations (Windsor, 2013).

- Diseases of the **respiratory system** are mainly caused by commensal organisms of the nasopharynx as *Mannheimia haemolytica*, *Mycoplasma ssp.*, *Bibersteinia trehalosi* and *Pasteurella multocida*. The bronchopneumonia caused by these pathogens usually need predisposing factors such as viral infections, immunosuppression or stress associated with management conditions. Vaccination schedule against *Mannheimia spp.* includes a primovaccination at two weeks of age, followed by a booster vaccine 3-4 months later and repeated vaccinations each 6-12 months (Lacasta *et al.*, 2015).

- Diseases of the **reproductive system** include abortions and metritis in ewes and epididymitis and orchitis in males. The main abortive agents in sheep are *Brucella spp.*, *Chlamydia abortus*, *Coxiella burnetii*, *Campylobacter ssp.*, *Salmonella spp.*, and *Toxoplasma gondii*. Vaccination should be performed every year before the mating period (Blasco and Molina-Flores, 2011; Rodolakis and Laroucau, 2015). Vaccination of pregnant or lactating animals against *Brucella spp.* may lead to

abortions or lactogenic shedding of the vaccine, respectively (Blasco and Molina-Flores, 2011).

- **Other common diseases** include caseous lymphadenitis, footrot, contagious echtyma, affections of the central nervous system as listeriosis and diseases of the mammary glands as contagious agalactia. The prophylactic measures against these conditions will vary depending on environmental conditions and infection pressure.

- **Emerging or re-emerging diseases** can modify health management programs as they can imply compulsory vaccination campaigns for effective control.

Although highly variable, in our local management conditions a single animal usually receive an estimated average of three vaccines per year, which implies more than fifteen vaccine injections during their whole lifespan.

1.3.2 Bluetongue Vaccination Campaigns

Bluetongue is a systemic disease caused by bluetongue virus (BTV), which is transmitted by midges of the genus *Culicoides.* BTV belongs to the genus *Orbivirus* (family *Reoviridae*) and includes more than 26 serotypes (Uzal, Plattner and Hostetter, 2016). Immunity against one serotype does not confer protection against other serotypes. On top of that, infection with a BTV serotype can produce sensitization, leading to a more severe disease after infection with other serotype (Uzal, Plattner and Hostetter, 2016). Sheep are highly susceptible to BTV and develop a systemic disease with mortalities ranging from 2% to 70% depending on the age, breed, immune status of the host, and the serotype of the virus. Goats and cattle are also susceptible to infection, but they usually remain subclinical (Maclachlan *et al.*, 2009).

Bluetongue is enzootic in Africa, Middle East, India, northern Australia and USA (Uzal, Plattner and Hostetter, 2016). Climate change is favoring the spread of *Culicoides* mites and outbreaks in the Iberian Peninsula, UK, Balkans, Corsica, and Sardinia have been reported during the last decades (Mellor *et al.*, 2008; Uzal, Plattner and Hostetter, 2016). In 2006, BTV serotype 8 was reported in Netherlands and quickly spread towards central and northern Europe, causing a state of emergency within the continent

(Carpenter, Wilson and Mellor, 2009). The compulsory vaccination campaign implemented in Europe (European Commission, 2008) led to the vaccination of the whole ovine population, usually including immunization against two different BTV serotypes, depending on each geographical area. In Spain, most common serotypes were BTV serotype 1, BTV serotype 4, and BTV serotype 8. One vaccine per each serotype was developed and animals were vaccinated against the two most prevalent serotypes in each region. Vaccines were applied through a simultaneous primovaccination followed by a booster inoculation after three to four weeks. As a result, animals received four vaccines in less than a month (Mellor *et al.*, 2008). The mass vaccination campaign successfully controlled the infection. However, several adverse effects were reported in different European countries. For instance, in the UK, vaccine-associated adverse events in 2008 increased by around 11% compared to 2007 (Dyer *et al.*, 2009). In France, reproductive problems (i.e., abortion and decreased fertility), weakness and anorexia were reported (Agence Française de Sécurité Sanitaire, 2009; Nusinovici *et al.*, 2011). Similar side effects were recorded in Germany and Switzerland although national surveys failed to clearly correlate them with BTV vaccines (Tschuor *et al.*, 2010; Probst *et al.*, 2011).

In Spain, a wide array of adverse effects was observed from 2008 onwards by farmers and veterinarians. Clinical signs were mainly characterized by ethological changes, neurologic signs and cachexia, and caused important economic losses (Asín *et al.*, 2018). Two independent research studies linked these clinicopathological changes with the intense vaccination schedule applied to animals (González *et al.*, 2010; Luján *et al.*, 2013). However, official reports of the Spanish government yielded inconclusive results: on one hand, they ruled out ovine diseases commonly associated with either cachexia or neurodegenerative lesions; on the other hand, no correlation with the bluetongue vaccination campaign was established (Pujols *et al.*, 2009; Sánchez-Vizcaíno, 2009). The media at the national level presented the situation and reflected the fear of farmers and their uncertainty (García, 2009; Rojo, 2009). Currently, farmers and veterinarians continue to express their fear regarding these vaccines as new BTV outbreaks has been recently reported and no clear explanation of the past adverse-effects have been found (Millán, 2017).

In 2013, Lujan *et al.* focused on aluminum-based adjuvants (ABAs) as the triggering factor of the clinicopathological findings observed and summarized them under the spectrum of the Autoimmune/autoinflammatory Syndrome Induced by Adjuvants (ASIA syndrome). This syndrome was first reported in humans to encompass several conditions (i.e.: Postvaccination phenomena; Macrophagic myofasciitis; Siliconosis; Gulf War syndrome; Sick building syndrome) developed as a consequence of the strong and persistent stimulation of the immune system triggered by adjuvants and similar products (Shoenfeld and Agmon-Levin, 2011; Agmon-Levin, Hughes and Shoenfeld, 2012). In humans, this syndrome can manifest myalgia, muscle weakness, chronic fatigue, sleep disturbances and neurological disorders (Watad *et al.*, 2017).

Ovine ASIA syndrome can appear as an acute phase in less than 0.5% of the flock, 2-6 days after vaccination with Aluminum (Al) containing vaccines, characterized by neurological signs such as low response to external stimuli, disorientation, transient blindness, prostration, and occasional convulsions. Animals usually recover a few days later. The acute phase is microscopically characterized by moderate to severe mixed-cell perivascular meningoencephalomyelitis associated with gliosis and neuronal death (Luján *et al.*, 2013). The chronic phase appears longtime after the vaccination with Al-containing vaccines and can affect a high proportion of animals. This phase is usually triggered by overlapping external stimuli as low temperatures, poor nutrition, and other stressful situations. The main clinical signs are pernicious cachexia and ethological changes as restlessness. Postmortem analyses show marked depletion of fat deposits, muscle wasting, liquid extravasation in cavities (i.e., hydrothorax and hydropericardium) and perineural edema. Main histopathological lesions are found in the gray matter of the lumbar spinal cord, which shows marked neuronal necrosis. Additionally, perineural myxedema with perineuritis and perivascular encephalitis with gliosis can also be observed (Luján *et al.*, 2013).

1.4 Types of vaccines

Vaccines require the activation of different elements of the innate and adaptive immunity to be effective. Vaccine development can rely on different concepts, each having advantages and limitations. In this scenario, the choice of the most suitable

vaccine should rely on the biology of the microbe and the pathogenesis of the disease to be prevented.

1.4.1 Whole-pathogen vaccines

Traditional vaccines consist of entire pathogens that have been markedly weakened or fully inactivated avoiding disease onset.

- **Live Attenuated Vaccines** are based on Jenner's principle that weakened pathogens, partially retain the ability to replicate within the host, thereby providing exogenous and endogenous antigens. The attenuation is usually achieved by serial passages through *in vitro* cultures or *in vivo* models as alternative resistant hosts. The best example is the BCG vaccine against tuberculosis. The main advantage is abundant antigen expression, including those associated with pathogen metabolism, thus inducing both cellular and humoral immune responses.

  Attenuated vaccines are highly effective against protozoan or helminth parasites since antigens are differentially expressed between parasite life cycle stages. For example, Toxovax® (MSD Animal Health) protect ewes against *Toxoplasma gondii* abortions and is based on an attenuated strain of the parasite (S48) that cannot form cysts an persist in the host (Chambers, Graham and La Ragione, 2016).

  A disadvantage of these vaccines is that the immune responses elicited are indistinguishable of those elicited by natural infections and generates false positive results in surveillance campaigns, as is the case of some vaccines against paratuberculosis as Silirum® (CZ Veterinaria) (Garrido *et al.*, 2013). Moreover, there is a risk of inactivated viruses to revert to a virulent form or recombine with field viruses and cause disease as reported with vaccines against bovine viral diarrhea virus (BVDV) in cattle, porcine reproductive and respiratory syndrome (PRRS) in swine and avian influenza in poultry (Chambers, Graham and La Ragione, 2016). Finally, there is also a risk of vertical transmission in pregnant animals that can lead to fetal abnormalities due to persistent infections (Ficken *et al.*, 2006). *In vitro* production of these vaccines requires an accurate diagnosis

of relevant pathogens since when eukaryotic systems are used, the risk of contamination with other pathogenic viruses should be considered as it can have highly detrimental consequences (Asín, Hilbe, *et al.*, 2020).

- **Inactivated vaccines** are produced by killing the pathogen with chemicals, heat, or radiation. The main advantage of these vaccines is that they are generally safe, stable and retain most of the structural antigens found in the native pathogen. However, due to low immunogenicity of pathogen-associated proteins they usually require booster immunizations and the use of adjuvants to strengthen immune responses. Moreover, inactivated whole-virus vaccines are a source of exogenous antigens and may not induce cross-protection between viral serotypes (e.g., Foot-and-mouth disease virus) as they work mainly via antibody-mediated immunity and induce weak cellular-mediated immunity (Chambers, Graham and La Ragione, 2016). This limitation may be overcome by including multiple serotypes within the same vaccine.

1.4.2 Subunit vaccines

These vaccines include only those components of a pathogen that stimulate the immune system. They rely on previous knowledge of the protective antigens. Including only the essential antigens in a vaccine can minimize side effects, what makes these vaccines safer and easy to produce. However, they are often poorly immunogenic and usually require the incorporation of adjuvants to elicit stronger and protective immune responses. Interestingly, subunit vaccines may relieve inhibition of the immune system in the presence of maternal antibodies (Newport *et al.*, 2004).

- **Recombinant protein vaccines** are based on recombinant DNA technology, which enables DNA from two or more sources to be combined. Plasmids containing the DNA insert of interest can be produced in prokaryotic or eukaryotic systems -depending on the glycosylation patterns needed during protein synthesis- to produce high amounts of the protein of interest. Recombinant protein vaccines allow the simultaneous protection against multiple bacterial serotypes as is the case of *Actinobacillus pleuropneumoniae* (Xiao *et al.*, 2020). However, immunization with purified protein antigens

typically results in the induction of a modest antibody response with little or no T cell responses (Reed, Fox and Orr, 2013).

- **Toxoid vaccines** elicit immune responses against disease-causing proteins, so-called exotoxins, secreted by bacteria (Plotkin, 2005). The antigens in these vaccines are chemically inactivated toxins, known as toxoids.

- **Virus-like particles (VLPs)** are robust protein cages with well-defined geometry, which mimic the outer shell of a native virus (Zhao *et al.*, 2011). VLPs are stable and highly immunogenic as they induce strong cellular and B-cell-mediated responses (Grgacic and Anderson, 2006). They lack viral genome and therefore VPLs are non-replicative and safe. Ingelvac CircoFLEX® (Boehringer Ingelheim), a vaccine against porcine circovirus was the first VLP-based vaccine in veterinary medicine. Calicivirus-like vaccines are highly effective in rabbits and pigs (Crisci *et al.*, 2012).

- **Polysaccharide-protein conjugated vaccines** are based on polysaccharides (i.e., sugars forming the outer coating of many bacteria) attached -or "conjugated"- to a protein antigen to offer improved protection. This vaccine formulation is mainly used in human vaccines to develop immunity against polysaccharides in young children. Vaccines against *Haemophilus influenzae type B* and *Staphylococcus aureus* are based on this concept (Schneerson *et al.*, 1980; Fattom *et al.*, 2004).

1.4.3 Nucleic acid vaccines

- **DNA plasmid vaccines** are based on the ability of plasmids to transfect and express vaccine antigens in host cells, mainly skin and muscle (Jazayeri and Poh, 2019). Plasmids are small circular molecules of DNA designed to carry genes from the pathogen of interest under the control of eukaryotic promoters. Plasmids are stable and do not need cold-chain and can be engineered to bias immune response towards a T_h1 or T_h2 profile. However, introduction of exogenous DNA into cells maybe troublesome, especially in immune cells. Delivery methods to enhance DNA vaccines immunogenicity are abundant and continuously evolving (Redding and Weiner, 2009). They are highly successful in fish as illustrated by

Apex-IHN® (Novartis) that protects Atlantic salmon against Infectious hematopoietic necrosis.

- **Messenger RNA (mRNA) vaccines** are based on the ability of mRNA to produce vaccine antigens after delivery into host cells. These vaccines have experienced a large expansion in basic and clinical research over the last 4 years due to the high efficacy, capacity for rapid development, low-cost of manufacture, and safe administration (Pardi *et al.*, 2018; Linares-Fernández *et al.*, 2020). They have reached outstanding recognition during the COVID-19 pandemics, since *BNT162b1* developed by BioNTech-Pfizer, was the first vaccine to be approved in Europe and USA.

1.4.4 Vector-based vaccines

Some vaccines use a harmless virus, bacterium or parasite as a vector/carrier to introduce genetic material into cells (Huang *et al.*, 2008). Recombinant poxviruses are particularly attractive vectors as they are genetically stable, environmentally robust, induce a strong immune response and can accommodate abundant foreign DNA. Ilustrative examples are: Proteq-Flu/Recombitek® (Merial) against equine influenza (i.e, horse flue), which is based on canarypox vector; and Vectormune FP-LT® (Ceva Animal Health) against avian infectious laryngotracheitis (ILT) in poultry, which is based on fowlpox vector (Coppo *et al.*, 2013).

More complex approaches employ the attenuated form of a pathogen as the vector to express proteins of other pathogens. This technology allows the creation of bivalent or chimeric vaccines as Vaxxitek HVT+IBD® (Merial) or Vectormune HVT-LT® (Ceva Animal Health) that confers protection against Marek's disease –cased by Gallid Herpesvirus type 2, GaHV2- and Infectious bronchitis disease (IBD) or ILT, respectively.

Vaccines using *Salmonella* vectors expressing *Camplylobacter* peptides and genetically-modified *Eimeria* parasites are nowadays being developed (Layton *et al.*, 2011; Blake and Tomley, 2014).

1.4.5 Plant-based vaccines

Genetic modification of plants to express recombinant vaccine antigens that induce immunity once they are consumed by animals is an attractive option to vaccinate large number of animals (Phan, Floss and Conrad, 2013). Plant-based vaccines have shown promising results and they are licensed and available against Newcastle disease of poultry, BTV of sheep and PRRS of swine (Loza-Rubio and Rojas-Anaya, 2010; Jacob *et al.*, 2013).

1.5 Vaccine Adjuvants: State-of-the-art and classification

Adjuvant derives from the Latin *adiuvare*, which means "to help". In the context of vaccines, adjuvants are components that contribute to enhance and/or to direct the vaccine-induced immune response (Reed, Fox and Orr, 2013). Most adjuvants are used in combination with inactivated or subunit vaccines (mainly protein subunit vaccines) due to their low immunogenic capacities (Montoya and Tchilian, 2021).

Vaccine adjuvants have multiple benefits (Figure Intro-1). Among others, they allow a rapid immune response, reduce the number of immunizations required (Levie *et al.*, 2002) and reduce the vaccine dose (i.e., dose sparing) in hard-to-produce vaccines (Girard *et al.*, 2011). Most adjuvants improve humoral responses broadening B-cell diversity (Wiley *et al.*, 2011) and increase antibody titers and their functionality (Kasturi *et al.*, 2011; McCluskie *et al.*, 2013). Some adjuvants, as agonists of Toll-like receptors (TLRs), influence the innate and cellular responses to elicit a more effective engagement of T_h cells (Reed, Fox and Orr, 2013).

Adjuvants have been traditionally classified based on their mode of action and split into two main categories: *immunostimulants*, which act directly on the immune system; and *vehicles*, which act primarily by delivering antigens to the immune system. However, this classification has been proven too simplistic as most of adjuvants display more than one mechanism of action (Plotkin *et al.*, 2018).

Figure Intro-1: Main features of the ideal adjuvant. Newly developed adjuvants should induce rapid responses agains vaccine antigens, broad the immune response agains pathogens, be appropiate for newborns and old individuals, induce humoral and cellular responses, allow dose sparing and reduce the total number of immunizations and the time between them.

1.5.1 Mineral salts

- **Aluminum (Al) salts.** This group include Al-phosphate (Adju-Phos®), Al-oxyhydroxide (Alhydrogel® and Rehydragel®), Al-potassium sulfate (Alum) and Al-hydroxycarbonate (ImjectTM Alum). They are widely used in veterinary and human vaccines. Al typically induce Th2 responses by inducing antigen depots and stimulating innate and adaptive responses (O'Hagan, 1998; Gradon and Lutwick, 1999; Marrack, McKee and Munks, 2009; Calabro et al., 2011).

- **Calcium salts**. They include mainly calcium phosphate (CaP), which also induce T_h2 responses. This adjuvant was used some decades ago and has been recently proposed as an alternative adjuvant to Al (Masson *et al.*, 2017).

1.5.2 Oil Emulsions

They are composed of a mixture of a lipid phase and an aqueous phase, stabilized by a surfactant. The lipid phase was initially a mineral oil but it has been currently replaced by vegetable or animal oils, which are safer although less effective (Aucouturier, Dupuis and Ganne, 2001).

- **Water-in-oil emulsions**. These adjuvants are microdroplets of an aqueous phase dispersed in oil. Vaccine antigens are released slowly, thus achieving good

long-term immunity (Aucouturier, Dupuis and Ganne, 2001). Injection-site reactions are quite severe, and this is the reason why these adjuvants are licensed in veterinary medicine but not in human medicine.

- **Oil-in-water emulsions**. This group include OW-14 (Galliher-Beckley *et al.*, 2015) emulsions and MF59® (Novartis). These are micro-droplets of oil dispersed in an aqueous phase. This emulsions release the antigen quite quickly and microdroplets of oil carry the vaccine antigens to the regional lymph nodes (Spickler and Roth, 2003). They achieve good short-term immunity (Leenaars *et al.*, 1994). These adjuvants are especially effective against viral infections (Galliher-Beckley *et al.*, 2015) as they are potent stimulators of both cellular and humoral immune responses.

- **Water-in-oil-in-water emulsions.** They are composed of microdroplets of water dispersed in droplets of oil, which are again dispersed in an aqueous phase. These adjuvants join the advantages of both methods and promote both short-term and long-term immunity (Macy, 1997; Aucouturier, Dupuis and Ganne, 2001).

1.5.3 Nanoparticles and microparticles

Solid particles that range from 10nm-1µm (nanoparticles) and 1-100µm (microparticles). They are composed either by biodegradable polymers (i.e. cyanoacrylates; polylactide-co-glycolide; polyphosphazenes) or biodegradable materials (i.e., CaP) and can encapsulate vaccine antigens and release them for up to several months (Eng *et al.*, 2010). These adjuvants induce both, cellular-mediated and humoral-mediated immune responses (Spickler and Roth, 2003).

1.5.4 Liposomes

They are vesicles of cholesterol and phospholipids resembling cell membranes that incorporate vaccine antigens in their lumen or the membrane. Liposomes fuse with endosomes and deliver antigens to cells. Antigens are processed as exogenous material and coupled to MHC-II pathway (Alving *et al.*, 2012). Liposomes have been also used for drug delivering with a good safety profile (Alving *et al.*, 2012; Schwendener, 2014).

Archeasomes are liposomes composed of lipids derived from archaea (unicellular prokaryotes similar to bacteria) that are associated with high stability and long-term immunity (Krishnan *et al.*, 2000).

1.5.5 Saponins and immuno-stimulating complexes (ISCOMs)

Saponins are complex chemical adjuvants extracted from plants, mainly the tree *Quillaia saponaria* (Sun, Xie and Ye, 2009). They induce strong T-cell responses, mediated by T_h1, T_h2 and cytotoxic T lymphocytes (Cox and Coulter, 1997; Gradon and Lutwick, 1999; Vogel, 2000). Quil A® and Spikoside are partially purified mixtures of saponins. Quil A® is widely used in veterinary vaccines (Macy, 1997) and has been shown to be well tolerated in sheep and cattle (Sjölander, Cox and Barr, 1998), although it can induce local inflammatory reactions (Kersten and Crommelin, 1995). Purified saponin fractions as QS21, COPREP 703 have lower toxicity (Spickler and Roth, 2003; Sun, Xie and Ye, 2009).

ISCOMs are cage-like structures composed of cholesterol, phospholipids and saponins that are associated with strong T_h1 and cytotoxic T-cell responses (Sjölander, Cox and Barr, 1998; Hu, Lövgren-Bengtsson and Morein, 2001). Novel adjuvants as ISCOMATRIX™ have shown promising results (Morelli *et al.*, 2012).

1.5.6 Bacterial products

Killed bacteria and their products may boost inflammatory responses and immunity associated with vaccines. A good example is Freund's complete adjuvant, which includes mycobacteria products. Moreover, bacterial cell wall components as muramyl dipeptide (MDP, the active component of a mycobacteria peptidoglycan) and their derivatives have been associated with strong T_h1 and T_h2 responses (Cox and Coulter, 1997). Bacterial toxins as *Escherichia coli* heat-labile toxin and lipopolysaccharide derivatives as 4'monophosphoryl lipid A are also linked to strong T_h1 responses (Cox and Coulter, 1997). Bacterial DNA induce strong immune responses due to the adjuvant activity of CpG oligonucleotides, shifting the response towards a T_h1 profile (Carson and Raz, 1997; Chu *et al.*, 1997).

1.5.7 Other adjuvants

- **Synthetic adjuvants** composed of hydrophobic polyoxypropylene and polyoxyethylene can be employed in aqueous buffers and emulsions and induce strong humoral responses (Brey, 1995).

- **Carrier proteins** act as haptens and improve the immunogenicity of antigens when they are linked to them (O'Hagan, 1998).

- **Immunological adjuvants** as cytokines can be used to direct immune responses. Cytokines are small proteins that orchestrate immune responses and other biological processes and some of them. Interferon gamma (IFN-γ), Interleukin 1 beta (IL-1β), IL-2 and IL-12 have been used as vaccine adjuvants (Vogel, 2000). Indeed, IL-1β and IL-12 have shown promising results in sheep and cattle (Lofthouse *et al.*, 1996). Cytokines have been considered in developing SRLV vaccines with good performances. The main disadvantages of immunological adjuvants are that they are species-specific. Furthermore, they usually have pleiotropic effects and can activate multiple signaling pathways simultaneously, thus making their specific effects difficult to predict (Hughes, 1998).

- **Complement proteins** bind foreign antigens, thus acting as opsonins and improving effector activities of antibodies and immune cells. C3d and C3b molecules have shown marked adjuvant activity by increasing immunogenicity of the vaccine antigen up to 1000-fold (Dempsey *et al.*, 1996; Test *et al.*, 2001). The main disadvantage of complement proteins is that they can potentially activate B cells and induce autoimmunity (Test *et al.*, 2001).

1.5.8 Combination of adjuvants

The complementary and synergistic effect of adjuvants has been demonstrated and the effects of adjuvant combination usually exceed the sum of their individual effects (Mutwiri *et al.*, 2011). This allow further dose reduction of each individual adjuvant, what reduces their corresponding toxicity. Recently, several new adjuvants have been developed following this criteria: MF59® (Novartis), AS03® (GlaxoSmithKline) and IC31® (Valneva) (Montoya and Tchilian, 2021).

1.6 Aluminum-Based Adjuvants (ABAs)

ABAs have been historically used in vaccines since their discovery in 1926 (Glenny *et al.*, 1926). Since then, Al salts are the most widely used adjuvants in human and veterinary vaccines (Lindblad, 2004; Plotkin *et al.*, 2018). Despite being used for such a long period of time, their mechanism of action has recently begun to be understood.

1.6.1 Physical structure and chemical properties

Several Al compounds are used as adjuvants in current vaccines:

- **Alhydrogel® and Rehydragel®** are prepared mixing an Al solution -usually [$AlCl_3$] or [$AlK(SO_4)_2$]- with sodium hydroxide [Na(OH)] followed by a dehydration process (Yau *et al.*, 2006). Their chemical structure is the crystalline Al-oxyhydroxide (AlOOH) rather than Al-hydroxide [$Al(OH)_3$] (Shirodkar *et al.*, 1990). The degree of crystallinity affects the adsorptive capacity and the dissolution rate *in vivo* and *in vitro*. Al-oxyhydroxide has a surface area[2] of 500m^2/g, which makes it an excellent adsorbent (Johnston, Wang and Hem, 2002). In comparison, Al-hydroxide shows lower surface area (20-50 m^2/g). Alhydrogel® is composed of primary fibers (5x5x200nm) that aggregate in micron-sized particles. The surface is composed of Al-OH (hydroxyl groups) and Al-O-Al groups (Plotkin *et al.*, 2018). The hydroxyl group can either accept or donate a proton and its point of zero charge (equivalent to the isoelectric point of proteins) is 11.4, therefore Al-oxyhydroxide shows positive surface charge at pH 7.4 (Figure Intro-2). This adjuvant has high affinity for phosphate and fluoride groups, moderate affinity for sulfate and low affinity for chloride and nitrate (HogenEsch, O'Hagan and Fox, 2018). In this sense, the adsorptive capacity of Alhydrogel varies depending on the composition of the surrounding medium.

- **Adju- Phos®** is prepared mixing an Al-solution -usually [$AlCl_3$] or [$AlK(SO_4)_2$]- with a basic solution of trisodium phosphate [$Na_3(PO_4)$], or mixing an Al salt with a phosphate solution and a precipitation with sodium hydroxide [Na(OH)]. Its

[2] Surface area: Area that the surface of the particle occupies, which is the sum of the areas of its faces and the place where vaccine antigens can bind.

chemical structure is Al-hydroxyphosphate [$Al(OH)_x(PO_4)_y$], which is an amorphous adjuvant with high surface area and great adsorptive capacity (HogenEsch, O'Hagan and Fox, 2018). Their hydroxyl groups are partially substituted by phosphate groups, so the surface is composed of Al-OH and Al-O-PO_3 groups (Plotkin *et al.*, 2018). The commercial Adju-Phos® has a P:Al ratio of 1.1-1.5 and a point of zero charge range between 4.5 to 5.5, thus providing a negative surface charge at pH 7.4 (Figure Intro-2).

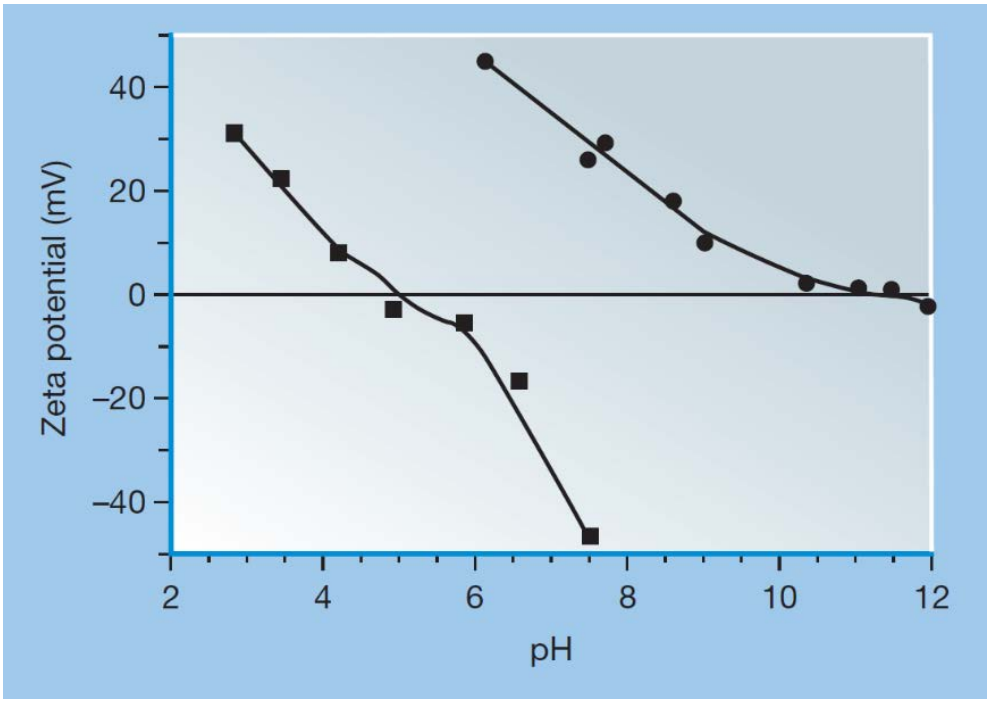

Figure Intro-2: Zeta potential* and point of zero charge# of Aluminum-based adjuvants. Squares represent the variation of the zeta potential of Al-oxyhydroxide at different pH. Circles represent the variation of the zeta potential of Al-phosphate at different pH (extracted from Plotkin *et al.*, 2018). *Zeta Potential: Electric charge of a solid particle in a colloidal dispersion. #Point of zero charge: pH at which the zeta potential is zero.

- **Imject™ Alum** is composed of amorphous Al-hydroxycarbonate and crystalline magnesium hydroxide (Hem, Johnston and HogenEsch, 2007). This compound induce weaker immune responses than Alhydrogel® and Adju- Plus® (Cain *et al.*, 2013).

- **Alum** is Al-potassium sulfate [$AlK(SO_4)_2$] and shows amorphous structure and similar adjuvant properties as Adju- Plus® (Hem *et al.*, 1996).

1.6.2 Adsorption mechanisms

There are three main mechanisms involved in adsorption of antigens by ABAs. The relevance of each mechanism varies depending on the nature of the antigen and the environmental conditions as pH, ionic strength and presence of surfactants (Rinella *et al.*, 1998; Rinella, White and Hem, 1998).

- **Electrostatic attraction**: This force depends on the surface charge of the antigen and the adjuvant (Seeber, White and Hem, 1991; Chang *et al.*, 1997). For example, at pH 7.4 Alhydrogel® (point of zero charge 11.4) adsorbs albumin (isoelectric point 4.8) but does not adsorb lysozyme (isoelectric point 11.0) whereas Adju-Phos® (point of zero charge 4.5 - 5.5) adsorbs lysozyme and not albumin (Seeber, White and Hem, 1991).

- **Hydrophobic forces**: Hydrophilic-hydrophobic interactions and van der Waals forces play a role in protein adsorption. These attractive forces are estimated by evaluating the effect of ethylene glycol on antigen adsorption (Al-Shakhshir *et al.*, 1995).

- **Ligand exchange**: Antigens containing phosphorylated groups (i.e. phosphorylated amino acids) interact with hydroxyl groups of the adjuvant leading to high-affinity binding (Iyer, HogenEsch and Hem, 2003).

1.6.3 Mechanism of action and immunity associated to ABAs

Despite ABAs being used for more than 70 years, mechanisms by which they booster the immune response are still poorly understood. First hypothesis pointed towards the “depot mechanism” as the dominant feature; secondly, the promotion of antigen uptake by dendritic cells and more recently, direct stimulation of the innate immunity were proposed as mechanisms of action. The most likely explanation is that all these mechanisms may be operating simultaneously, thus making ABAs as effective as hard to understand.

1.6.3.1 <u>Depot mechanism</u>

Vaccine antigens remain adsorbed to ABAs at the site of injection and they are released slowly, delaying clearance from the injection site, and allowing recruitment of

antigen presenting cells (APCs) (Gupta *et al.*, 1995; Marrack, McKee and Munks, 2009). This hypothesis was developed by Glenny et al. and remained dogmatically accepted for more than 60 years (Glenny, Buttle and Stevens, 1931). It was supported by experiments in which antigens strongly bound to ABAs induced higher antibodies levels than those bond weakerly to the adjuvants. Other studies showed that, 7 weeks after inoculation of guinea pigs with ABAs, injection-site nodules could be excised and employed to immunize a second guinea pig (Harrison, 1935). Latter studies performed in rabbits showed that abundant B lymphoblast appeared in the draining lymph node about 7 days after immunization - indicating active antigen presentation - and remained up to 3 weeks after immunization (White, Coons and Connolly, 1955).

On the contrary, studies in mice showed that complete excision of the injection site shortly after inoculation have no effect on the immune response elicited (Hutchison *et al.*, 2012). However, these studies raised relevant immunological concerns as they were performed by inoculation in the pinnae and its excision could have released danger signals eliciting similar immune responses as the injection nodule itself.

1.6.3.2 Effects on Innate immunity

- **Antigen presenting cells uptake**

  Soluble antigens are captured by APCs by pinocytosis. However, when adsorbed to ABAs, antigens are recognized as particulate and captured by APCs by phagocytosis (Mannhalter *et al.*, 1985; Morefield *et al.*, 2005). Additionally, *in vitro* experiments evinced that antigen presentation is upregulated by ABAs in monocytes as shown by increased expression of MCH class II molecules, CD40 and CD86 (Ulanova *et al.*, 2001). Similar results have been found in dendritic cells although these results are still under discussion (Sokolovska, Hem and HogenEsch, 2007; Sun, Pollock and Brewer, 2003).

- **Sensors of Pathogen-associated molecular patterns (PAMPs)**

  Studies in mice analyzing the role of TLRs as sensors of ABAs showed that neither TLRs nor their downstream adaptor molecules (MyD88[3] and TRIF[4]) are involved

[3] MyD88: Myeloid differentiation primary response 88
[4] TRIF: TIR-domain-containing adapter-inducing interferon-β (TIR: Toll-interleukin-1 receptor)

in the signaling pathways of these adjuvants (Schnare *et al.*, 2001; Gavin *et al.*, 2006).

- **Sensors of Danger associated molecular patterns (DAMPs)**

  Recent studies show the central role of NLRP3[5]-dependent inflammasome activation in the immunity elicited by ABAs (Eisenbarth *et al.*, 2008; HogenEsch, 2012). NLRP3 - also known as NALP3 - is an intracellular protein that is activated by potassium efflux after lysosomal damage/rupture and cathepsin B release (Figure Intro-3) (Kool, Pétrilli, *et al.*, 2008). When activated, NLRP3 associates with ASC[6] and activates Caspase-1, which cleaves the precursors of IL-1β, IL-18 and IL-33 and generate active cytokines inducing a strong pro-inflammatory stimulus (Li, Nookala and Re, 2007; Li *et al.*, 2008).

  As stated before, lysosomal damage is a key step in this process and it can be achieved by two alternative ways:

  Indirect activation model: ABAs induce cytotoxicity leading to cell death and release of abundant DAMPs as uric acid. Uric acid saturates and precipitate in the extracellular millieu as monosodium urate (MSU) crystals, which interact with cholesterol in plasma membranes and are phagocytosed by macrophages and dendritic cells (Ng *et al.*, 2008). Indeed, MSU crystals has been employed as adjuvants and have shown to increase antibody responses in mice by themselves (Behrens *et al.*, 2008).

  Direct activation model: Phagocytic cells engulf ABAs within phagolysosomes, leading to lysosome destabilization and release of antigens and lysosomal products (i.e., cathepsin B) into the cytoplasm (Hornung *et al.*, 2008). It is unknown if ABAs interact with cell surface through specific but not known receptors or via non-specific adsorption to plasma membrane as MSU crystals (Marrack, McKee and Munks, 2009; Flach *et al.*, 2011).

[5] NLRP3: NLR family pyrin domain containing 3 (NLR: NOD Like Receptor)
[6] ASC: Apoptosis-associated speck-like protein containing a CARD (CARD: Caspase activation and recruitment domain)

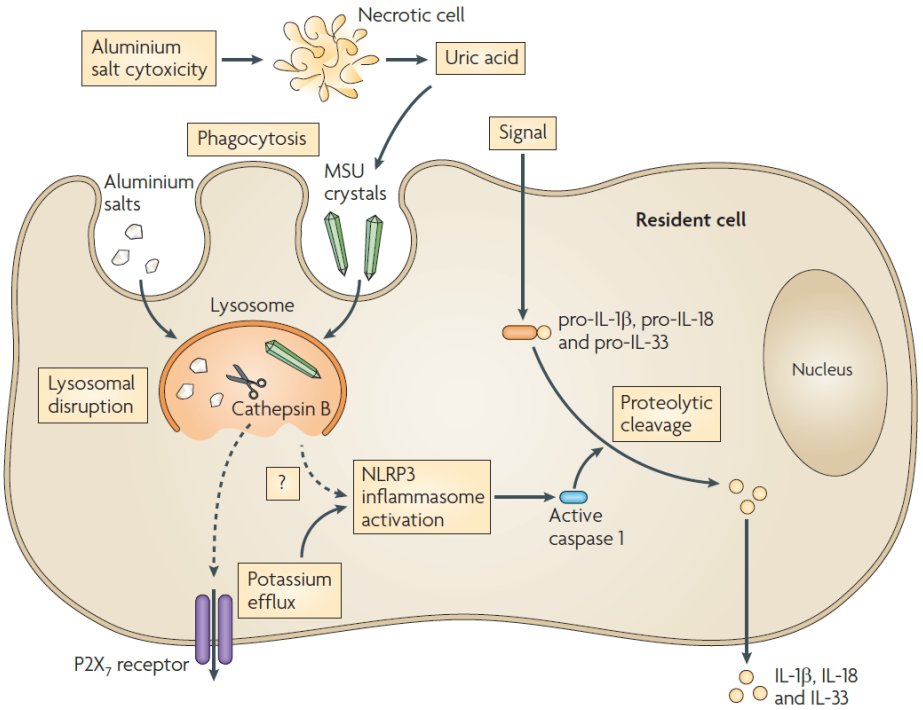

Figure Intro-3: Proposed mechanism of activation of the NLRP3 inflammasome by Aluminum-based adjuvants (ABAs). (1) ABAs are directly phagocytosed by the resident cells. **(2)** ABAs cytotoxicity leads to cell death and release of DAMPs uric acid, which forms monosodium urate (MSU) and is phagocytosed by adjacent cells. **(3)** Phagocytosed ABAs and/ or MSU crystals disrupt lysosomes, which results in the release of cathepsin B. **(4)** Cathepsin B induce potassium efflux, which activates the NLRP3inflammasome. **(5)** Caspase 1, which is activated by the NLRP3 inflammasome, cleaves pro-IL-1β, pro-IL-18 and pro-IL-33, thereby inducing the release of the active cytokines and promoting their secretion. (Extracted from Marrack *et al.,* 2007)

1.6.3.3 Effects on Adaptive immunity

ABAs preferentially induce T_h2 cells (Grun and Maurer, 1989). This subset of T lymphocytes induces the differentiation of B cells that secrete specific immunoglobulins (Ig). IL-4 is the main cytokine in T_h2-biased responses induced by ABAs, promoting Ig production and inhibiting T_h1 cell responses (Brewer *et al.*, 1996, 1999). Eosinophils arrive to the injection site within 24 hours and are the main producers of IL-4, enhancing B-cell proliferation and IgM synthesis (McKee *et al.*, 2008; Wang and Weller, 2008).

1.6.3.4 Controversial points

An important part of the information concerning stimulation of the immune system by ABAs belongs to *in vitro* studies that inconsistently correlate with *in vivo* results (Ghimire, 2015). For example, uric acid seems to be required *in vivo* but not *in vitro* for Al-induced inflammation (Kool, Soullié, *et al.*, 2008).

Additionally, the link between inflammasome activation by ABAs and T_h2-cell responses and antibody production is still in debate. Some studies clearly demonstrate that these adjuvants require NLRP3, ASC and Caspase 1 activation to induce IgG1 production (Eisenbarth *et al.*, 2008; Li *et al.*, 2008). Other studies have shown that NLRP3 or Caspase-1 deficiency have no impact on antibody production (Franchi and Núñez, 2008) or partial impact in T_h-cells differentiation and IgE synthesis but not in IgG1 production (Kool, Pétrilli, *et al.*, 2008). These differences have been proposed to be related to the genetic background of mice strains, immunization schedule and the type and dose of ABAs employed (Marrack, McKee and Munks, 2009).

1.6.4 Injection-site reactions

Inoculation of ABAs induce the recruitment of inflammatory cells to the injection site leading to transient swelling and local irritation (Goto *et al.*, 1997). In mice, pro-inflammatory mediators such as IL-1β, CCL2, CCL11, histamine and IL-5 are released at the injection site shortly after inoculation of ABAs (Sharp *et al.*, 2009). These cytokines trigger the early recruitment of innate immune cells as neutrophils, eosinophils, immature dendritic cells, monocytes and macrophages (McKee *et al.*, 2009). Few hours after intramuscular or intraperitoneal vaccination in mice, neutrophils are the dominant inflammatory cell at the injection site, beginning with the phagocytosis of particles ranging from 0.5µm to 5µm (McKee *et al.*, 2009; Akinc and Battaglia, 2013). Phagocytosis is enhanced after monocyte arrival and differentiation into macrophages, which starts 24 hours after inoculation and it is massive after 7 days (McKee *et al.*, 2009; Lu and HogenEsch, 2013). Marked increase in eosinophils and MHC-II-positive dendritic cells is observed 7 days after immunization, contributing to humoral-mediated immunity and enhancing antibody production (Lu and HogenEsch, 2013; Quandt *et al.*, 2015).

In sheep, subcutaneous inoculation of ABAs invariably induces sterile injection-site nodules that can persist for more than 15 months (Asín *et al.*, 2019). These nodules persist longer and show higher severity when ABAs are inoculated as part of a vaccine. In contrast, ABAs are cleared quicker when inoculated alone, evincing the contributing role of vaccine antigens to the immune reaction elicited (Asín *et al.*, 2019). Field studies carried out in commercial sheep flocks revealed the presence of Al-induced

subcutaneous nodules in 56% of the animals, with some animals having more than 5 nodules (Rodríguez-Largo *et al.*, 2021).

Injection-site nodules are so-called injection-site granulomas as they are composed of a central sterile necrotic core surrounded by abundant epithelioid macrophages with abundant granular cytoplasm and indented nuclei (Asín *et al.*, 2019). Macrophages are associated with moderate amounts of multinucleated giant cells, peripheral lymphocyte aggregates and rimmed by a fibrous capsule. Electron microscopy revealed enlarged phagolysosomes with micron-sized aggregates of needle-shaped, electron-dense material. This material was identified as Al by energy-dispersive X-ray spectroscopy (Asín *et al.*, 2019).

These injection-site granulomas have also been reported in cows (Marcato, 1990), pigs (Valtulini *et al.*, 2005), macaques (Chamaza, 2012), mice (Crépeaux *et al.*, 2017) and humans (Gherardi *et al.*, 2001). Additionally, ABAs has been linked to the development of injection-site sarcomas in cats (Hendrick *et al.*, 1992). Similar processes have been reported in dogs and ferrets (Munday, Stedman and Richey, 2003; Vascellari *et al.*, 2003).

1.6.5 Clearance and biodistribution of ABAs

The first *in vivo* study focusing on ABAs kinetics was conducted in rabbits at the end of the last century (Flarend *et al.*, 1997). Blood levels and urine excretion were monitored for 4 weeks showing that 1 hour after intramuscular injection, Al could be detected in blood. At necropsy, biodistribution studies revealed higher levels of Al in the kidneys followed by the spleen, the liver, heart, lymph nodes and the brain. Al levels recorded in blood and urine are likely the result of Al dissolved under the influence of interstitial fluid, whereas Al found in lymph nodes and spleen can be associated with Al particles phagocytosed and distributed within APCs (Lindblad and Duroux, 2017). However, the work conducted by Flarend *et al.* raise important biological concerns as they used a low sample sized of animals, they did not look for Al at the injection site and other methodological issues.

In sheep, ABAs can reach the regional lymph node and accumulate within macrophagic aggregates (Asín *et al.*, 2019). In mice, translocation of intramuscularly

inoculated ABAs to the central nervous system (CNS) has been demonstrated (Khan *et al.*, 2013). Most Al particles were shown within astrocytes and microglial cells, reinforcing the idea of an Al entering the CNS via Al-loaded macrophages. Indeed, hematogenous dissemination of Al-loaded macrophages from the regional lymph node via the efferent lymphatics and the thoracic duct has been demonstrated (Khan *et al.*, 2013). Interestingly, in mice Al accumulation in CNS is inversely correlated to the persistence of the intramuscular post-vaccination granuloma, what point towards the key role of the Al particle size in systemic Al biodistribution and accumulation in the CNS (Khan *et al.*, 2013; Crépeaux *et al.*, 2017).

1.6.6 Aluminum localization in tissues

Fluorescent Morin stain. This fluorophore [2',3,4',5,7-Pentahydroxyflavone] is the traditional method to detect Al in plant and animal tissues. Morin interacts 3:1 with the metal ion, producing a green fluorescence emission at 420nm (Shardlow, Mold and Exley, 2018). This stain can interact with other organic minerals and limiting the specificity of the technique.

Fluorescent lumogallion stain: this fluorophore [4-chloro-3-(2,4-dihydroxyphenylazo)-2-hydroxybenzene-1-sulphonic acid] was originally used for Al detection in natural waters and biota (Hydes and Liss, 1976). Lumogallion interacts 1:1 with the Al ion (Al^{3+}) via its two phenolic oxygen ions and the azo group producing a yellow-orange fluorescence emission at 590nm. This interaction 1:1 between lumogallion and Al is much more specific than Morin stain. Indeed, there is no detectable interaction with calcium or magnesium at physiological concentrations (Wu *et al.*, 1995; Ren *et al.*, 2001). Although lumogallion can bind to iron, this results in the formation of a non-fluorescent complex (Wu *et al.*, 1995). Recently, lumogallion stain was adapted to human tissues resulting in a highly specific binding and selective detection of Al in the CNS (Mirza *et al.*, 2016). Furthermore, labelling was further validated in a variety of animal species (Martinez *et al.*, 2017; Asín *et al.*, 2019; Mold, Cottle and Exley, 2019) and proved to be useful for detecting Al adjuvants (Mold *et al.*, 2014; Mile *et al.*, 2015).

Transmission electron microscopy: This technique allows the visualization of ultrastructural components of cells and extracellular matrix, thus providing high

resolution images to localize Al. In ovine macrophages, ABAs are located within phagolysosomes and appear as round micron-sized aggregates composed of interlacing nano-sized acicular particles (Asín *et al.*, 2019).

Fluorescent functionalized nanodiamonds: These particles have nitrogen-vacancy centers that emit a specific and photostable fluorescence, which allow their detection at very low levels and over a very long-term period (Faklaris *et al.*, 2009). They can be coupled to Al via hyperbranched polyglycerol leading to stable complexes that are comparable to particles contained in vaccines in terms of size and zeta potential (Eidi *et al.*, 2015).

2 SMALL RUMINANT LENTIVIRUSES

2.1 The Genus Lentivirus

The genus Lentivirus belongs to the family *Retroviridae*, subfamily *Orthoretrovirinae*. The main feature of this family is the enzyme retrotranscriptase (RT), which allows retrotranscription of viral RNA to viral DNA that will be integrated within the host genome. By following this strategy, retroviruses cause persistent infections that can remain subclinical for a long time. Indeed, the term lentivirus include the Latin prefix "lenti-", making reference to the characteristically slow onset of the associated disease (Painter and Collins, 2019). Based on their cellular tropism, lentiviruses can be divided in two main groups (Painter and Collins, 2019):

- **Viruses with tropism for the lymphocytic and the mononuclear-phagocyte systems.** They infect and dysregulate CD4+ T-lymphocytes, causing severe depletion of T lymphocytes and leading to acquired immunodeficiency syndrome. To this group belongs the Human Immunodeficiency Virus 1 (HIV-1), HIV-2, the Simian Immunodeficiency Virus (SIV) and the Feline Immunodeficiency Virus (FIV).
- **Viruses with tropism for mononuclear-phagocyte system.** They do not infect lymphocytes, and mainly replicate in macrophages leading to a chronic multi-organic disease. To this group belongs the Equine Infectious Anemia Virus (EIAV), Small Ruminant Lentiviruses (SRLV) and Bovine Immunodeficiency Virus (BIV).

2.2 Visna/maedi virus and Caprine arthritis encephalitis virus

Visna/maedi virus (VMV) was first isolated in Iceland in 1960, twenty-seven years after the dramatic importation of 20 Karakul rams from Germany to improve the Icelandic sheep production (Sigurdsson, Pálsson and Grímsson, 1957; Pálsson, 1990; Minguijón *et al.*, 2015). Indeed, the name of the virus belongs from the Icelandic words "visna" and "maedi", meaning wasting and breathlessness, respectively. Caprine

Arthritis Encephalitis Virus (CAEV) was isolated in USA in 1980 (Cork *et al.*, 1974; Crawford and Adams, 1981). VMV and CAEV were considered as species-specific viruses for a long time. However, after the discovery of cross-infections between sheep and goats and phylogenetic analysis, both viruses were grouped together under the umbrella of SRLV.

2.3 Viral Structure and Genetics

SRLV genome is composed of two positive single-stranded RNA chains of 8.4-9.2 Kb (Figure Intro-4) that encode 3 structural (*env, gag, pol*) and three accessory genes (*vif, vpr-like, rev*). When viral RNA is retrotranscribed to proviral DNA, the 6 genes are flanked at their ends by two non-coding regions called Long Terminal Repeats (LTR).

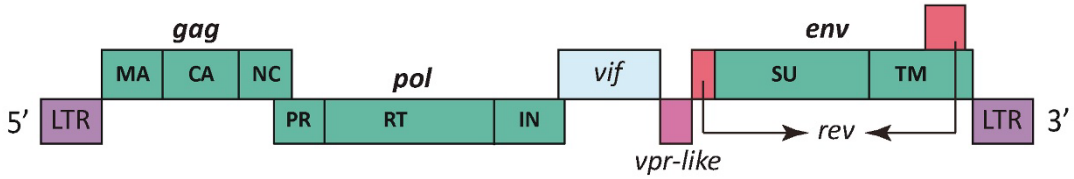

Figure Intro-4: Genome organization in small ruminant lentiviruses (SRLV). LTRs, gag, pol, vif, vpr-like, rev and env genes are represented. SRLV genes are organized in different Reading frames, allowing the virus to produce higher number of proteins using less genomic information, thus reducing its size.

Virions of SRLV are 80-100nm in diameter and they are composed of approximately 60% of protein, 35% of lipids, 3% of carbohydrates and 1% of RNA (Petursson, Andresdottir and Andresson, 1992). Figure Intro-5 provides a schematic design of a virion particle. From the outer to the inner part, their structure is characterized by:

- **Outer envelope (Env)** composed of a lipid bilayer, which derives from the host plasma membrane and is stuffed with viral glycoproteins (i.e., transmembrane protein and surface protein) (Murphy *et al.*, 1999).
- **Capsid (Ca)** and Protein **Matrix (Ma**) that are 60nm in diameter and have icosahedral morphology.
- **Nucleocapsid (Nc)** contain the viral genome, approximately 30 RT enzymes and other viral proteins such as protease (Pr) and integrase (In).

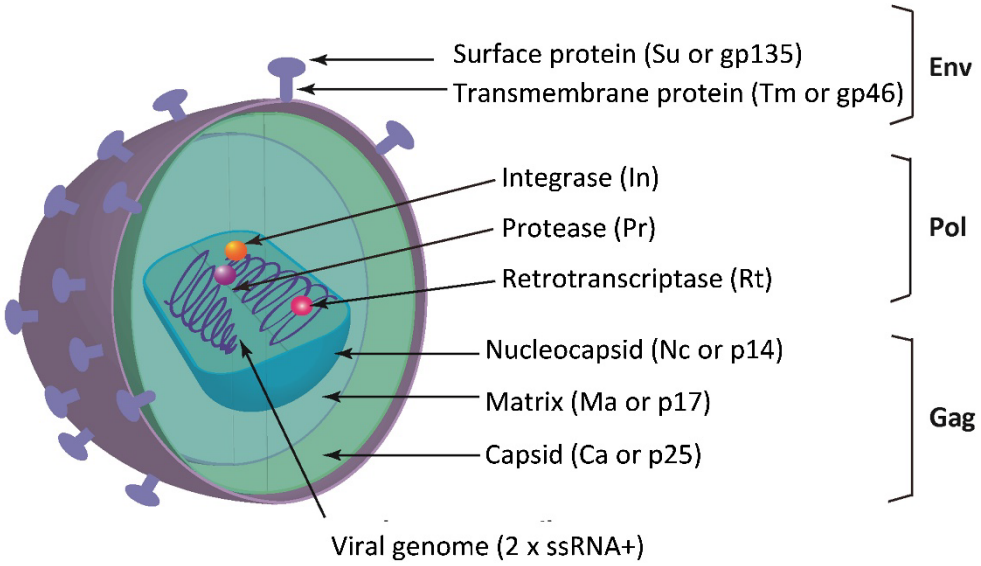

Figure Intro-5: Virion structure of small ruminant lentiviruses. Viral particle is composed of a nucleocapsid core containing two ssRNA+ (viral genome) and enzymes as integrase, protease and retrotranscriptase. The nucleocapsid is surrounded by the viral matrix and the capsid. The outer enveloped is composed of a lipid bilayer (derived from host plasma membrane) an integrated viral proteins as transmembrane protein and surface protein.

2.4 Viral cycle

The replicative cycle of SRLV can be divided in 6 consecutive stages as represented in Figure Intro-6.

2.4.1 Entrance into the host cell

Main target cells *in vivo* are dendritic cells and monocytes/macrophages. Surface glycoprotein of the virion (Su or gp135) binds to a receptor located in the host target cell. This binding triggers the fusion between viral and cellular lipid membranes. After fusion, the viral capsid is released into the cytoplasm. Several target receptors in the host cell have been proposed but a consensus is lacking (Crane, Buzy and Clements, 1991; Bruett, Barber and Clements, 2000; Crespo *et al.*, 2011).

2.4.2 Virus retrotranscription

The viral capsid and nucleocapsid disintegrates and release their content within the host cytoplasm, including viral RNA and the enzymes RT, In and Pr. The cytoplasmic assembly of the pre-integration complex leads to RNA retrotranscription and transport

to the nuclei. First, negative complementary DNA (cDNA-) is synthesized from retrotranscription of viral RNA, leading to a DNA-RNA hybrid. Then, RNA is degraded, and positive complementary DNA (cDNA+) is synthesized. The double-stranded DNA flanked by the LTR regions is known as proviral DNA or provirus. RT enzyme has no verification step of the retrotranscription process, leading to the accumulation of abundant mutations within the SRLV genome.

2.4.3 Provirus integration

The proviral DNA is transported into the nucleus and randomly integrates within the host genome thanks to In protein. Only a single provirus integrates within each host cell and remains stable flanked by host DNA without interfering with cellular activity. SRLV integrated DNA can remain in a latent stage for long periods of time. Provirus strategy successfully enables persistent infection since integrated proviral DNA is not recognized by the host immune system (Peluso *et al.*, 1985). Monocyte precursors in bone marrow remain latently infected by SRLV, thus acting as reservoirs and constantly providing new infected cells. Differentiation of monocytes into macrophages in tissues will activate cellular metabolism, triggering transcription and virus replication (Blacklaws, 2012). Cell division transmits proviral DNA to both daughter cells (Coffin M., 1996).

2.4.4 Provirus transcription

Transcription depends on the host cell enzymatic machinery, mainly the RNA polymerase II. Viral replication is highly susceptible to environmental stimuli. Indeed, viral transcription is low to absent in bone-marrow myeloid precursor cells and blood monocytes, which are known as non-permissive cells. However, monocyte maturation to macrophages, which are known as permissive cells, leads to abundant viral transcription (Blacklaws, 2012). Viral transcription is regulated by factors expressed in the infected cells but also in neighboring cells (Narayan *et al.*, 1983; Gendelman *et al.*, 1986). Indeed, pro-inflammatory stimuli are linked to increased viral replication due to the presence of enhancer elements in the LTR regions for pro-inflammatory cytokines such as GAS or TAS, respectively (Murphy *et al.*, 2007, 2012).

2.4.5 *Viral translation*

Synthesis of viral proteins is compartmentalized in two cellular regions. Gag and Pol proteins are produced in cytoplasmic polyribosomes and released to the cytosol. Proteins of the outer membrane are synthesized in the rough endoplasmic reticulum, modified in the Golgi and transported to the cytoplasmic membrane (Murphy *et al.*, 1999).

2.4.6 *Viral maturation and release*

Viral Gag and cellular proteins orchestrate viral assembly. Gag and Pol proteins assemblage around the viral genome and bind to viral glycoproteins inserted into the cytoplasmic membrane (Coffin M., 1996). Virus can be then released to the extracellular milieu by cellular membrane budding. Indeed, the outer membrane of the virus is composed of the plasma membrane with associated viral proteins. Viral maturation confers the virion an infective status (Murphy *et al.*, 1999). SRLV virions differ from other lentiviruses as they have a short cell-free viremia and need to rapidly infect neighboring target cells (Blacklaws, 2012).

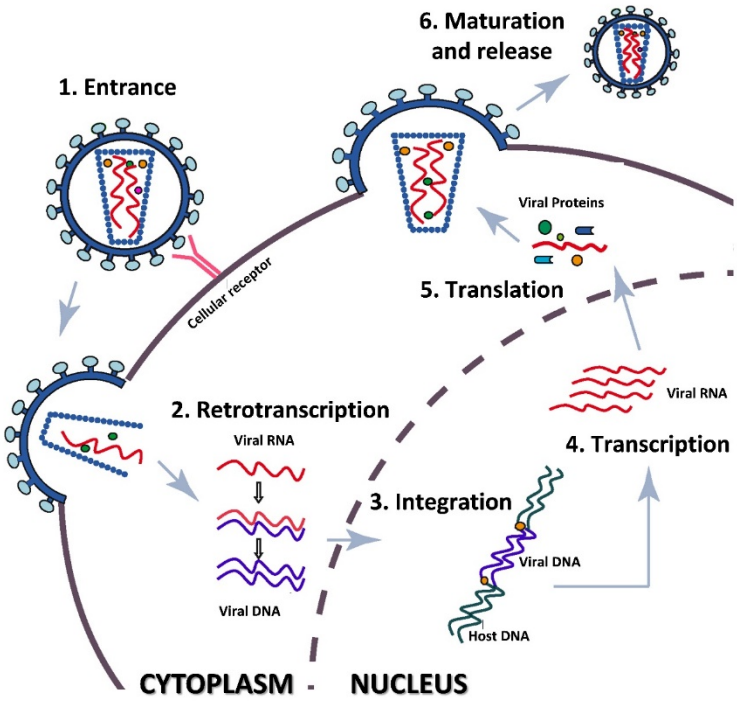

Figure Intro-6: Viral cycle of Small Ruminant Lentiviruses (SRLV). 1) The viral cycle starts with binding of the SRLV to a cellular receptor, fusion of lipid membranes and release of genetic material into the cytosol. 2) Viral RNA retrotranscription is carried out by Retrotranscriptase enzyme in the cytosol. 3) Proviral DNA is integrated into the host genome and replicate synchronously with the host cell. 4) Viral transcription is triggered by external stimuli. 5) Viral translation is performed in the polyribosomes and the rough endoplasmic reticulum. 6) Viral particle assembly leads to viral maturation and release.

2.5 Genetic Variability of SRLV

SRLV are characterized by a marked genetic and antigenic heterogeneity (Ramírez *et al.*, 2013) hampering the development of universal diagnostic tests and commercial vaccines to control SRLV infection.

2.5.1 Sources of SRLV variability

There are different mechanisms for the overwhelming variability of SRLV:

- **RNA retrotranscription** is carried out by RT enzyme, which lacks proof-reading activity to correct mismatches between the original RNA strand and the newly synthesized DNA strand. The lack of a verification step in this process yields to the accumulation of abundant mutations in the viral genome. Indeed, the mutation rate of SRLV is thought to between 0.1 and 2 mutations per replicative cycle (Roberts, Bebenek and Kunkel, 1988).

- **Viral recombination** can happen when a single cell is co-infected by two virions at the same time. In these scenario, RT can switch from the first RNA template to the second, originating a mixed DNA strand that will integrate within the host genome (Smyth, Davenport and Mak, 2012). This mechanism confers an important evolutionary advantage due to the important properties eventually acquired, potentially enlarging host-range, drug resistance or immunological escape (L'Homme *et al.*, 2015).

- **Host immune system** tries to fight against the infection, but at the same time, natural selective pressure purifies the most adapted viral strains. Antiviral proteins of the immune system such as APOBEC3 can also induce mutations in the viral genome during retrotranscription (Vartanian *et al.*, 1991; Bishop *et al.*, 2004). Finally, neutralizing antibodies pressure may also favor the so-called escape mutants, against which antibodies are inefficient (Torsteinsdóttir *et al.*, 2007).

2.5.2 Phylogenetic classification of SRLV

Several SRLV classifications have been proposed along the last decades (Quérat *et al.*, 1984; Zanoni, 1998; Rolland *et al.*, 2002). Currently, SRLV are classified following the rules proposed for the HIV classification, which consist on the sequencing of two genome fragments of 1.8Kb (*gag-pol* genes) and 1.2Kb (*pol* gene) (Shah *et al.*, 2004). Based on this classification, SRLV are grouped in 4 main genotypes (A, B, C and E) that differ in 25-40% of their nucleotide sequence (Figure Intro-7). There are multiple subtypes within genotypes, which differ 15-25% in their nucleotide sequence. Genotype A gather strains classically associated with MVV and includes 21 subtypes (A1-A21). Genotype B gather strains classically associated with CAEV and include 5 subtypes so far (B1-B5).

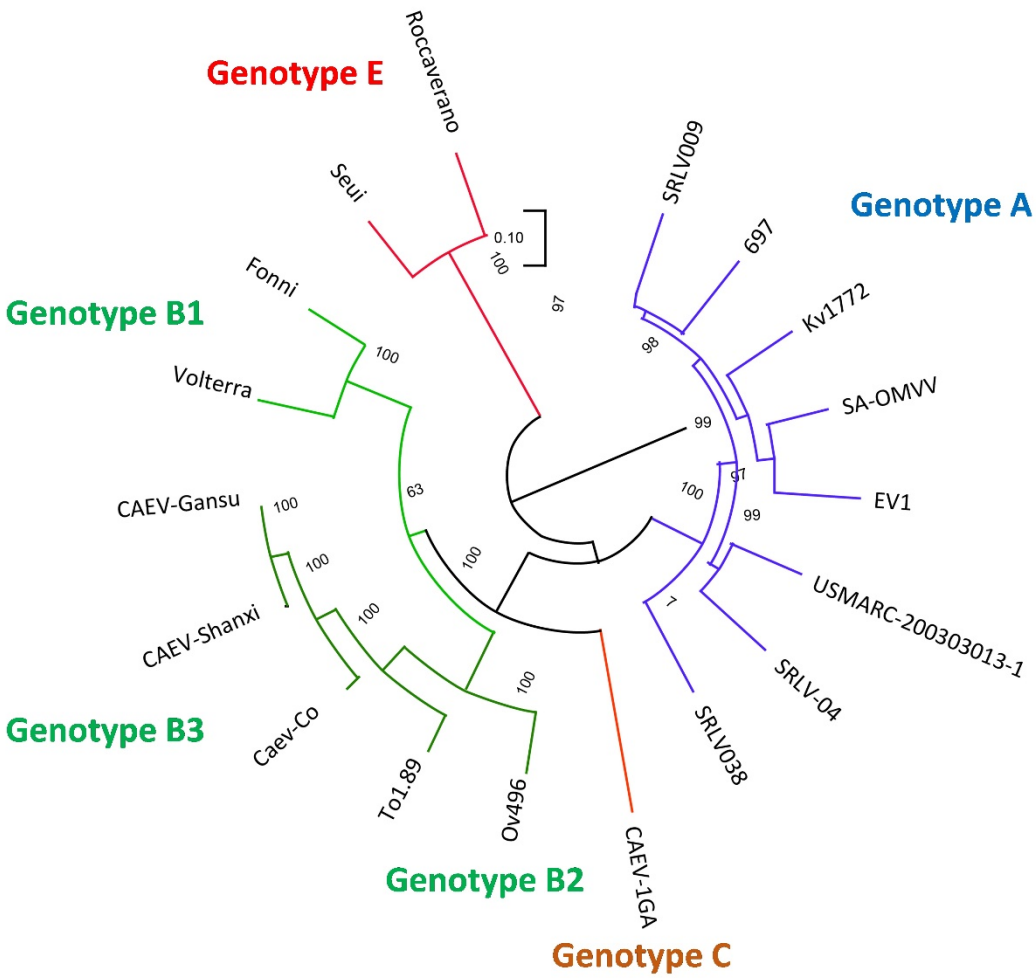

Figure Intro-7: Phylogenetic classification of small ruminant lentiviruses. Four main genotypes (A, B, C and E) with multiple subtypes have been reported.

This genetic variability has notable effects in the spectrum of susceptible-species, target tissues and virulence. Genotype A and B are widely distributed across the world. Genotype A includes well-known strains as the Ev1 strain, isolated in UK (Sargan *et al.*, 1991) and the strain 697, isolated in Spain from a neurological outbreak in sheep (Glaria *et al.*, 2012). Genotype B includes the strain CAEV-Co, isolated in USA from a goat with leucoencephalitis (Cork et al., 1974; Saltarelli et al., 1990) and the strain Ov496, isolated from an outbreak of arthritic sheep in Spain (Glaria *et al.*, 2009). Genotype E only affect goats and shows important biologic and phylogenetic differences (Grego *et al.*, 2009; R. Reina *et al.*, 2009; Juganaru *et al.*, 2010; Reina *et al.*, 2010).

2.6 Pathogenesis

Main transmission routes of SRLV are direct contact between animals and the colostrum (Luján, Begara and Watt, 1994; Blacklaws *et al.*, 2004; Peterhans *et al.*, 2004). Oronasal route is the most effective way to transmit the SRLV infection, which is favored by animal crowding (Houwers, Visscher and Defize, 1989; Blacklaws *et al.*, 2004; Leginagoika *et al.*, 2006; Leginagoikoa *et al.*, 2010; Barquero *et al.*, 2013). This transmission route is influenced by numerous environmental, demographic and management factors (APHIS, 2003; Minguijón *et al.*, 2015). Lactogenic transmission during colostrum and milk ingestion also plays an important role in SRLV dissemination, with up to 16% of lambs born from seropositive ewes being infected during their first day of life (Álvarez *et al.*, 2005; Hasegawa *et al.*, 2017). SRLV have been detected in colostral macrophages and also in the gut-associated lymphoid tissue of the neonate within hours after colostrum intake (Preziuso *et al.*, 2004; Álvarez *et al.*, 2005). Animals infected through colostrum may delay seroconversion for several months (Blacklaws *et al.*, 2004).

Viral infection dynamics starts with the colonization of mucosal dendritic cells that migrate towards regional lymph nodes allowing SRLV to infect medullar macrophages. These infected macrophages escape lymph nodes via afferent lymphatics, reach the blood via the thoracic duct and disseminate throughout the body. SRLV-infected cells can reach the bone marrow, favoring the infection of myeloid precursor cells that will transmit the virus to their progeny (i.e., monocytes) and

establishes a latent and persistent infection (Gendelman *et al.*, 1986; Grossi *et al.*, 2005). Infected monocytes released from the bone marrow into the bloodstream may migrate to tissues and maturate into macrophages.

As myeloid precursor cells and monocytes are non-permissive cells, they can be infected by SRLV but viral transcription and translation is not accomplished. This strategy allow SRLV to hide from the immune system until maturation of monocytes into tissue macrophages, the so called *Trojan horse* mechanism of dissemination (Peluso *et al.*, 1985). As macrophages are permissive cells, upon their maturation SRLV transcription and translation are triggered leading to an active infection. Expression of viral particles induce the recruitment of immune cells leading to the mononuclear inflammatory cell infiltrates seen in SRLV-induced lesions (Narayan *et al.*, 1983).

2.6.1 Host vs Virus interaction

After SRLV infection, a short-term viremia is usually detected, allowing SRLV antigens to be processed by the immune system leading to antibody production. These antibodies are eventually able to neutralize viral particles but are inefficient against integrated SRLV provirus (Blacklaws, 2012). Initial viremia is followed by a period of latent infection that can last weeks to years. During this period SRLV is present as integrated provirus but neither viral particles nor clinical signs can be detected (Minguijón *et al.*, 2015). Triggered by multiple and sometimes unknown factors, SRLV infection reactivates, leading to production of infective viral particles that stimulates the immune response and disease onset. Indeed, lentiviral diseases are considered immune-mediated processes as the main cause of organ dysfunction is the chronic immune response of the host against the virus (Blacklaws, 2012). Chronic infection with SRLV has also been related with moderate immunodeficiency, associated with immune exhaustion linked to a drop in the co-stimulatory molecules needed for appropriate antigen presentation (Reina *et al.*, 2007).

2.7 Clinical forms and histopathological lesions

SRLV infection finally leads to disease manifestations that are usually confined to four anatomic compartments: lungs, mammary glands, joints (mainly carpus and tarsus)

and CNS. Only pulmonary and nervous affection entail animal death, whereas articular and mammary conditions usually imply a premature culling. The specific tropism of SRLV for these organs is not well understood, however specific interaction with cellular receptors is considered to play a key role. SRLV disease is usually confined to a single anatomic location, however lesions in two or more organs are also quite usual (Gayo *et al.*, 2018). Lesion severity and distribution largely depend on host factors (e.g., genetic basis, age and immune status), viral factors (e.g., viral strain and infective dose) and environmental conditions (e.g., management system).

Microscopic SRLV lesions are characterized by infiltration of abundant lymphocytes (with low CD4+/CD8+ ratio), macrophages and plasma cells, associated with variable degree of fibrosis (Pépin *et al.*, 1998). In spite of being an immune mediated disease, higher levels of viral RNA and proteins correlate with higher lesion degree in the CNS (Stowring *et al.*, 1985). Similarly, higher viral load in lungs correlate with higher pulmonary lesion severity (Brodie *et al.*, 1993; Zhang *et al.*, 2000) and higher viral load in PBMCs is related to increased clinical signs in target organs (Ravazzolo *et al.*, 2006; Herrmann-Hoesing *et al.*, 2009). In this scenario, viral DNA quantification has been proposed as biomarker of disease progression in sheep (Zhang *et al.*, 2000).

2.7.1 Respiratory syndrome

Lungs are commonly affected organs in SRLV-infected sheep. Clinical signs are characterized by marked dyspnea and tachypnea together with progressive weight loss leading to cachexia (Thormar, 2013). This presentation is usually seen in animals older than 2 years old but it can be occasionally observed in younger animals (Luján *et al.*, 2019).

Pulmonary gross lesions are characterized by diffuse increase in lung volume. Lungs fail to collapse when the thoracic cavity is opened and shows a rubbery consistence, pale color and occasional presence of subpleural, multifocal darker miliary areas. Microscopically, alveolar septa are markedly thickened by abundant mononuclear cells, mainly CD8+ T lymphocytes, macrophages and plasma cells (Watt *et al.*, 1992; Caswell and Williams, 2016). Additionally, moderate septal fibrosis and occasional smooth muscle hyperplasia can be present (Dawson, 1980; Lujan *et al.*, 1991). Studies

performed in bronchoalveolar lavage fluid (BAL) revealed increased expression of MHC class II receptors in macrophages and lymphocytes. Immunohistochemistry studies showed the co-localization of viral particles with CD163 and CD172 receptors, evincing the role of M2-differentiated macrophages in the pulmonary form of SRLV (Cadoré *et al.*, 1996; Herrmann-Hoesing, 2010). SRLV strains from different genotypes preferentially infect M2-antiinflammatory macrophages determining T_h2-biased responses and healing responses. Contrarily, M1-proinflammatory macrophages seem to be refractory to infection (Crespo *et al.*, 2011).

2.7.2 Articular syndrome

Articular form has been mostly considered the hallmark of the disease in goats but there are also abundant references in sheep (Oliver *et al.*, 1981; Cutlip *et al.*, 1985; Pérez *et al.*, 2015). Main joints affected are carpi, with occasional affection of stifle (Watt, Cott and Collie, 1994; Pinczowski *et al.*, 2017). Clinical signs are distinguished by asymmetrical swelling of the affected joint together with lameness.

Articular gross lesions are characterized by thickening of carpal and tarsal synovial membranes, hygromas, increased synovial fluid and fibrosis of articular capsule with articular cartilage erosion and subchondral bone thickening (Craig, Dittmer and Thompson, 2016). Microscopically there is variable hypertrophy and hyperplasia of synoviocytes with finger-like projections and marked infiltration of lymphocytes, plasma cells and macrophages with occasional carpal mineralization and periosteal growth (Narayan *et al.*, 1992).

2.7.3 Nervous syndrome

Affection of the CNS is usually reported in animals older than two years although occasional cases in younger animals have also been reported (Brahic and Haase, 1981; Benavides *et al.*, 2007). There are two main clinical forms: the first one centered on the brainstem leading to ataxia, circling gait and tremors (Christodoulopoulos, 2006), and the second one characterized by affection of the spinal cord with progressive paralysis of the hind limbs (Benavides *et al.*, 2006).

Nervous gross lesions are rarely reported although occasionally encephalomalacia and myelomalacia have been reported (Christodoulopoulos, 2006; Benavides *et al.*, 2006). Microscopically, lesions are characterized by non-suppurative chronic leucoencephalitis with marked demyelination. Occasional, neuronal death with neuronophagia are also present (Sigurdsson, Pálsson and Grímsson, 1957; Cantile and Youssef, 2016).

2.7.4 Mammary syndrome

Mammary gland affection is usually reported in SRLV-infected sheep (Lujan *et al.*, 1991) and is considered the main cause of important productive loses in dairy sheep since a marked decreased milk production and increased number of somatic cells are consistently observed in affected flocks (Echeverría *et al.*, 2020; Juste *et al.*, 2020). Clinical signs are characterized by diffuse bilateral indurative mastitis (van der Molen and Houwers, 1987). Interestingly, production losses are associated to diseased but also infected asymptomatic animals (Echeverría *et al.*, 2020).

Mammary gross lesions are characterized by increased consistency of the gland with no remarkable changes at cut section (Dawson, 1987; Zink and Johnson, 1994). Microscopically, there is marked mononuclear interstitial mastitis with atrophy of glandular acini. Some studies reported increased amounts of fibrous tissue while in others this feature is not consistently present (Bolea *et al.*, 2006).

2.8 SRLV diagnosis

There are multiple techniques to detect SRLV infection and SRLV-associated diseases. However, due to intrinsic factors of the host and the virus, there is no defined "gold standard" technique as all of them have their advantages and disadvantages.

2.8.1 Clinical diagnosis

Clinical signs of the disease include: dyspnea (pulmonary form); carpal swelling (articular form); ataxia or weakness of the hind limbs (nervous form); increased consistency and decreased secretion of the mammary gland (mammary form). Clinical examination is an easy an economic *in-field* approach. However, due to the low onset of

clinical signs and the high number (usually around 80%) of SRLV-infected but subclinical animals, clinical diagnosis has limited success (Crawford and Adams, 1981; Woodard *et al.*, 1982). Indeed, clinical signs usually remain overlooked until the infection prevalence in a flock exceeds 30% (Ritchie and Hosie, 2014).

2.8.2 Imaging diagnosis

The use of medical imaging techniques as echography, radiography, termography, computered tomography (CT) and magnetic resonance imaging allows early detection of the disease and follow-up disease progression (Cadoré *et al.*, 1997; Castells *et al.*, 2019). Radiographies and CT of the lungs revealed increased radiopacity caused by interstitial pneumonia. Echography showed increased echogenicity of pulmonary tissue (Castells *et al.*, 2019).

2.8.3 Pathology

Macroscopic lesions observed in SRLV-associated diseases are quite characteristic in lungs and joints, but absent or at least unspecific in CNS and mammary gland. The histopathological hallmark is the chronic inflammatory infiltrate in target organs with occasional formation of tertiary lymphoid follicles. Complementary techniques are usually needed to confirm SRLV etiology and to rule out other pathogens that induce similar lesions.

Immunohistochemistry (IHC) allows detection of viral proteins and correlation with lesions. A primary antibody specifically binds to SRLV proteins in tissue preparations, and then is targeted by a species-specific secondary antibody that is revealed by enzymatic or fluorescence methods. This technique is highly specific and very useful to trace the virus within cells and to confirm the infection in organs with minimal histologic lesions (Gayo *et al.*, 2019). However, IHC as a diagnostic tool for SRLV is usually limited to the research field. On top of that, IHC approaches in SRLV-infected animals are only applicable after euthanasia.

2.8.4 Serologic diagnosis

Detection of the immune response against SRLV is the most common and feasible approach for epidemiologic surveys and control programs. Antibodies against

SRLV can be detected from weeks to months after infection. It is widely accepted that SRLV-antibody titers sharply fluctuate along time, but they usually remain detectable during the whole life of the animal. However, intermittent humoral responses have been reported (Rimstad *et al.*, 1993) and may be responsible of failed control programs around the world. Additionally, severely-affected clinical animals can markedly decrease their humoral response yielding low levels of antibodies (Torfason, Gudnadóttir and Löve, 1992). Finally, serologic tests may yield poor results short after infection due to delayed seroconversion (Lacerenza *et al.*, 2006).

Agar gel immunodiffusion assay (AGID) was set-up against SRLV in 1977 (Cutlip, Jackson and Laird, 1977; Winward, Leendertsen and Shen, 1979) and has been widely used to detect SRLV antibodies despite low sensitivity reported and subjective interpretation. This technique consists on placing target serum in the agar gel and the solution containing SRLV antigen nearby. Both, target serum and the solution containing SRLV antigen passively diffuse into the agar gel. If the serum sample contains antibodies against SRLV, they mix with SRLV antigens forming antigen-antibody complexes that precipitate on the agar gel. The precipitate is visible to the unaided eye as a thin white line.

Enzyme-linked immunosorbent assay (ELISA) is based on solid-phase proteins (either antigen or antibody) fixed on the base of a plate well to detect their ligand (either antibody or antigen, respectively) in a liquid sample. In SRLV diagnosis, indirect ELISA based on whole virus, recombinant proteins or synthetic peptides are the most commonly employed (De Andrés *et al.*, 2005). Additionally, competitive ELISA have been described (Fevereiro, Barros and Fagulha, 1999). Over the last decades, specificity and sensibility of SRLV ELISA test have been notably improved (Houwers, Gielkens and Schaake, 1982; Houwers and Schaake, 1987; Pépin *et al.*, 1998). Currently, different commercial ELISAS are available, each with different combinations of synthetic peptides and/or recombinant proteins:

- *Eradikit™ SRLV Screening Kit* (IN3 DIAGNOSTIC): Based on a mix of antigens encoded in *gag* and *env* genes. Detection of Genotypes A, B and E.

- *INgezim Maedi Screening* (INGENASA): Based on a monoclonal antibody and 2 peptides encoded in the *env* gene. Detection of MVV and CAEV.
- *Elitest-MVV/CAEV* (Hyphen Biomed): Based on Gag-derived recombinant protein and Env-like synthetic peptide. Detection of Genotype A.
- *ID Screen® MVV/CAEV Indirect* (ID.VET): Based on peptides derived from Tm and Gag. Detection of MVV and CAEV.
- *IDEXX MVV/CAEV p28 Ab Screening Test* (IDEXX France): Based on Gag-derived recombinant protein and Env-like synthetic peptide.
- *CAEV/MVV Total Ab Test* (IDEXX Switzerland): Based on whole virus
- *SRLV antibody test kit, cELISA* (VMRD): Based on monoclonal antibody of Env. Detection of Genotype B.
- *PrioCHECK™ Maedi-visna & CAEV Ab Serum Kit* (Thermo Fisher Scientific): Based on monoclonal antibody of Env. Detection of MVV and CAEV.

Indeed, ELISA is the most widespread method due to low cost, high-reproducibility, and quick results (De Andrés *et al.*, 2005). However, the phylogenetic diversity of SRLV implies a marked antigenic heterogeneity, which can impact the sensitivity of diagnostic tests (Grego *et al.*, 2002).

Other indirect techniques as complement fixation (Gudnadóttir and Kristinsdóttir, 1967), passive hemagglutination (Karl and Thormar, 1971), indirect immunofluorescence (De Boer, 1970), radioimmunoprecipitation assay (Gogolewski *et al.*, 1985), Western blotting (Houwers and Nauta, 1989) and seroneutralization (Sigurdardóttir and Thormar, 1964) are rarely used nowadays due to their complexity and cost.

2.8.5 Viral isolation

This technique is usually performed from tissue explants (e.g., spleen biopsies) or biologic fluids (e.g., BAL). Cells are grown in tissue culture and if present, SRLV lead to a cytopathic effect consisting in the formation of syncytia that can be observed from

days to weeks after onset of cultures. Usually, clinical samples are co-cultured with indicative cells in which syncytia formation is evident.

As not all SRLV strains induce cytopathic effect, complementary methods such as detection of RT enzymatic activity in cell culture supernatants have been developed. This technique consists of the detection of RT activity present within virions by revealing cDNA synthesis (Pizzato *et al.*, 2009; Vermeire *et al.*, 2012). A further modification known as SYBR-Green based RT-qPCR[7] (SG-PERT) provides an artificial RNA template for the viral RT enzyme to synthesize cDNA, which is detected by qPCR. Additionally, SRLV-strains with low capacity to replicate in common target cells used *in vitro* can be overlooked (Colitti *et al.*, 2019).

2.8.6 Molecular detection

Molecular techniques based on PCR consist on amplification of viral sequences by using specific synthetic oligonucleotices (so-called primers) and *Taq* polymerase, that replicate DNA fragments within primers. PCR is usually applied to DNA from PBMCs with diagnostic purposes, but other cells and tissues can be employed (Rimstad *et al.*, 1993; Leroux *et al.*, 1997; Extramiana *et al.*, 2002).

Diagnostic PCRs use primers targeted to highly conserved regions of the SRLV genome. It allows early viral detection as a positive result can be obtained 15 days after the infection, overcoming the limitations of serologic testing that usually requires 40 to 60 days to yield a positive result. Another clue advantage is avoiding of false positive results in neonates due to the presence of colostral antibodies when mothers are infected (Herrmann-Hoesing *et al.*, 2007; Li *et al.*, 2013).

Main disadvantages involve the genetic heterogeneity of SRLV and the low number of infected cells in the blood. Indeed, it is estimated that just 1 out of 10^6 blood leucocytes contain the provirus (Haase, 1986; Dolfini *et al.*, 2015). Degenerated primers that can bind to regions of DNA with single nucleotide polymorphisms between viral

[7] RT-qPCR: Quantitative reverse transcription PCR (PCR : Polymerase chain reaction).

strains may increase PCR sensitivity as well as nested PCR strategies when low DNA quantities are to be amplified (Chassalevris *et al.*, 2020).

As no gold diagnostic standard for SRLV has been reached, mixed strategies that combine serologic testing and PCR may increase the diagnostic sensitivity (Modolo et al., 2009; Brinkhof et al., 2010). Optimal differentiation between infected and non-infected animals is crucial in eradication campaigns, in studies analyzing productive parameters or when applying strategies based on genetic control (Echeverría *et al.*, 2020).

JUSTIFICATION AND OBJECTIVES

This PhD Thesis has been developed between the Department of Animal Pathology (Veterinary Faculty of the University of Zaragoza) and the Institute of Agrobiotechnology (IdAB) of the Spanish National Research Council (CSIC) and the Government of Navarra. Both groups have been working as one for more than 20 years and they have a long-established tradition in studying SRLV with an accredited experience in the research of sheep and goat immunology. The Veterinary Faculty is a reference international center in sheep pathology and medicine and it is an official training site for both, the European College of Veterinary Pathologist (ECVP) and the European College of Small Ruminant Health Management (ECSRHM). The IdAB is a highly-reputed research institute with deep experience on virology, immunology and molecular techniques applied to animal health. Furthermore, the research work presented in this PhD thesis has been mostly developed in Aragón and Navarra, both regions national references in sheep husbandry with a history linked to ovine production with their own local breeds (i.e: Rasa Aragonesa, Latxa Navarra, Ojinegra). Remarkably, Spain is the second European country in sheep population with more than 24 million of animals, the sixth in the world producing lamb meat and the ninth in the world producing sheep milk (Kilgour *et al.*, 2008; Gilbert *et al.*, 2018).

To understand the context in which this work has been performed one need to go back to 2006, when a new serotype of bluetongue virus was reported in the Netherlands and rapidly spread to other European countries. This unexpected event led to a European emergency state and triggered the implementation of a compulsory continental vaccination campaign (European Commission, 2008). This strategy was highly effective and controlled virus expansion and disease but, at the same time, adverse events linked to the vaccination were reported all over Europe. In Spain, these events were observed in different geographic areas with diverse production systems and they were characterized by cachexia and neurological disorders, among others. At that time, there were multiple studies addressing the event with a plethora of diverse results but a final consensus on the etiology was never reached. In this scenario, our research group tried to unravel the enigma behind these adverse reactions in sheep and pointed out towards ABAs as the main causative triggering factor. In 2013, the research group received funding from the Spanish Ministry of Economy and Industry to: i) Evaluate the

effect of repetitive inoculations with Al-based vaccines in sheep and dissect the role of Al-oxyhydroxide, which is the most common vaccine adjuvant in sheep; ii) Study the interaction between Al-based vaccines and the pathogenesis of SRLV in sheep. Four PhD students (Javier Asín, Lorena de Pablo, Irache Echeverría and Endika Varela-Martínez) were highly involved during the first years and their work has been essential to the optimal development of the present PhD Thesis. In this sense, Chapter 1 is based on the analysis and interpretation of productive, clinical and histopathological parameters in groups of lambs subcutaneously inoculated with Al-oxyhydroxide, either alone or as part of commercial vaccines. Chapter 2 studies the presence of Al deposits in the CNS of these lambs using lumogallion, a fluorescence microscopy-based technique specific for Al detection in tissues. Chapter 3 focuses on the immunity induced by Al-oxyhydroxide based vaccines via mRNA quantification performed at the injection-site granuloma and secondary organs of the immune system (i.e., regional lymph node and spleen). The striking discovery that each Al-oxyhydroxide inoculation (either alone or as part of a vaccine) can induce the formation of a highly persistent granuloma, together with the scientific breakthrough that SRLV can replicate within these granulomas, has opened a door to an unknown field of research. In these sense, Chapter 4 of this PhD thesis address the inference of Al-induced granulomas in the SRLV pathogenesis and the host response and Chapter 5 provides rational basis on the broad distribution and high prevalence of SRLV worldwide.

In the light of the aforementioned facts, this PhD Thesis is focused on sheep, SRLV and Al-oxyhydroxide adjuvants and have five main objectives:

- **Objective 1:** To study the long-term effects and postmortem changes induced by the repetitive inoculation of Al-oxyhydroxide, either alone or in vaccines, in lambs raised under different environmental conditions and productive systems.
- **Objective 2:** To determine the presence and location of Al in the CNS of lambs after the repetitive inoculation of Al-oxyhydroxide, either alone or in vaccines.
- **Objective 3**: To assess the molecular expression pattern triggered in injection-site granulomas, regional lymph node and spleen of lambs after the repetitive inoculation of Al-oxyhydroxide, either alone or in vaccine.

- **Objective 4:** To study the effect of Al-induced injection-site granulomas in the SRLV pathogenesis based on viral load, antibody titers, location of SRLV in granulomas, and progression of SRLV-induced lesions.
- **Objective 5:** To estimate and compare the global SRLV prevalence by performing a systematic review and meta-analysis of the articles published during the last 40 years (1981-2020) complemented by a comprehensive description of the diagnostic tests used.

GLOBAL MATERIAL AND METHODS

The experimental work of this PhD Thesis is based on two main experiments, numbered 1 and 2. Chapters 1, 2 and 3 are based on data and samples obtained in Experiment 1. Chapter 4 is based on data and samples obtained from animals in Experiment 2. The *in vivo* phase and the histopathological analyses of animals included in Experiment 1 were carried out before the arrival of the PhD student to the research group.

ETHICAL STATEMENT

Requirements of the Spanish Policy for Animal Protection (RED53/2013) and the European Union Directive 2010/63 on the protection of experimental animals were always fulfilled. The Ethical Committee of the University of Zaragoza approved and licensed all the experimental procedure (ref. PI15/14).

EXPERIMENT 1:

LAMBS REPETITIVELY INOCULATED WITH AL-OXYHYDROXIDE CONTAINING VACCINES, AL-OXYHYDROXIDE ONLY OR PBS FOR 15 MONTHS

Animals

A total of 84, three-month-old, neutered male lambs were divided into four flocks of 21 animals each. Each flock was located in different geographical areas and characterized by different production systems and sheep breed (Table M&M-1).

Flock 1 originated from a Rasa Aragonesa breed-accredited commercial farm ("Masía El Chantre, Teruel, Spain), which is free of the most important sheep diseases. These animals were placed in a research facility (Experimental farm, Veterinary Faculty, University of Zaragoza) and were raised indoor with optimal conditions of diet, management and housing.

Animals from flocks 2, 3, and 4 were born, selected, and raised in commercial sheep farms located in different geographical areas. They remained integrated in their original herd for the entire duration of the experiment.

Table M&M-1: Characteristics of the lambs and flocks used in the experiment.

Flock	Breed	Management	Shepherding	Location
1	Rasa Aragonesa purebred	Experimental farm[1]	No	41°41'N 0°52'W
2	Rasa Aragonesa x Romanov crossbred	Intensive[2]	No	41°31'N 0°32´W
3	Rasa Aragonesa x Romanov crossbred	Extensive[2]	Yes	42°90'N 0°12´W
4	Rasa Aragonesa purebred	Extensive[2]	Yes	41°36'N 0°41´W

[1]Treatment groups not in contact. Flock free of ovine diseases.
[2]Treatment groups pooled together and mixed with the rest of the flock

Treatment groups

Each flock of 21 lambs was split into three treatment groups of 7 animals each: Vaccine group, which was inoculated with commercial vaccines; Adjuvant-only group, which received the equivalent dose of Al-oxyhydroxide (Alhydrogel®, CZ Veterinaria), and Control group, which was injected with phosphate-buffered saline (PBS).

Six animals (i-vi) died along the experiment for reasons unrelated to the treatments:

i. Flock 3. Control group. Diagnosis: Urolithiasis and hydronephrosis.

ii. Flock 3. Control group. Diagnosis: Aspiration pneumonia

iii. Flock 3. Adjuvant-only group. Diagnosis: Urolithiasis and hydronephrosis

iv. Flock 3. Vaccine group. Diagnosis: Urolithiasis and hydronephrosis.

v. Flock 4. Adjuvant-only group. Diagnosis: septicemia by *Pasteurella spp.*

vi. Flock 4. Vaccine group. Diagnosis: sheep bloat

The final number of animals in each flock was: Flock 1: n=21; Flock 2: n=21; Flock 3: n=17; and Flock 4: n=19. Therefore, when all flocks were grouped together, each treatment group (Vaccine, Adjuvant-only, and Control) consisted of 26 animals at the end of the experiment. Data derived from animals dying during the experiment were not considered for any of the parameters evaluated.

Inoculation Schedule

An intensive vaccination schedule was applied. The goal was to reproduce, within an acceptable time frame for a 3-year research project, the local management field conditions. Animals received a total of 19 subcutaneous inoculations, which mimic the amount of Al that animals can receive during their productive lifespan (a mean of seven years). The last injection was applied 5 days prior to euthanasia in the four flocks. Inoculation schedule and the experimental procedures performed on animals are described in Figure M&M-1. The complete study lasted 15 months, ranging from 432 to 470 days, depending on each flock.

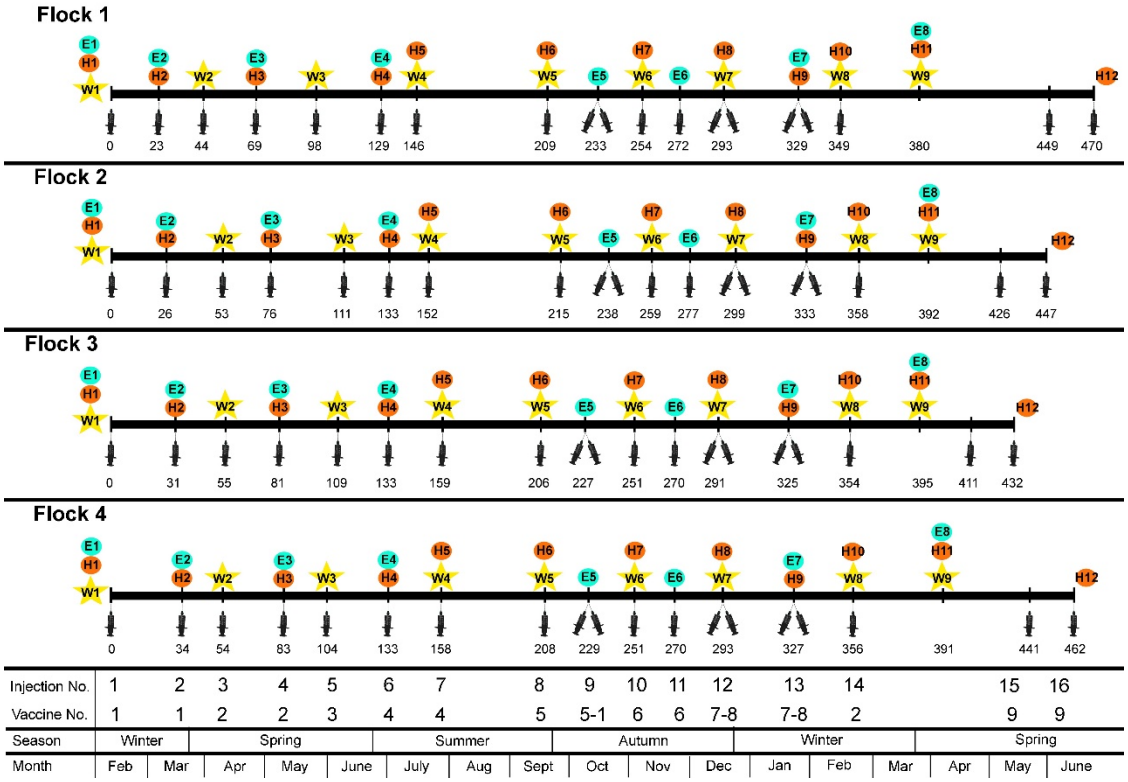

Figure M&M-1: Inoculation schedule in Experiment 1 for each individual flock. All flocks were subjected to the same inoculation schedule and experimental procedures. Differences in the number of days between inoculations in the different flocks and other experimental procedures are shown. Each injection date is indicated by a syringe symbol. W: Weight measurement. E: Clinical examination. H: Hematological analysis. Information about the vaccines used is presented in Table M&M-2.

Inocula used

- **Vaccine group:** Inoculated with commercial vaccines against common ovine diseases. The application recommendations and period between vaccines were always fulfilled. The Al content in each vaccine was established by Inductively Coupled Mass Spectrometry (ICP-MS). Animals received a total of 81.29 mg of Al along the experiment. Vaccines used are summarized in Table M&M-2.

Table M&M-2: Vaccines used in Experiment 1 and inoculation dates. Aluminum (Al) content was established by inductively coupled mass spectrometry (ICP-MS) and calculated as milligrams (mg) per total dose.

Vaccine Number	Commercial Name	Antigen/s	Inoculation date (Fig M&M-1)	Al per dose (mg)
1	Heptavac P Plus	*Pasteurella multocida* *Mannheimia haemolytica* *Clostridium spp.*	1, 2, 9	7.5
2	Autogenous vaccine	*Staphylococcus aureus spp. anaerobius*	3, 4, 14	1.644
3	Vanguard R	Rabies virus	5	1.025
4	Agalaxipra	*Mycoplasma agalactiae*	6, 7	6.764
5	Ovivac CS	*Chlamydia abortus* *Salmonella abortus ovis*	8, 9	5.6
6	Autogenous vaccine	*Corynebacterium pseudotuberculosis*	10, 11	1.32
7	Bluevac-1	Bluetongue virus Serotype 1	12, 13	4.18
8	Bluevac-4	Bluetongue virus Serotype 4	12, 13	4.16
9	Bluevac BTV8	Bluetongue virus Serotype 8	15, 16	4.4

- **Adjuvant-only group:** Injected with Al-oxyhydroxide (Alhydrogel®, CZ Veterinaria). The concentration of Al in each inoculum was adjusted to the Al quantity in the corresponding vaccine. Al-oxyhydroxide was diluted in PBS. The volume administrated at each date was identical to the corresponding vaccine. Animals received a total of 81.29 mg of Al along the experiment.

- **Control group:** Injected with PBS. The volume administrated at each date was identical to the corresponding vaccine. Al content of PBS inocula was calculated by ICP-MS and was always under the limit of detection of the technique (0.074 µg/mL).

EXPERIMENT 2:

ADULT ARTHRITIC SHEEP NATURALLY INFECTED BY SRLV AND REPETITIVELY INOCULATED WITH AL-OXYHYDROXIDE CONTAINING VACCINES, AL-OXYHYDROXIDE ONLY OR PBS FOR 75 DAYS.

Animals

Fifteen adult (>4 year-old) female commercial Rasa Aragonesa sheep naturally infected by SRLV and showing bilateral arthritis were selected from different flocks of Aragón (Spain). Sheep were lodged at the experimental farm of the University of Zaragoza and were raised indoor with optimal conditions of diet, management and housing.

Treatment groups

Sheep were divided into 3 treatment groups of 5 animals each: Vaccine group, which was inoculated with commercial vaccines; Adjuvant-only group, which received Al-oxyhydroxide (Alhydrogel ®, CZ Veterinaria); Control group, which was injected with PBS.

Six animals were excluded after the quarantine period for being either pregnant or diagnosed with concomitant diseases. These included one animal in the Vaccine group, two animals in the Adjuvant-only group and three animals in the Control group.

The final number of animals in each group was: Vaccine group (n=4; animals V-1, V-2, V-3 and V-4); Adjuvant-only group (n=3; animals A-5, A-6 and A-7); Control group (n=2; animals C-8 and C-9).

Additionally, a Vaccine-extra group (n=2; animals V-10 and V-11) was included at the end of the experiment to clarify and complement the molecular results obtained.

Inoculation Schedule

Vaccination schedule of these animals prior to the study was unknown. Animals of Vaccine, Adjuvant-only and Control groups received 8 subcutaneous inoculations at different time points and were euthanized 75 days after the first inoculation (dpi). Animals of Vaccine-extra group were subjected to the same injection protocol and were euthanized earlier (at 40 dpi), therefore receiving four inoculations in total**.** Inoculation schedule and the experimental procedures performed are described in Figure M&M-2.

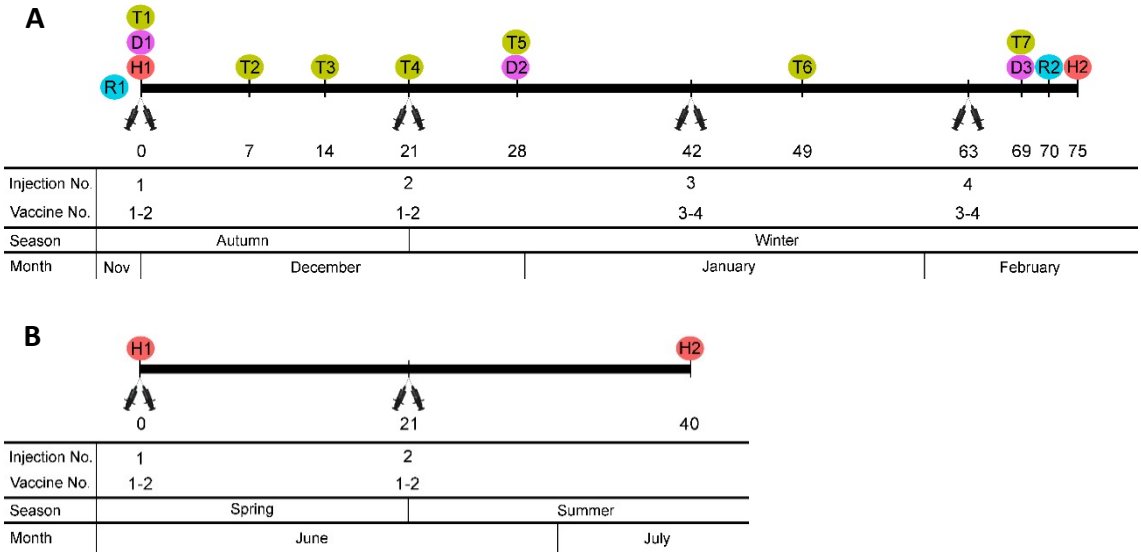

Figure M&M-2: Inoculation schedule of Experiment 2. Information on the injection and vaccines number, season, and month is also provided. Each injection date is indicated by a syringe symbol. Thermographies (green T), radiographies (blue R), measurement of carpal diameter (Purple D) and blood sampling for hematology, serology and molecular techniques (Red H) were performed at the provided days post first inoculation (dpi). **(A)** Vaccine group, Adjuvant-only group and Control group were subjected to the same inoculation schedule and experimental procedures. Inoculations were administered at 0, 21, 42 and 63 dpi. Animals were euthanized at 75 dpi. **(B)** Vaccine-extra group. Inoculations were administered at 0 and 21 dpi. Animals were euthanized at 40 dpi. Information about the vaccines used is presented in Table M&M-3.

Inocula used

- **Vaccine group:** Inoculated with commercial vaccines against common ovine diseases. The application recommendations and period between vaccines were always fulfilled. The Al content in each vaccine was established by ICP-MS. Animals of Vaccine group received a total of 43.36 mg of Al along the experiment. Animals of Vaccine-extra group received a total of 17.16 mg of Al along the experiment. Vaccines used are summarized Table M&M-3.

Table M&M-3: Vaccines used in Experiment 2 and inoculation dates. Aluminum (Al) content was established by inductively coupled mass spectrometry (ICP-MS) and calculated as milligrams (mg) per total dose.

Vaccine Number	Commercial Name	Antigen/s	Inoculation date (Fig M&M-2)	Al per dose (mg)
1	Bluevac-1	Bluetongue virus Serotype 1	1, 2	4.18
2	Bluevac BTV8	Bluetongue virus Serotype 8	1, 2	4.4
3	Heptavac P Plus	*Pasteurella multocida* *Mannheimia haemolytica* *Clostridium spp.*	3, 4	7.5
4	Ovivac CS	*Chlamydia abortus* *Salmonella abortus ovis*	3, 4	5.6

- **Adjuvant-only group:** Injected with Al-oxyhydroxide (Alhydrogel®, CZ Veterinaria). The concentration of Al in each inoculum was adjusted to the Al quantity in the corresponding vaccine. Al-oxyhydroxide was diluted in PBS. The volume administrated at each date was identical to the corresponding vaccine. Animals received a total of 43.36 mg of Al along the experiment.

- **Control group:** Injected with PBS. The volume administrated at each date was identical to the corresponding vaccine. Al content of PBS inocula was calculated by ICP-MS and was always under the limit of detection of the technique (0.074 µg/mL).

CHAPTER 1

Growth performance and clinicopathological analyses in lambs repetitively inoculated with aluminum-oxyhydroxide containing vaccines or aluminum-oxyhydroxide alone

ABSTRACT

Al-oxyhydroxide is an effective adjuvant used in sheep vaccines. However, ABAs have been implicated as potential contributors of a severe wasting syndrome in sheep, the so-called ovine ASIA syndrome. This chapter aimed to characterize the effects of the repetitive injection of Al-oxyhydroxide containing products in lambs. Four flocks (Flocks 1-4; n=21 each) kept under different conditions were studied. Three groups of 7 lambs (Vaccine, Adjuvant-only, and Control) were established in each flock. Mild differences in average daily gain and fattening index were observed, indicating a reduced growth performance in Vaccine groups likely related to short-term episodes of pyrexia and anorexia. Clinical and hematological parameters remained within normal limits. Histology showed no significant differences between groups, although there was a tendency to present higher frequency of hyperchromatic, shrunken neurons in the lumbar spinal cord in the Adjuvant-only group. Although Al-oxyhydroxide was linked to granulomas at the injection site and ethological changes in sheep, results of the present experimental work indicate that injected Al-oxyhydroxide is not enough to fully reproduce the wasting presentation of the ASIA syndrome. Other factors such as sex, breed, age, production system, diet or climate conditions could play a role.

INTRODUCTION

Vaccines are indispensable tools in animal production to control diseases and increase production rates (Greenwood, 2014). In sheep husbandry, vaccination protocols differ depending on a variety of factors such as production system, geographical location, climate, and/or disease prevalence (Lacasta *et al.*, 2015). Furthermore, health management programs can be modified by compulsory vaccination campaigns to fight against emerging or re-emerging epizootics (Morens, Folkers and Fauci, 2004). A recent example was the compulsory vaccination campaign against bluetongue virus that took place in most European countries during the first decade of the 21st century (European Commission, 2008; Mellor *et al.*, 2008). This immunization campaign effectively controlled virus circulation and stopped disease progression. However, the repetitive vaccination caused diverse side-effects of variable intensity that affected productive parameters and animal health in several countries (Agence Française de Sécurité Sanitaire, 2009; Dyer *et al.*, 2009; González *et al.*, 2010; Nusinovici *et al.*, 2011; Asín *et al.*, 2018). Interestingly, a wasting syndrome associated with neurological signs was described and ABAs that the used vaccines contained were incriminated as the potential triggering etiology (Luján *et al.*, 2013). The name ASIA syndrome was proposed for this process (Shoenfeld and Agmon-Levin, 2011; Luján *et al.*, 2013).

In veterinary medicine, Al-oxyhydroxide is a widely employed vaccine adjuvant that efficiently boosters immune responses against the vaccine antigens (Burakova *et al.*, 2017; Shardlow, Mold and Exley, 2018). Therefore, Al is currently present in most ovine commercial vaccines. Previous publications demonstrated that subcutaneous inoculation of Al-oxyhydroxide adjuvants induces the formation of persistent, sterile granulomas composed of abundant Al-laden macrophages in the experimental animals used in the present study (Asín *et al.*, 2019). These macrophages can reach regional lymph nodes and potentially disseminate Al throughout the body (Asín *et al.*, 2019).. Moreover, Al-oxyhydroxide was linked to the development of an array of ethological changes in a group of the same lambs (Asín *et al.*, 2020). The evaluation of productive and clinical parameters together with a comprehensive pathological analysis in the

animals included in the aforementioned publications have never been reported. Moreover, whether repetitive inoculation of Al-oxyhydroxide may induce an ovine wasting syndrome has never been addressed in a large-scale experiment.

In accordance with the Objective 1 of the PhD Thesis. The aim of this chapter was to study the clinical long-term effects and postmortem changes induced by the repetitive injection of Al-oxyhydroxide, either alone or combined into commercial vaccines, in lambs kept under different environmental conditions and productive systems.

MATERIAL AND METHODS

Experimental design

A total of 84, three-month-old, neutered male lambs were divided into four flocks of 21 animals each. Flock 1 originated from a Rasa Aragonesa breed-accredited commercial farm and was placed in a research facility (Experimental farm, University of Zaragoza) under previously described conditions (Global Material and Methods; Experiment 1). Animals from flocks 2, 3, and 4 were born, selected, and raised in commercial sheep farms located in different geographical areas. Flocks 2, 3, and 4 remained integrated in their original herd for the entire duration of the experiment. Detailed information of the production systems and climatological parameters is provided in Table M&M-1 and Appendix Ch1-1, respectively.

Each flock of 21 lambs was split into three treatment groups of 7 animals each: Vaccine group, which was inoculated with commercial vaccines; Adjuvant-only group, which received the equivalent dose of Al-oxyhydroxide (Alhydrogel®, CZ Veterinaria), and Control group, which was injected with PBS. Six animals (i-vi) died for reasons unrelated to the treatments and the final number of animals in each flock was: Flock 1: n=21; Flock 2: n=21; Flock 3: n=17; and Flock 4: n=19. Therefore, when all flocks were grouped together, each treatment group (Vaccine, Adjuvant-only, Control) consisted of 26 animals at the end of the experiment. Data derived from dead animals were not considered for any of the parameters evaluated.

An accelerated vaccination schedule was applied. The goal was to reproduce, within an acceptable time frame for a 3-year research project, the management field conditions that led to the ovine ASIA syndrome. Animals received a total of 19 subcutaneous inoculations, which mimic the amount of Al that animals can receive during their productive lifespan. The last injection was applied 5 days prior to euthanasia in the four flocks. Global inoculation schedule is described in Figure Ch1-1. Details of the vaccines used are described in Table M&M-2. Vaccine and Adjuvant-only groups received a total of 81.29 mg of Al. The complete study lasted 15 months, ranging from 432 to 470 days, depending on each flock.

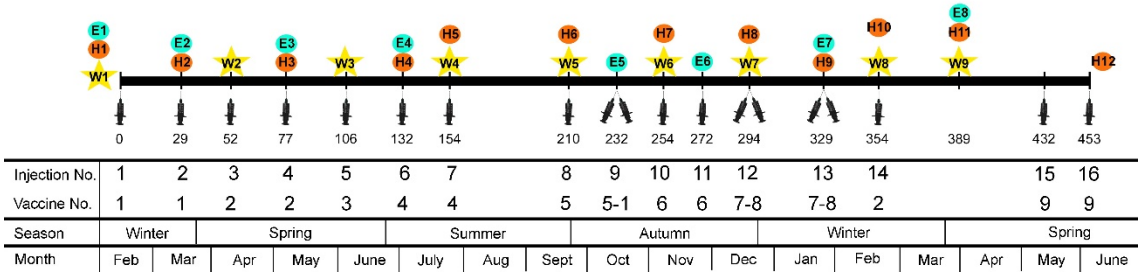

Figure Ch1-1: Global inoculation schedule. Each injection date is indicated by a vertical line and a number (mean value of dpi of the four flocks). W: Weight measurement. E: Clinical examination. H: Hematological analysis. Information on the injection and vaccines number, season, and month is also provided. Inoculation schedule for each individual flock is provided in Figure M&M-1. Information about the vaccines used is presented in Table M&M-2.

Productive and Clinical parameters

In order to analyze animal growth, lamb weight was recorded nine times along the experiment, days between each measurement ranged from 31 to 63 (Figure Ch1-1, W1 to W9). Partial and global average daily gain (ADG) were calculated. Partial ADG included all the weighing dates; global ADG was calculated using the first and the last weights and dividing the difference by the number of days between them. General clinical examination was performed periodically (Figure Ch1-1), 18 to 41 days after previous inoculation date and just prior to the application of the next inoculation. It included rectal temperature, heart rate, and respiratory rate. As part of the general examination, blood samples were obtained by jugular venipuncture with 6 mL EDTA tubes (BD Vacutainer®) and a hematological panel including white blood cell count, red blood cell count, hematocrit, hemoglobin, and platelet count was performed (scil Vet

abc Plus ™ Animal Blood Counter). Additionally, animals from Flock 1 were subjected to two rounds of ethological tests (one in summer and another in winter) and these results were previously reported (Asín, Pascual-Alonso, *et al.*, 2020). Urine was analyzed just after euthanasia with a biochemical strip to test pH, glucose, and protein.

Post-mortem studies

Euthanasia was performed by intravenous injection of an overdose of barbiturate solution (Dolethal®, Vetoquinol). Complete post-mortem examinations were performed. Perirenal, mesenteric, pericardial, thoracic, and subcutaneous fat deposits were scored from 0-3 (0: Absence of fat; 1: Scarce fat deposition; 2: Moderate fat deposition; 3: Normal fat deposition), and a fattening index was calculated as the mean value of these five scores. Additionally, thickness of subcutaneous sternal fat was measured.

Systematic sampling of all tissues was performed. CNS and peripheral nervous system (PNS) were sampled following a previously-described protocol (Vandevelde, Higgins and Oevermann, 2012). Tissues were fixed in 10% neutral-buffered formalin for 48-72h. Samples were routinely processed for paraffin embedding and production of 4µm, hematoxylin-eosin (HE)-stained slides. Histopathological analysis of different areas of the CNS (brain: frontal cortex-caudate nucleus, parietal cortex, thalamus-hypothalamus; spinal cord: cervical, thoracic, and lumbar segments), PNS (subcutaneous-thoracic, sciatic, tibial, and radial nerves), liver, kidney, pancreas, spleen, adrenal glands, thyroid, and thymus were performed by a single pathologist that was blinded to the treatment group. Histopathological features evaluated and scoring system used in each tissue are described in Appendix Ch1-2.

Statistical analysis

All statistical analyses were performed using IBM SPSS 19.0 for Windows (IBM Corp., Armonk, NY, USA). Quantitative variables (i.e., body weight, ADG, fattening index, sternal fat deposits) were analyzed by Shapiro-Wilk test to assess normality of data. Levene's test was used to test the equality of variances. When data followed a normal distribution and had homogeneous variances, a parametric test as ANOVA was used

followed by Duncan's multiple range test as a *post-hoc*. In normally-distributed quantitative variables with unequal variances, Welch's t-test was used. In non-normal quantitative variables, a non-parametric test as Kruskal-Wallis was used followed by Dunn's test as *post-hoc*. In qualitative variables (i.e., histopathological analyses), assessment of the association between groups was carried out using Pearson's Chi-square test or alternatively Likelihood ratio test and Fisher's exact test when needed. Statistical significance was considered when p value < 0.05. Statistical tendency was considered when p value ≤ 0.1.

RESULTS AND DISCUSSION

Body weight and average daily gain

Results on body weight and ADG are presented in Appendix Ch1-3 and Appendix Ch1-4, respectively. Mild to moderate differences in ADG were observed between treatment groups in each one of the individual flocks. Global ADG of each flock is represented in Figure Ch1-2 and indicated a moderate growth rate reduction in Vaccine groups in contrast with Control groups. Adjuvant-only groups showed lower ADG than Control groups but higher ADG than Vaccine groups. This data distribution was observed for the ADG values of all flocks, although Flock 2 was the only one where these differences were statistically significant (p=0.045). Moreover, when all flocks were grouped together, this tendency was maintained although it did not reach significance (p=0.072).

This lower ADG for Vaccine and -to a lesser extent- Adjuvant-only groups could be explained by transient, short-term, post-vaccination events, including brief periods (24-48 h) of fever after vaccinations and associated decreased appetite (Troxel *et al.*, 2001; Cerviño *et al.*, 2011). Indeed, it has been observed that booster vaccinations against respiratory pathogens in fattening lambs can cause moderate growth retardation, with animals reaching their optimal sacrifice weight 5 days later than control animals (JM Gonzalez, personal communication). The lambs included in this work likely suffered repetitive episodes of hyperthermia and decreased daily intake, which could have affected ADG and absolute weight at the end of the experiment. In

fact, the acute-phase response elicited by vaccination is essential for optimal development of the immune response (Arthington *et al.*, 2013; Silva *et al.*, 2018). This response increases nutrient demands so they are redistributed to support the immune system instead of growing, which may lead to reduced growth performance and feed efficiency (Reeds and Jahoor, 2001; Moriel and Arthington, 2013). Moreover, stimulation of immune response can activate mTOR pathway and thus affect metabolic routes involved in reduced anabolism (Arts *et al.*, 2016; O'Neill, Kishton and Rathmell, 2016). The latter is in accordance with energy consumption due to vaccination and may affect body condition in specific vaccination strategies, especially in negatively energy balanced feedlot animals. In such scenario, the presence of more severe inflammatory reactions in the injection sites of animals in the Vaccine groups (Asín *et al.*, 2019) might also help to explain the differences between Vaccine and Adjuvant-only groups. None of the lambs injected with the adjuvant only or with Al-containing vaccines unequivocally developed a wasting syndrome as the one described after the compulsory vaccination campaigns against bluetongue (Luján *et al.*, 2013).

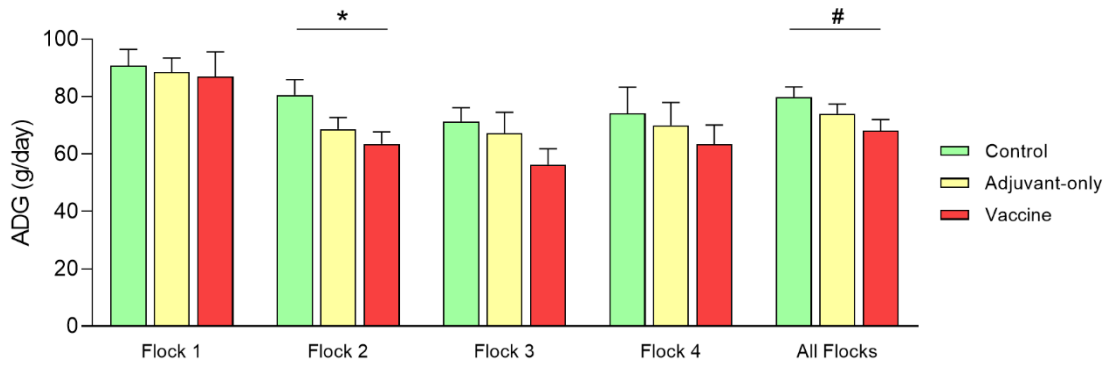

Figure Ch1-2: Global average daily gain (ADG) along the experiment in Control (green), Adjuvant-only (yellow), and Vaccine groups (red), both in each individual flock and in all flocks grouped together (All Flocks). Data represented as mean and Standard Error. *: statistical significance ($p<0.05$); #: statistical tendency ($p\leq0.1$).

Analysis of partial variations in ADG revealed significant differences between weight measurements at dates W4 and W5 (Appendix Ch1-4), coinciding with the summer (Figure Ch1-1). In Flocks 1 and 2, Vaccine groups showed a significantly lower ADG than Control and Adjuvant-only groups (Flock 1: p=0.02; Flock 2: p=0.049). Flock 4 showed similar, although non-significant (p=0.055) results. No statistically significant

variation was observed in Flock 3. When the four flocks were considered altogether, these variations in the Vaccine group also reached statistical significance (p=0.045). Globally, these variations in ADG are likely associated with the high temperatures reached during this period and detailed in Appendix Ch1-1. High environmental temperatures induce heat stress and negatively alter lamb growth due to lower feed intake and activation of thermoregulatory mechanisms (Macías-Cruz *et al.*, 2018). Thermoregulatory capacity and productive performance in fattening lambs with heat stress depends on breed, production system, diet, and age (Al-Dawood, 2017). Perhaps these effects were more marked in the Vaccine group because they combined with preexisting stressors in these animals, i.e., persistent injection-site reactions (Asín *et al.*, 2019). Interestingly, transcriptomic studies performed in Flock 1 of the present work demonstrated that Al adjuvants significantly increased the expression of pro-inflammatory cytokines and genes of the NF-κB and apoptotic pathways (Varela-Martínez *et al.*, 2018). Activation of these pathways may potentially interfere with optimal thermoregulatory mechanisms.

Clinical and hematological examination

Rectal temperatures, heart and respiratory rates, and urine analyses showed no relevant differences between groups in any of the flocks individually or when all flocks were grouped together. Transient pyrexia is a common and expectable post-vaccination effect in feedlot lambs and calves, especially after booster vaccinations (Troxel *et al.*, 2001; Cerviño *et al.*, 2011). In our study, rectal temperature was recorded 18 to 41 days after the previous inoculations (Figure Ch1-1), as the main objective was to measure the cumulative, long-term effect of the repetitive injections rather than short-term variations. In this context, it is likely that those transient differences were missed.

Hematological results of the three treatment groups of the four flocks grouped together are detailed in Appendix Ch1-5. There were point differences between groups both at individual flock level and when all flocks were considered together, but data were always within normal ranges for sheep. Marked normochromic, non-regenerative anemia was reported as part of the wasting syndrome described after the compulsory bluetongue vaccination campaign (Luján *et al.*, 2013), but this phenomenon was not

observed in this experimental work. This might be due to different factors influencing the development of that particular feature, as experimental conditions in the present study probably could not reproduce the exact scenario that fueled the appearance of the wasting presentation of the ovine ASIA syndrome.

Post-mortem studies

Necropsy findings revealed mild differences in the fattening index and sternal fat deposits (Table Ch1-1) when all flocks were considered together. For both parameters, Vaccine group showed lower values than Control group, whereas values in the Adjuvant-only group were higher than Vaccine group and lower than Control group. These results parallel the mild differences observed in the ADG of these animals. Therefore, decreased fat deposition at the end of the experiment in the Vaccine group may be also the result of transient periods of anorexia. Sternal fat deposits play an important role in thermogenesis in sheep (Henry *et al.*, 2017). There were no other gross abnormalities in any of the treatment groups apart from those previously described (Asín *et al.*, 2019).

Table Ch1-1: Fattening index and sternal fat deposits in Control, Adjuvant-only, and Vaccine groups (n=26 each) when all flocks were considered together. Data represented as mean, standard deviation (SD), and interquartile range (IQR).

Group	Fattening index				Sternal fat deposits			
	Mean	SD	IQR	*p*	Mean	SD	IQR	*p*
Control	2.83	0.17	2.80-3.00[a]		3.74	0.38	3.50-4.00[a]	
Adjuvant-only	2.71	0.31	2.60-3.00[a]		3.58	0.70	3.00-4.27[ab]	
Vaccine	2.52	0.38	2.30-2.80[b]	**0.003**[KW]*	3.32	0.52	3.00-3.50[b]	**0.008**[KW]*

[a,b]: Statistically significant differences between groups based on *post-hoc* test.

[KW]: Kruskal-Wallis test.

*: Statistically significant ($p<0.05$).

Histopathological results of the four flocks grouped together are detailed in Table Ch1-2 and Appendix Ch1-6. Evaluation of CNS and PNS showed point differences between treatment groups when each flock was analyzed individually but they were heterogeneous between flocks and not clearly liked to treatments. However, when all flocks where grouped together only a statistical tendency (p=0.100) in the Adjuvant-only group to present higher numbers of dark neurons in the lumbar spinal cord (Table Ch1-2) was observed. The term "dark neuron" defines a hyperchromatic, shrunken neuron (Kherani and Auer, 2008; Zimatkin and Bon', 2018). This histological finding should be

interpreted cautiously as it may be just an artifact (Zimatkin and Bon', 2018). Degenerated to necrotic neurons tend to be brightly acidophilic rather than basophilic/dark, although sometimes these two appearances are difficult to differentiate. Furthermore, ischemic neurons in peracute stages of degeneration may be indistinguishable from dark neurons (Jortner, 2006; Garman, 2011).

Table Ch1-2: Histopathological findings in the central nervous system in Control, Adjuvant-only (Adjuvant), and Vaccine groups (n=26 each) of all flocks grouped together. Data provided as animals with the referred histological lesion relative to the total number of animals analyzed. Methodology of histopathological evaluation is detailed in Appendix Ch1-2.

Location	Group	Perivascular Cuffing	Meningitis	Glial Nodules	Microglial Activation	Dark Neurons
Frontal cortex & Caudate nucleus	Control	8/26	0/26	19/26	6/26	22/26
	Adjuvant	10/26	2/26	19/26	4/26	23/25
	Vaccine	7/26	2/26	14/26	2/26	22/26
	p	0.662^{Xi}	0.187^{LR}	0.236^{Xi}	0.239^{LR}	0.645^{LR}
Parietal cortex	Control	7/26	1/26	3/26	6/26	21/26
	Adjuvant	6/26	2/26	2/26	4/26	22/26
	Vaccine	2/26	2/26	2/26	3/26	22/26
	p	0.177^{Xi}	0.808^{Xi}	0.859^{LR}	0.528^{LR}	0.913^{LR}
Thalamus & Hippothalamus	Control	8/26	0/26	3/26	7/26	24/26
	Adjuvant	4/26	0/26	1/26	12/26	25/26
	Vaccine	7/26	1/26	4/26	11/26	24/26
	p	0.495^{Xi}	0.329^{LR}	0.335^{LR}	0.311^{LR}	0.793^{LR}
Cervical spinal cord	Control	3/26	2/26	1/26	0/26	9/26
	Adjuvant	2/26	1/26	0/26	0/26	12/26
	Vaccine	1/26	0/26	0/26	0/26	11/26
	p	0.568^{LR}	0.240^{LR}	0.329^{LR}	-	0.690^{Xi}
Thoracic spinal cord	Control	0/26	0/26	0/26	0/26	17/26
	Adjuvant	1/26	0/26	0/26	0/26	16/26
	Vaccine	0/26	0/26	0/26	0/26	10/26
	p	0.329^{LR}	-	-	-	0.108^{Xi}
Lumbar spinal cord	Control	1/26	0/26	0/26	24/26	13/26
	Adjuvant	1/26	0/26	1/26	25/26	20/26
	Vaccine	0/26	0/26	0/26	24/26	14/26
	p	0.439^{LR}	-	0.329^{LR}	0.793^{LR}	**0.100$^{Xi\#}$**

^{Xi}: Pearson's chi square test.
^{LR}: Likelihood ratio test.
$^{\#}$: Statistical tendency ($p \leq 0.1$)

Interestingly, analytical measurements performed in the CNS of animals from Flock 1 revealed increased levels of Al in the lumbar spinal cord of the Adjuvant-only group. Perhaps this tendency in the number of dark neurons in the spinal cord of the Adjuvant-only group is related to Al accumulation in the same location. Remarkably, this global absence of histological lesions in the encephalon was observed in animals from Flock 1, which showed significant ethological alterations in a previous study (Asín,

Pascual-Alonso, *et al.*, 2020). Furthermore, transcriptomic studies performed in the encephalon of these animals revealed dysregulation of genes related to neurological function and mitochondrial energy metabolism (Varela-Martínez *et al.*, 2020). Most likely, these clinical and molecular differences did not induce structural abnormalities that could be detected with basic histological methods such as HE.

Pancreas showed a significantly (p=0.012) increased presence of multifocal and/or periductal lymphoplasmacytic inflammatory infiltrates in the Adjuvant-only group when all flocks were considered together (Table Ch1-3). Interestingly, pancreatic changes have been reported in guinea pigs inoculated with Al-oxyhydroxide adjuvants either subcutaneously or intraperitoneally (Goto, Ueno and Iwasa, 1987). Histopathological results obtained in the rest of organs are presented in Appendix Ch1-7. There was a positive tendency (p=0.078) in the number of lambs with thyroid follicular cell hypertrophy in Adjuvant-only and Vaccine groups, and a significant (p=0.043) decrease in number of lambs showing thymic germinal center hyperplasia in the Adjuvant-only and Vaccine groups. No significant differences were found in any of the parameters analyzed in liver, kidney, spleen, and adrenal gland.

Table Ch1-3: Inflammation in the pancreas (i.e., interstitial and/or periductal aggregates of lymphocytes, plasma cells, and/or histiocytes) in Control, Adjuvant-only, and Vaccine groups (n=26 each) of all flocks grouped together. Data provided as animals with the histological lesion relative to the total number of animals analyzed. Methodology of histopathological evaluation detailed in Appendix Ch1-2.

	Control	Adjuvant-only	Vaccine	*p*
Inflammation	1/26	8/26	2/26	**0.012^{LR}***

^{LR}: Likelihood ratio test.
*: Statistical significance (p<0.05).

Study limitations

The interpretation of these results has some limitations intrinsic to the study design and experimental procedures performed. First, the number of animals used could have limited some of the statistical analyses. Second, most of the descriptions of the wasting syndrome that occurred after the bluetongue vaccination campaigns included adult animals, generally ewes in full production (Luján *et al.*, 2013). The animals used in this experiment were growing, male neutered, young lambs, which perhaps limited the capacity of the inoculations to induce severe weight loss. A similar study using adult

sheep with stable body weight at the beginning of the experiment could help to clarify this aspect. Lastly, the number of inoculations performed overrates the normal vaccination schedule for sheep in a year. In fact, the wasting syndrome occurred just with four doses in around a month, with an amount of 16 mg of Al inoculated per animal (Luján *et al.*, 2013; Asín *et al.*, 2018). Most likely, in addition to Al, other parameters such as sex, breed, age, productive system, diet, and/or climate conditions (winter cold) are necessary co-factors for the full development of the devastating wasting presentation of the ovine ASIA syndrome.

CONCLUSIONS

This work summarizes the results obtained on the growth performance and clinicopathological parameters in lambs subjected to repetitive inoculations with saline solution (Control group), Al-oxyhydroxide adjuvants (Adjuvant-only group) or Al-oxyhydroxide-based vaccines (Vaccine group) either under experimental or in field conditions. Mild differences in ADG and fattening index were reported in the Vaccine group and were likely associated with transient post-injection hyperthermia with anorexia and/or intense inflammatory reactions occurring at the injection sites (Asín *et al.*, 2019). Clinical, hematological, and histopathological analyses revealed minimal abnormalities, even knowing that previous ethologicl and transcriptomic studies performed in one of the flocks here studied revealed significant alterations in Adjuvant-only and/or Vaccine groups (Asín, Pascual-Alonso, *et al.*, 2020; Varela-Martínez *et al.*, 2020). Despite previously-observed results showing the effects of repetitive inoculations of Al-oxyhydroxide containing vaccines and adjuvants in sheep (Varela-Martínez *et al.*, 2018, 2020; Asín *et al.*, 2019; Asín, Pascual-Alonso, *et al.*, 2020), the results or this experimental study seem to indicate that injected Al may be necessary but not sufficient to reproduce all the productive and clinicopathological characteristics of the ovine wasting syndrome (ovine ASIA syndrome) (Luján *et al.*, 2013).

APPENDICES

- **Appendix Ch1-1:** Climate conditions along the experiment.
- **Appendix Ch1-2:** Histopathological features evaluated in the experimental lambs in central and peripheral nervous systems, liver, kidney, pancreas, spleen, adrenal glands, thyroid, and thymus.
- **Appendix Ch1-3:** Body weight (W) along the experiment in Control, Adjuvant-only, and Vaccine groups.
- **Appendix Ch1-4:** Average daily gain (ADG) between weighing dates (W) along the experiment in Control, Adjuvant-only, and Vaccine groups.
- **Appendix Ch1-5:** Hematological results along the experiment in Control, Adjuvant-only, and Vaccine groups.
- **Appendix Ch1-6:** Histopathological findings in the peripheral nervous system in Control, Adjuvant-only and Vaccine group.
- **Appendix Ch1-7:** Histopathological results in liver, kidney, spleen, adrenal gland, thyroid gland, and thymus in Control, Adjuvant-only and Vaccine group.

Appendix Ch1-1: Climate conditions along the experiment. Higher and lower temperatures during the experiment are highlighted in red and blue, respectively. Higher relative humidity along the experiment is indicated in green. Data obtained from AEMET (http://www.aemet.es/).

Month-Year	FLOCK 1 & 4								FLOCK 2								FLOCK 3							
	T. mean[3]	T. min[1]		T. max[2]		N0[4]	N30[5]	RH[6]	T. mean	T. min		T. max		N0	N30	RH	T. mean	T. min		T. max		N0	N30	RH
		Mean	Abs	Mean	Abs					Mean	Abs	Mean	Abs					Mean	Abs	Mean	Abs			
January-2015	7.1	2.5	-2.0	11.6	16.7	7	0	66	6.0	1.1	-5.6	10.8	16.9	14	0	N/A[7]	5.9	1	-1.8	10.7	17.5	10	0	75
February-2015	7.1	2.8	-2.9	11.3	18.4	7	0	61	6.9	2.1	-4.9	11.8	18.4	10	0	N/A	6.3	1.2	-5.2	11.4	16.7	8	0	65
March-2015	11.8	6.7	1.3	16.9	24.0	0	0	56	11.6	6.1	-1.1	17.1	23.7	3	0	N/A	11.4	5.6	0.1	17.1	23.0	0	0	61
April-2015	15.6	9.4	4.6	21.8	27.9	0	0	46	14.5	7.6	1.3	21.4	26.8	0	0	54	14.5	7.9	2.4	20.9	25.5	0	0	52
May-2015	20.1	13.5	9.4	26.5	36.4	0	9	43	19.1	11.1	4.6	27	34.0	0	7	47	18.9	11.2	5.0	26.6	35.1	0	6	43
June-2015	25.2	17.5	14.0	32.9	41.6	0	20	38	23.4	15.2	11.7	31.6	39.1	0	20	50	23.4	15.6	10.6	31.2	38.6	0	19	44
July-2015	27.9	20.2	16.2	35.5	43.7	0	27	38	26.7	18.5	13.3	34.7	42.8	0	28	48	27.3	19.1	12.5	35.5	42.1	0	29	39
August-2015	25.5	18.8	14.2	32.1	37.2	0	24	45	24.2	17.3	11.0	31.1	36.8	0	21	58	24.2	17.2	11.8	31.3	36.5	0	21	49
September-2015	20.5	14.9	10.7	26.1	30.4	0	2	48	19.1	12.8	6.8	25.4	30.2	0	1	59	19	12.8	8.5	25.1	30.1	0	2	59
October-2015	16.6	11.5	4.9	21.7	28.3	0	0	58	15.4	9.6	2.3	21.2	27.4	0	0	66	15.8	10.3	2.7	21.3	26.3	0	0	66
November-2015	12.2	8	1.7	16.4	24.8	0	0	73	10.9	7	-3.6	14.8	22.1	3	0	80	10.9	7.2	-0.9	14.6	23.8	1	0	81
December-2015	7.6	3.9	-0.2	11.2	16.6	1	0	82	7.3	3.4	-1.0	11.1	16.4	4	0	87	8.6	4.7	-0.9	12.5	17.9	3	0	84
January-2016	9.6	5.9	0.2	13.3	20.5	0	0	70	7.8	3.4	-2.1	12.2	18.4	4	0	78	7.8	4.1	-1.7	11.5	16.5	2	0	81
February-2016	9.5	4.7	-0.8	14.2	21.2	2	0	60	8	2.5	-4.1	13.5	19.6	9	0	70	8.2	3.2	-3.9	13.1	18.7	4	0	71
March-2016	10.3	5.5	0.7	15.1	24.9	0	0	58	9.2	3.5	-1.9	14.8	24.2	2	0	67	9.3	3.6	-1.3	15.1	22.4	2	0	66
April-2016	14	8.5	2.6	19.4	26.9	0	0	51	12.8	6.4	1.0	19.3	26.5	0	0	60	12.3	6.2	1.5	18.4	23.8	0	0	62
May-2016	17.9	12.1	6.8	23.7	31.3	0	2	48	16.4	9.4	2.2	23.3	29.5	0	0	57	15.8	9.1	1.2	22.5	30.2	0	1	57
June-2016	23.4	16.4	11.3	30.3	37.0	0	17	40	22.2	14.2	8.4	30.1	34.9	0	16	47	21.7	13.9	7.7	29.5	35.9	0	14	42

[1]T. min: Minimum temperature (Mean: Mean of the minimum temperature / Abs: Lowest value for a specific month).

[2]T. max: Maximum temperature (Mean: Mean of the maximum temperature / Abs: Highest value for a specific month).

[3]T. mean: Mean temperature for a specific month.

[4]N0: Number of days with the minimum temperature under 0°C.

[5]N30: Number of days with the maximum temperature over 30°C.

[6]RH: Relative humidity.

[7]N/A: Not available.

Appendix Ch1-2: Histopathological features evaluated in the experimental lambs in central and peripheral nervous systems, liver, kidney, pancreas, spleen, adrenal glands, thyroid, and thymus.

Histopathological features evaluated in the **central nervous system** (brain: frontal cortex-caudate nucleus, parietal cortex, thalamus-hypothalamus; spinal cord: cervical spinal cord, thoracic spinal cord, lumbar spinal cord).

Features	Evaluation	Description
Perivascular cuffing	P/A[1]	At least one blood vessel surrounded by >2 layers-thick perivascular cuff of lymphocytes, plasma cells, and/or histiocytes.
Meningitis	P/A	Aggregates of lymphocytes, plasma cells, and/or histiocytes in the meninges
Glial nodules	P/A	At least one nodular aggregate of glial cells in the neuropil
Microglial activation	P/A	Aggregates of rod shaped glial cells in the neuropil
Dark neurons	P/A	Deeply hyperchromatic, shrunken neurons

[1]P/A: Presence/Absence

Histopathological features evaluated in the **peripheral nervous system** (subcutaneous-thoracic, sciatic, tibial, and radial nerves).

Features	Evaluation	Description
Perineural, perivascular cuffing	P/A[1]	At least one, ≥1 layer thick, perivascular aggregate of lymphocytes, plasma cells, and/or histiocytes in the tissues adjacent to the nerve
Intraneural inflammation	P/A	Aggregates of lymphocytes, plasma cells, and/or histiocytes within the peri- or endoneurium

[1]P/A: Presence/Absence.

Histopathological features evaluated in the **liver**.

Features	Evaluation	Description
Portal/periportal inflammation	P/A[1] Type	Inflammatory infiltrates in or around portal spaces LP: Lymphoplasmacytic LP + E: Lymphoplasmacytic and eosinophilic
Hepatocellular degeneration	P/A	Swollen hepatocytes with vacuolated or feathery cytoplasm
Hepatocellular necrosis	P/A	Shrunken eosinophilic hepatocytes with pyknotic nucleus
Hepatocellular atrophy	P/A	Shrunken hepatocyte cords with distended sinusoids

[1]P/A: Presence/Absence.

Histopathological features evaluated in the **kidney**.

Features	Evaluation	Description
Glomeruli: Proteinuria	P/A[1]	Protein globules in the Bowman's space
Tubules: Degeneration	P/A	Swollen tubular epithelium with vacuolated or feathery cytoplasm
Tubules: Hyaline droplets	P/A	Deeply eosinophilic, 1-3 □m intracytoplasmic droplets
Interstitium: Inflammation	P/A	Aggregates of lymphocytes, plasma cells, and/or histiocytes
Medulla: Mineralization	P/A	Foci of tubulointerstitial mineralization

[1]P/A: Presence/Absence.

Histopathological features evaluated in the **pancreas**.

Features	Evaluation	Description
Inflammation	P/A[1]	Interstitial and/or periductal aggregates of lymphocytes, plasma cells, and/or histiocytes

[1]P/A: Presence/Absence.

Histopathological features evaluated in the **spleen**.

Features	Evaluation	Description
White pulp hyperplasia	P/A[1]	Prominent lymphoid follicles with increased numbers of lymphocytes/blasts
Perifollicular PMs[2]	P/A	Aggregates of neutrophils and/or eosinophils around the lymphoid follicles

[1]P/A: Presence/Absence.
[2]PMs: Polymorphonuclear leukocytes.

Histopathological features evaluated in the **adrenal gland**.

Features	Evaluation	Description
Cortical hyperplasia	P/A[1]	Thickened adrenal cortex
	Localization	Fascicular Reticular Both
Cortical inflammation	P/A	Aggregates of lymphocytes, plasma cells, histiocytes, and/or neutrophils in the cortex

[1]P/A: Presence/absence.

Histopathological features evaluated in the **thyroid gland.**

Features	Evaluation	Description
Inflammation	P/A[1]	Aggregates of lymphocytes, plasma cells, and/or histiocytes in the interstitium
Follicular cells hyperplasia	P/A	Increased numbers of follicular cells
Follicular cells hypertrophy	P/A	Increased size of follicular cells
C cells hyperplasia/hypertrophy	P/A	Increased number and/or size of C cells

[1]P/A: Presence/absence.

Histopathological features evaluated in the **thymus**.

Features	Evaluation	Description
Germinal centers	P/A[1]	Presence of conspicuous germinal centers in >80% of the follicles
Degree of involution	0	No involution: Well-formed follicles.
	1	Mild involution: Smaller follicles.
	2	Moderate involution: Smaller follicles with fat-filled areas between them.
	3	Severe/total involution: Rare thymic remnants

[1]P/A: Presence/absence.

Appendix Ch1-3: Body weight (W) along the experiment in Control, Adjuvant-only, and Vaccine groups in each of the four flocks individually (Flock 1-4) and all flocks grouped together (All Flocks). Data represented as mean and standard deviation (SD).

		Flock 1			Flock 2			Flock 3			Flock 4			All Flocks		
	Group	n=21			n=21			n=17			n=19			n=78		
		Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*
W1	Control	31.68	3.7		38.26	3.4		38.30	2.7		38.61	2.2		36.59	4.2	
	Adjuvant	31.28	4.6		37.71	4.4		38.08	3.4		38.03	2.6		36.14	4.7	
	Vaccine	31.83	3.4	0.942[KW]	40.74	4.8	0.381[A]	38.67	3.9	0.956[A]	38.78	2.6	0.857[A]	37.41	5.0	0.611[A]
W2	Control	43.69	4.6		45.93	4.2		49.80	2.1		50.29	2.7		47.24	4.4	
	Adjuvant	43.16	4.9		43.57	3.2		49.25	4.6		50.08	4.7		46.27	5.2	
	Vaccine	43.66	2.9	0.965[A]	46.14	4.6	0.433[A]	48.50	3.6	0.839[A]	51.17	3.2	0.856[A]	47.18	4.4	0.709[A]
W3	Control	49.95	5.2		51.43	5.7		48.10	3.0		53.29	3.3		50.89	4.6	
	Adjuvant	49.33	4.4		49.79	4.9		48.83	3.6		54.08	5.8		50.43	4.9	
	Vaccine	49.28	3.4	0.524[KW]	52.43	6.1	0.677[A]	46.42	4.4	0.535[A]	52.58	5.2	0.865[A]	50.23	5.2	0.885[A]
W4	Control	53.39	6.1		55.00	4.9		52.50	2.7		48.14	5.5		52.24	5.5	
	Adjuvant	53.65	4.9		52.21	4.9		53.17	4.3		46.25	5.9		51.44	5.6	
	Vaccine	55.69	4.2	0.661[A]	56.29	7.0	0.408[A]	51.50	5.4	0.805[A]	48.91	5.6	0.708[A]	53.32	6.1	0.499[A]
W5	Control	54.22	6.5		58.00	4.4		56.00	1.7		52.36	4.2		55.08	4.9	
	Adjuvant	54.54	4.1		54.93	5.3		56.25	5.7		51.25	5.6		54.28	5.2	
	Vaccine	52.19	3.2	0.622[A]	56.50	8.7	0.672[A]	56.00	6.3	0.994[KW]	50.83	4.6	0.839[A]	53.92	6.2	0.736[A]
W6	Control	57.76	7.2		61.43	4.2		59.10	4.6		54.29	5.9		58.07	6.0	
	Adjuvant	58.01	5.8		58.64	4.8		59.25	7.4		52.83	6.1		57.27	6.2	
	Vaccine	57.31	4.4	0.660[KW]	57.86	8.3	0.52[A]	56.25	8.0	0.714[A]	52.33	6.1	0.833[A]	56.06	6.8	0.465[KW]
W7	Control	59.43	7.6		60.93	4.8		60.00	5.6		56.79	5.9		59.23	6.0	
	Adjuvant	57.89	6.9		58.14	4.4		59.00	6.1		55.67	7.1		57.7	5.9	
	Vaccine	58.57	5.9	0.915	56.93	8.2	0.462[A]	55.25	8.4	0.488[A]	54.00	6.6	0.744[A]	56.31	7.1	0.242[KW]
W8	Control	63.01	7.0		66.71	5.3		62.40	3.0		64.36	7.9		64.25	6.1	
	Adjuvant	62.57	7.0		61.93	6.6		61.33	6.9		62.67	10		62.13	7.4	
	Vaccine	62.85	6.0	0.992[A]	63.36	8.1	0.416[A]	58.08	5.9	0.433[A]	62.17	6.5	0.887[A]	61.73	6.6	0.355[A]
W9	Control	66.21	7.4		69.86	6.3		66.50	3.8		67.64	9.1		67.63	6.9	
	Adjuvant	64.95	7.0		64.64	6.4		64.67	8.0		65.42	9.2		64.91	7.2	
	Vaccine	64.85	7.6	0.468[KW]	65.64	8.4	0.366[A]	60.92	7.9	0.423[A]	63.58	7.5	0.705[A]	63.86	7.6	0.158[A]

[KW]: Kruskal-Wallis test.

[A]: ANOVA.

Appendix Ch1-4: Average daily gain (ADG) between weighing dates (W) along the experiment in Control, Adjuvant-only, and Vaccine groups in each of the four flocks individually (Flocks 1-4) and all flocks grouped together (All Flocks). Data represented as mean and standard deviation (SD).

	Group	Flock 1 n=21			Flock 2 n=21			Flock 3 n=17			Flock 4 n=19			All Flocks n=78		
		Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*	Mean	SD	*p*
ADG1	Control	273	45		145	78		209	64		216	59		211	76	
(W2-W1)	Adjuvant	270	44		111	46		203	25		223	43		201	72	
	Vaccine	269	35	0.982^{A}	102	52	0.394^{A}	179	35	0.472^{A}	229	48	0.898^{A}	194	77	0.719^{A}
ADG2	Control	116	28		95	38		-31	90		60	22		67	69	
(W3-W2)	Adjuvant	114	23		107	36		-8	65		80	59		76	66	
	Vaccine	104	43	0.763^{A}	108	78	0.772^{KW}	-39	44	0.715^{A}	28	54	0.189^{A}	55	81	0.227^{KW}
ADG3	Control	72	53		87	46		88	70		-95^{ab}	57		34	96	
(W4-W3)	Adjuvant	90	43		59	70		87	39		-145^{a}	22		27	1075	
	Vaccine	134	88	0.164^{KW}	94	82	0.605^{A}	102	48	0.865^{A}	-68^{b}	42	**0.023^{A}***	69	102	0.288^{KW}
ADG4	Control	13^{a}	57		48^{a}	28		74	35		84^{ab}	45		53^{a}	50	
(W5-W4)	Adjuvant	14^{a}	21		43^{a}	28		66	43		100^{a}	18		54^{a}	42	
	Vaccine	-56^{b}	69	**0.020^{KW}****	3^{b}	46	**0.049^{KW}***	96	43	0.528^{KW}	38^{b}	54	**0.055$^{A\#}$**	17^{b}	76	**0.045^{KW}***
ADG5	Control	79	92		78^{a}	26		69	87		45	64		67	68	
(W6-W5)	Adjuvant	77	48		84^{a}	34		67	62		37	32		67	46	
	Vaccine	114	99	0.610^{KW}	31^{b}	35	**0.011^{A}***	6	69	0.270^{KW}	35	44	0.928^{A}	48	76	0.146^{A}
ADG6	Control	43	53		-13	27		23	61		60	53		29	54	
(W7-W6)	Adjuvant	-3	57		-13	54		-6	55		67	58		10	61	
	Vaccine	32	102	0.205^{KW}	-23	24	0.827^{A}	-25	65	0.451^{A}	40	56	0.675^{A}	6	71	0.384^{A}
ADG7	Control	64	32		98	31		38	66		120	73		83	58	
(W8-W7)	Adjuvant	84	51		64	66		37	42		111	89		74	65	
	Vaccine	76	21	0.608^{A}	109	26	0.435^{KW}	45	52	0.962^{A}	130	28	0.897^{A}	90	44	0.589^{A}
ADG8	Control	103	53		92	80		100	81		94	76		97	68	
(W9-W7)	Adjuvant	77	60		80	52		81	38		79	154		79	81	
	Vaccine	65	71	0.522^{KW}	67	99	0.838^{A}	69	58	0.792^{KW}	40	74	0.662^{A}	61	74	0.372^{A}
Global	Control	91	15		81^{a}	14		71	11		74	24		80^{a}	18	
ADG	Adjuvant	89	13		69^{ab}	11		67	18		70	19		74^{ab}	17	
(W9-W1)	Vaccine	87	23	0.913^{A}	64^{b}	11	**0.045^{A}***	56	14	0.229^{A}	63	16	0.759^{KW}	68^{b}	20	**0.072$^{A\#}$**

^{A}: ANOVA.

^{KW}: Kruskal-Wallis test.

^{a,b}: Statistically significant differences between groups based on *post-hoc* test.

*: Statistical significance ($p<0.05$).

#: Statistical tendency ($p\leq0.1$).

Appendix Ch1-5: Hematological results along the experiment in Control, Adjuvant-only, and Vaccine groups (n=26 each) of all Flocks grouped together. Data represented as mean and standard deviation (SD). H: Hematology date. A reference threshold is provided at the end of the Table.

	Group	WBC1 (x103/mm3)		RBC2 (x103/mm3)		Hematocrit (%)		Hemoglobin (g/dl)		Platelets (x103/mm3)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
H1	Control	7.43	1.88	11.56	0.97	35.52	3.01	11.72	1.04	666	145
	Adjuvant	7.60	2.31	11.65	0.87	35.09	2.95	12.15	0.99	616	183
	Vaccine	7.64	1.86	11.18	0.74	34.53	2.57	11.65	0.69	631	157
H2	Control	7.70	1.89	10.93	0.95	34.29	2.76	11.32	0.93	601	223
	Adjuvant	8.67	2.54	11.14	0.64	34.42	2.80	11.72	0.83	618	233
	Vaccine	7.56	1.31	11.20	0.88	35.21	2.77	11.71	1.00	602	215
H3	Control	7.28	1.48	11.08	1.13	34.96	3.60	11.06	1.22	552	197
	Adjuvant	7.95	2.80	10.96	0.95	34.24	2.93	11.04	1.02	525	198
	Vaccine	7.46	1.98	11.31	0.76	35.68	2.80	11.28	0.78	521	139
H4	Control	8.69	2.03	10.47	0.93	32.49	3.01	10.55	0.95	458	156
	Adjuvant	8.41	1.87	10.61	0.71	32.62	2.18	10.58	0.73	469	146
	Vaccine	8.22	1.62	10.51	0.74	32.51	2.38	10.53	0.77	460	118
H5	Control	7.70	1.46	10.72	0.95	33.50	3.27	10.56	0.75	680	338
	Adjuvant	8.06	1.94	10.80	0.77	33.38	2.76	10.77	0.82	672	379
	Vaccine	7.40	1.56	10.79	0.90	33.59	3.15	10.70	0.90	776	385
H6	Control	6.87	1.70	10.79	1.77	34.64	5.07	10.85	1.58	622	290
	Adjuvant	7.66	2.37	10.97	1.62	34.70	4.93	11.10	1.60	645	398
	Vaccine	6.96	2.06	10.94	1.31	35.11	4.42	11.03	1.14	667	316
H7	Control	7.29	2.50	10.03	1.71	32.58	5.26	10.64	1.62	396	128
	Adjuvant	8.13	2.14	10.40	1.17	33.57	3.60	11.04	1.21	363	242
	Vaccine	6.88	1.52	9.94	1.55	32.33	4.44	10.56	1.35	462	153
H8	Control	7.65	1.90	10.36	1.57	34.40	5.14	11.18	1.29	638	368
	Adjuvant	8.11	1.80	10.67	0.97	35.02	3.38	11.70	0.77	524	292
	Vaccine	7.53	2.19	10.19	1.27	33.70	4.56	11.08	1.11	553	312
H9	Control	8.08	2.31	10.40	1.61	34.72	4.86	11.04	1.68	469	140
	Adjuvant	8.42	2.18	10.57	0.97	34.71	3.71	11.08	1.11	477	210
	Vaccine	8.03	2.39	10.57	1.01	35.02	3.33	11.04	1.01	461	132
H10	Control	8.35	2.71	10.41	1.42	34.95	5.10	10.89	1.36	357	122
	Adjuvant	8.10	1.91	10.45	1.57	34.51	3.97	10.78	1.17	437	191
	Vaccine	8.49	1.98	10.89	1.58	36.10	4.51	11.13	1.22	425	152
H11	Control	9.60	2.51	9.80	1.18	32.67	3.92	10.58	1.31	370	129
	Adjuvant	8.95	1.62	10.14	1.21	33.20	3.43	10.58	1.25	385	134
	Vaccine	9.56	3.00	9.83	0.98	32.49	2.64	10.42	0.90	382	151
H12	Control	7.63	2.18	10.07	1.70	32.97	5.49	10.09	1.76	496	171
	Adjuvant	7.83	1.60	10.48	1.03	33.71	3.28	10.47	1.03	447	192
	Vaccine	7.32	1.23	10.40	0.96	33.69	2.98	10.39	0.94	476	171
Reference Treshold		**4-12 x10^3/mm^3**		**9-14 x10^6/mm^3**		**28-40 %**		**8-15 g/dl**		**250-750 x10^3/mm^3**	

1WBC: White blood cell count. 2RBC: Red blood cell count

Appendix Ch1-6: Histopathological findings in the peripheral nervous system in Control, Adjuvant-only and Vaccine groups of all Flocks grouped together. Data provided as animals with the referred histological lesion relative to the total number of animals analyzed. Methodology of histopathological evaluation is detailed in Appendix Ch1-2.

Location	Group	Perivascular cuffing	Inflammation
		Presence	Presence
Subcutaneous thoracic nerve	Control	16/24	1/25
	Adjuvant	15/26	1/26
	Vaccine	17/25	2/26
	p	0.706[Xi]	0.790[LR]
Sciatic nerve	Control	12/25	1/26
	Adjuvant	14/26	0/26
	Vaccine	16/26	0/26
	p	0.622[Xi]	0.320[LR]
Tibial nerve	Control	11/26	1/26
	Adjuvant	15/26	1/26
	Vaccine	12/26	1/26
	p	0.513[Xi]	1.000[LR]
Radial nerve	Control	15/24	1/24
	Adjuvant	13/26	0/26
	Vaccine	13/23	0/23
	p	0.672[Xi]	0.324[LR]

[Xi]: Pearson's chi square test.
[LR]: Likelihood ratio test

Appendix Ch1-7: Histopathological results in liver, kidney, spleen, adrenal gland, thyroid gland, and thymus of Control, Adjuvant-only, and Vaccine groups of all Flocks grouped together. Data provided as animals with the referred histological lesion relative to the total number of animals analyzed. Methodology of histopathological evaluation is detailed in Appendix Ch1-2.

Histopathological findings in the **liver**.

Location	Group	Portal/periportal inflammation			Hepatocytes		
		Presence	Type		Degeneration	Necrosis	Atrophy
			LP[1]	LP+E[2]			
Liver	Control	9/26	8/9	1/9	13/26	1/26	14/26
	Adjuvant	12/26	6/12	6/12	15/26	1/26	9/26
	Vaccine	12/26	9/12	3/12	10/26	0/26	12/26
	p	0.62[LR]	0.13[LR]		0.377[Xi]	0.439[LR]	0.374[Xi]

[1]LP: Lymphoplasmacytic.
[2]LP + E: Lymphoplasmacytic and eosinophilic.
[LR]: Likelihood ratio test.
[Xi]: Pearson's chi square test.

Histopathological findings in the **kidney**.

Location	Group	Glomeruli	Tubules		Interstitium	Medulla
		Protein	Degeneration	Hyaline droplets	Inflammation	Mineralization
Kidney	Control	15/26	2/26	10/26	8/26	10/26
	Adjuvant	16/26	2/26	9/26	11/26	10/26
	Vaccine	15/26	4/26	12/26	10/26	9/26
	p	0.948[Xi]	0.589[LR]	0.687[Xi]	0.681[Xi]	0.947[Xi]

[Xi]: Pearson's chi square test.
[LR]: Likelihood ratio test.

Histopathological findings in the **spleen**.

Location	Group	White pulp hyperplasia	Perifollilular PMs[1]
Spleen	Control	11/26	24/26
	Adjuvant	12/26	25/26
	Vaccine	10/26	23/26
	p	0.854[Xi]	0.568[LR]

[1]PMs: Polymorphonuclear leukocytes (i.e., neutrophils. eosinophils).
[Xi]: Pearson's chi square test.
[LR]: Likelihood ratio test.

Histopathological findings in the **adrenal gland.**

Location	Group	Cortical hyperplasia				Inflammation
		Presence	Localization			Presence
			Fascicular	Reticular	Both	
Adrenal Gland	Control	13/26	4/12	1/12	7/12	4/26
	Adjuvant	15/26	7/15	3/15	5/15	5/26
	Vaccine	18/26	9/18	1/18	8/18	8/26
	p	0.365[Xi]		0.558[LR]		0.376[Xi]

[Xi]: Pearson's chi square test.
[LR]: Likelihood ratio test.

Histopathological findings in the **thyroid gland.**

Location	Group	Inflammation	Follicular cells hyperplasia	Follicular cells Hypertrophy	C Cells Hypertrophy
Thyroid Gland	Control	8/26	16/26	0/26	4/26
	Adjuvant	11/26	15/26	3/26	4/26
	Vaccine	4/26	13/26	3/26	7/26
	p	0.102[Xi]	0.694[Xi]	**0.078[LR#]**	0.489[LR]

[Xi]: Pearson's chi square test.
[LR]: Likelihood ratio test. [#]: Statistical tendency (p≤0.1)

Histopathological findings in the **thymus.**

Location	Group	Germinal centers	Degree of involution			
			0	1	2	3
Thymus	Control	4/26	13/25	10/25	0/25	2/25
	Adjuvant	1/26	9/26	16/26	0/26	1/26
	Vaccine	0/26	11/26	11/26	0/26	4/26
	p	**0.043[LR]***		0.364[LR]		

[LR]: Likelihood ratio test.
*: Statistical significance (p<0.05).

CHAPTER 2

Detection of aluminum in lumbar spinal cord of lambs repetitively inoculated with aluminum-oxyhydroxide containing vaccines or aluminum-oxyhydroxide alone

ABSTRACT

The use of vaccines containing ABAs is widespread in ovine production. ABAs induce an effective immune-response but lead to the formation of Al-induced injection-site granulomas from which Al can disseminate. This chapter aims to study the accumulation of Al in the CNS of sheep subcutaneously inoculated with Al-oxyhydroxide containing products. Lumbar spinal cord and parietal lobe from 21 animals inoculated with 19 doses of Vaccine (n=7), Adjuvant-only (n=7) or PBS as Control (n=7) were analyzed with lumogallion staining for Al tisular localization. In the lumbar spinal cord, Al median content was higher in both the Adjuvant-only (p=0.001) and Vaccine group (p=0.002) compared with the Control group. Deposits were significantly more abundant in the lumbar spinal cord than in the parietal lobe in the Adjuvant-only group (p=0.027) and they showed a marked trend in the Vaccine group (p=0.054). In the parietal lobe, Al deposits were higher in the Vaccine group compared with Control group (p=0.017) and Adjuvant-only group (p=0.017). In the lumbar spinal cord, lumogallion reactive Al deposits were more abundant in the gray matter than in the white matter in both Vaccine (p=0.034) and Adjuvant-only groups (p=0.017) and Al deposits were mostly associated with glial-like cells (p= 0.042). In the parietal lobe, few Al deposits, which were sometimes related to vessels, were found. In sheep, Al-oxyhydroxide adjuvants inoculated in the subcutaneous tissue selectively accumulates in the lumbar spinal cord.

INTRODUCTION

Vaccines are key elements for controlling diseases in animal populations. From the 1930s, vaccines have made a major contribution to improving sheep health, welfare and productivity (Responsible Use of Medicines in Agriculture Alliance, 2009). The ovine health programs in South-Europe usually involve vaccination against common reproductive, digestive and respiratory pathogens (Lacasta *et al.*, 2015). The vaccination schedule of each flock largely depends on management system, production type, breed and local climate (Lacasta *et al.*, 2015). In our local conditions (meat-producing breeds managed in semi-intensive to intensive systems that often implies shepherding), animals usually receive between 2-4 vaccines per year during their whole lifespan.

The majority of vaccines need an adjuvant to strengthen the humoral and cellular immune responses induced against vaccine antigens (Ruwona *et al.*, 2016). Most ovine vaccines use Al-oxyhydroxide as adjuvant, which induces a fast and effective immune response against vaccine antigens. This adjuvant consists of primary nano-sized particles that spontaneously aggregate forming micron-sized agglomerates subjected to marked size variations depending on Al-oxyhydroxide concentration, ionic strength of the diluent and antigen absorption (Eidi *et al.*, 2015; Shardlow, Mold and Exley, 2016).

In sheep, Al-oxyhydroxide is phagocytosed by macrophages inducing the formation of persistent sterile subcutaneous granulomas, from which intracytoplasmic Al-oxyhydroxide can translocate by leukocyte trafficking to the regional lymph node (Asín *et al.*, 2019). In mice, lymph node plays a key role in Al biodistribution enabling intramacrophagic Al to reach distant tissues such as the spleen and CNS (Khan *et al.*, 2013; Crépeaux *et al.*, 2017). Whether Al-oxyhydroxide can reach other locations in the sheep body after subcutaneous injection of Al-oxyhydroxide alone or formulated in Al-based vaccines is unknown. In accordance with the Objective 2 of the PhD Thesis, the aim of this chapter is to determine the presence of Al in the CNS of lambs after subcutaneous injection of Al-oxyhydroxide containing products and to study the localization of this Al within the neuroparenchyma.

MATERIAL AND METHODS

Animals

Animal samples analyzed in this work belonged to a previous, wider study carried out by our group that aimed to study the local and systemic effects caused by repetitive subcutaneous inoculation of Al-containing adjuvants in sheep (Asín *et al.*, 2019). Animals analyzed were those of Flock 1 (Global Material and Methods, Experiment 1). Briefly, twenty-one, three-month-old, neutered male purebred Rasa Aragonesa lambs were selected from a pedigree flock of certified good health and lodged indoor at the experimental farm of the University of Zaragoza, with optimal conditions of housing, management and diet for 15 months.

Based on the received treatment, animals were divided into 3 groups of 7 animals each and were inoculated with different substances: Vaccine group was treated with commercial vaccines, Adjuvant-only group was inoculated with Al-oxyhydroxide (Alhydrogel®, CZ Veterinaria, Spain) and Control group received PBS. Animals had never been vaccinated or inoculated with any other substance prior to the experiment. Lambs underwent an accelerated vaccination scheduled to mimic, in an acceptable time frame for an experimental project, the Al load that these animals can receive in field conditions during their lifespan. Inoculations were performed in the subcutaneous tissue of the flank. A total of 19 inoculations were distributed in 15 injection dates. The schedule of injections for the three groups and the vaccines used are detailed in Figure M&M-1 and Table M&M-2, respectively. Al content of each inocula was mesured by inductively coupled plasma mass spectrometry. Vaccine and Adjuvant-only groups received a total of 81.29 mg of Al per animal. Al content of PBS inocula was always under the limit of detection of the technique (0.074 µg/mL). At the end of the study animals were eutanished and systematic tissue sampling was performed.

Aluminum localization in lumbar spinal cord and parietal lobe

Lumogallion staining was performed in formalin-fixed paraffin-embedded tissues of the lumbar spinal cord and parietal lobe following a recently validated method which is highly specific for Al detection in tissues (Mirza *et al.*, 2016). Briefly, 5 µm-thick tissue sections carefully protected from environmental Al contamination were dewaxed, rehydrated and incubated for 45 minutes with a 1 mM solution of lumogallion (Tokyo Chemical Industry buffered in 50 mM 1,4-Piperazine-diethanesulfonic acid buffer (PIPES-buffer) pH 7.4. Serial sections from each tissue were used as controls for evaluation of tissue autofluorescence. The control sections followed the same protocol but they were incubated only with PIPES-buffer solution. After incubation, all slides were washed 6 times with PIPES-buffer solution, rinsed in ultrapure water, mounted with an aqueous medium and stored at 4ºC overnight prior to analysis. Lumogallion and control autofluorescence analyses were performed using a fluorescence microscope (Zeiss AxioVert 200M, Germany) with a bandpass excitation filter (470/40nm), beam splitter (495nm) and bandpass emission filter (590/33nm). Excitation of the Al-lumogallion complex emits characteristic yellow-orange fluorescence. Autofluorescence of immediately adjacent serial sections confirmed lumogallion fluorescence as indicative of Al staining. Images were taken with AxioCam HR and processed with analyses software package AxioVision 4.6.3. Total evaluated area was approximately 200 mm^2 in the parietal lobe and approximately 50 mm^2 in the lumbar spinal cord.

Statistical analysis

All statistical analyses were performed using IBM SPSS 19.0 for Windows (IBM Corp., Armonk, NY, USA). Al deposits is a quantatitave discrete variable that was described and represented using mean and standard error mean.

In non-normal non-paired variables (i.e., comparisons of Al deposits in the CNS among the three groups) association of a non-normal quantitative variable with a qualitative variable with three or more categories was assessed by Kruskal-Wallis test followed by *post-hoc* Dunn's test (Daniel and Cross, 2013). In non-normal paired variables

(i.e., comparisons of Al deposits in the gray matter and white matter) the association between variables was assessed by Wilcoxon test (Daniel and Cross, 2013).

RESULTS

Details of Al deposits found in the CNS (lumbar spinal cord and parietal lobe) are shown in Appendix Ch2-1 and Appendix Ch2-2. Lumogallion reactive deposits, showing a yellow-orange fluorescence emission were observed in both, lumbar spinal cord and parietal lobe and they were identified as Al after autofluorescence verification. The vast majority of Al deposits were located in the gray matter, they were micron-sized (1-10µm approx.) and were mostly cell-associated. Deposits were significantly more abundant in the lumbar spinal cord than in the parietal lobe in the Adjuvant-only group (p=0.027) and they showed a marked trend in the Vaccine group (p=0.054). All animals of the Adjuvant-only group and Vaccine group showed at least one Al deposit in the lumbar spinal cord. The total number of deposits at lumbar spinal cord were similar in Vaccine and Adjuvant-only groups and they significantly differed (p=0.002 and p=0.001 respectively) from the Control group (Figure Ch2-1).

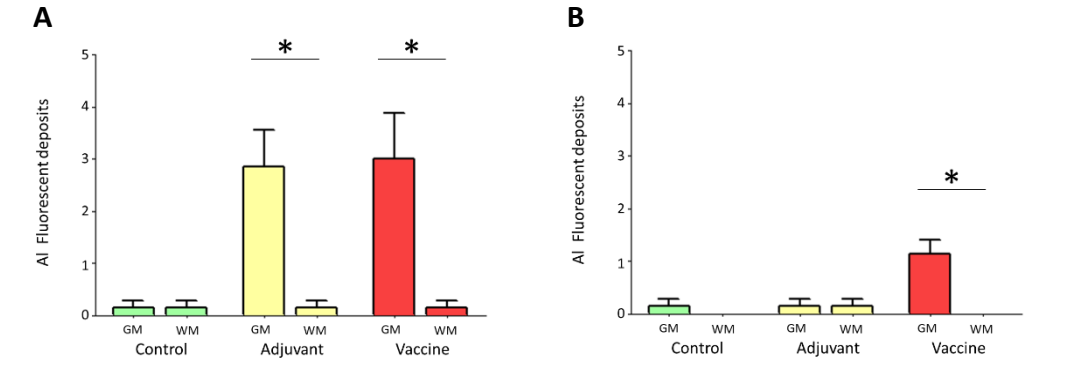

Figure Ch2-1: Aluminum deposits found by fluorescence microscopy after lumogallion stain. (A) Lumbar spinal cord. There are more Al deposits in the gray matter (GM) than in the white matter (WM) in Vaccine and Adjuvant-only groups. **(B)** Parietal lobe. Al deposits are more abundant in the gray matter (GM) than in the white matter (WM) in Vaccine group.

In the lumbar spinal cord, Al deposits were significantly more abundant in the gray matter (Figure Ch2-2) than in the white matter in both Vaccine (p= 0.034) and Adjuvant-only (p= 0.017) groups. Al deposits in the gray matter of the lumbar spinal cord were

observed mostly associated with morphologically compatible glial cells (Figure Ch2-3:a,b) and only a few deposits were non-cell associated (Figure Ch2-3:c,d), this difference being statistically significant (p= 0.042).

In the parietal lobe, Al was mostly found in the gray matter of the Vaccine group, in sharp contrast to Adjuvant-only (p=0.017) and Control groups (p=0.017). These deposits were cell-associated and sometimes closely related to vessels (Figure Ch2-4). The Al deposits observed in the parietal lobe was strickingly lower in contrast with the deposits found in the lumbar spinal cord (Appendix Ch2-1 and Appendix Ch2-2).

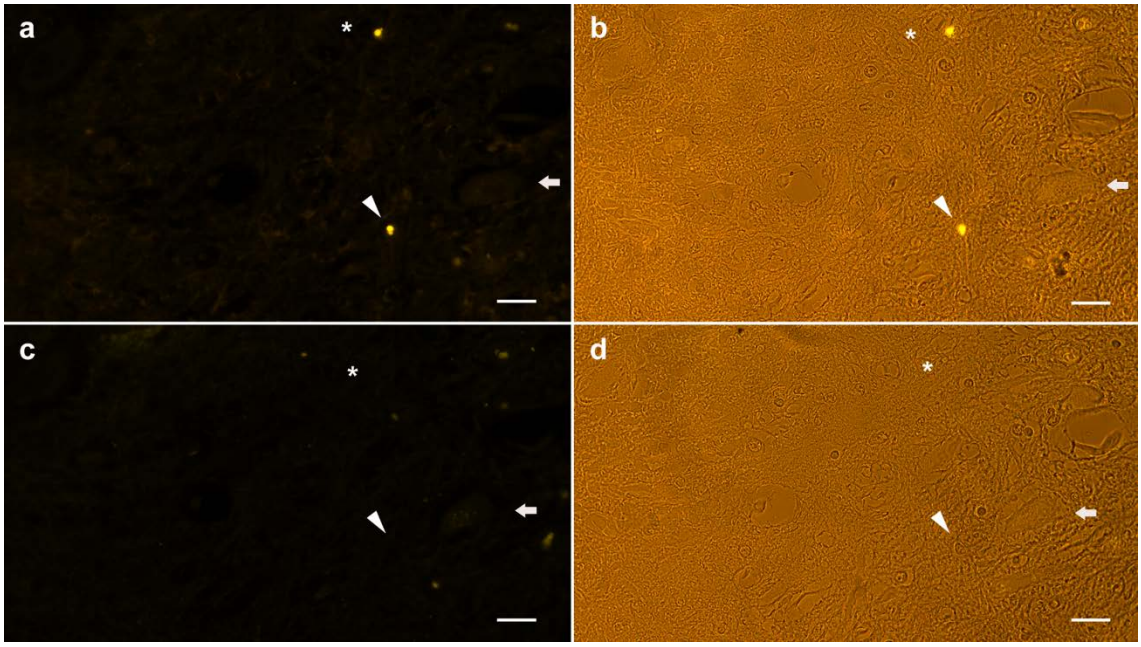

Figure Ch2-2: Sheep 111, Adjuvant-only group, gray matter of the lumbar spinal cord. **(A-B)** Lumogallion staining. Two intense yellow-orange fluorescent aluminum (Al) deposits (asterisk and arrow head) depicting the fluorescence channel (A) and bright field overlay (B). **(C-D)** Sequential unstained sections for autofluorescence evaluation (asterisk and arrow head indicates the same area as Al deposits were found with lumogallion). A green autofluorescence emission background was identified in non-stained sections, confirming the specific staining of Al deposits found in (A) and (B), respectively. Note weakly autofluorescent intraneuronal pigments of lipofuscin (arrow) shown in both the lumogallion stained (A) and the non-stained sections (C) under fluorescent channel. Scale bar: 20µm.

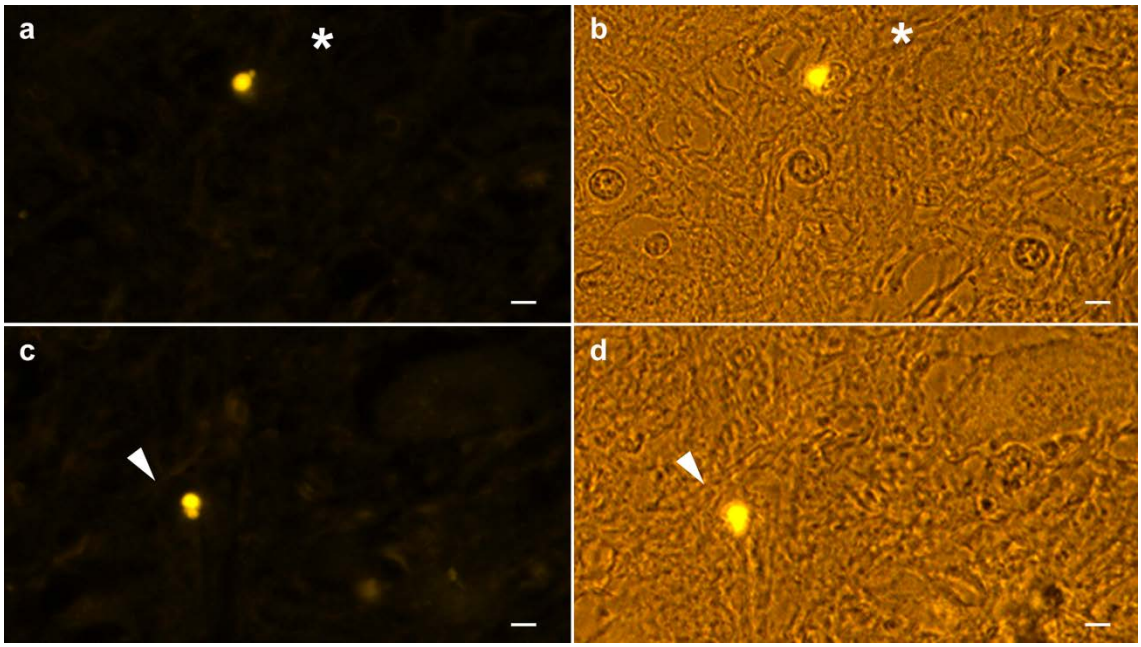

Figure Ch2-3: Sheep 111, Adjuvant-only group, gray matter of the lumbar spinal cord. Higher magnification of deposits seen in Figure Ch2-2. Lumogallion staining. Two intense yellow-orange fluorescent aluminum (Al) deposits (asterisk and arrowhead) are seen with the fluorescence channel **(A-C)** and bright field overlay depicted **(B-D)**. One of the deposits (asterisk) is cell-associated with a glial-like cell (A-B) whereas the other (arrowhead) is shown to be non-cell associated within the neuroparenchyma (C-D). A green autofluorescence emission background was identified in non-stained sections, confirming the specific staining of Al deposits found (shown in Figure 2). Scale bar: 5µm.

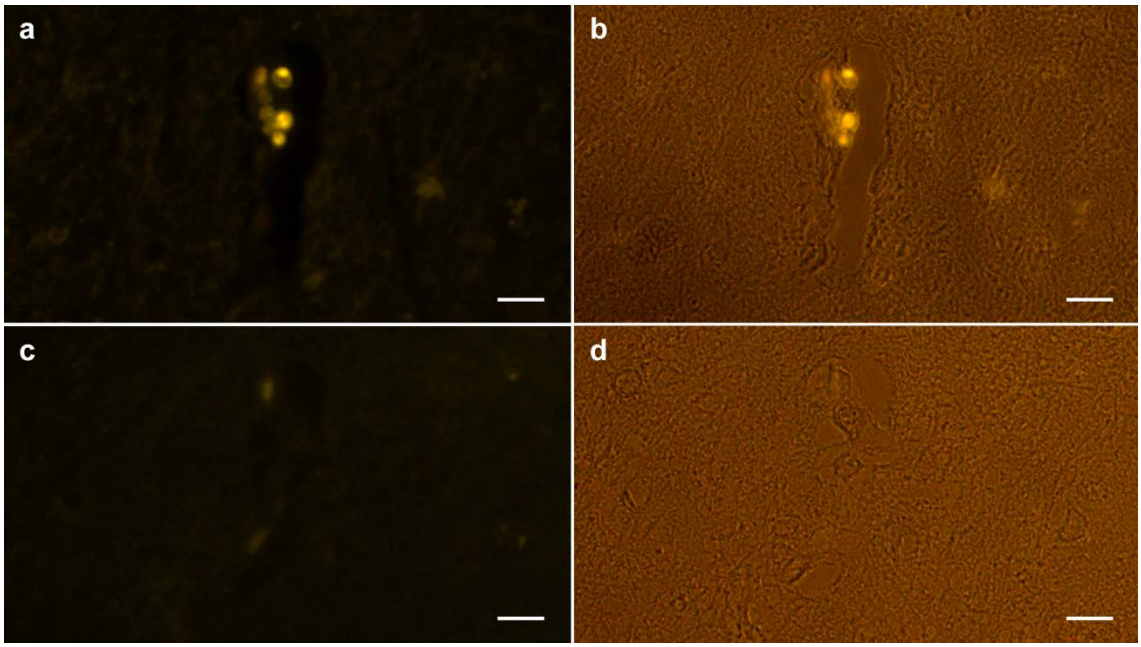

Figure Ch2 - 4: Sheep 114, Adjuvant-only group, gray matter of the parietal lobe. **(A-B)** Lumogallion staining. Cell-associated, intense yellow-orange fluorescent Al deposits close to a blood vessel with the fluorescence channel (A) and bright field overlay depicted (B). **(C-D)** Sequential unstained sections for autofluorescence evaluation. A green autofluorescence emission background was identified in non-stained sections, confirming the specific staining of Al deposits found in (A) and (B), respectively. Scale bar: 10µm.

DISCUSSION

In sheep, Al-containing products lead to the formation of subcutaneous granulomas from where intramacrophagic Al-oxyhydroxide can reach the regional lymph node and potentially disseminate to other organs (Asín *et al.*, 2019). This work studies accumulation of Al in the CNS of sheep after subcutaneous, repeated injections of Al-containing products. Al was localized by lumogallion staining in lumbar spinal cord and parietal lobe.

Lumogallion is a highly specific fluorescent stain for Al localization in CNS (Mirza *et al.*, 2016) that has been widely validated in a variety of animal species (Martinez *et al.*, 2017; Asín *et al.*, 2019; Mold, Cottle and Exley, 2019) and is useful for detecting Al adjuvants (Mold *et al.*, 2014; Mile *et al.*, 2015). In this study, lumogallion reactive Al deposits were more abundant in the lumbar spinal cord than in the parietal lobe. This results correlate with analytical measurements performed in these animals by transversely heated graphite atomic absorption spectroscopy (TH-GFAAS). These measurements were performed by Javier Asín and showed that Al levels in the parietal lobe were within the same limits in the three treatment groups (Adjuvant-only: median=0.2 μg/g, IQR=0.11-0.73; Vaccine: median=0.1 μg/g, IQR=0.01-0.35; Control: median=0.1 μg/g, IQR=0.01-0.26) whereas Al levels in the lumbar spinal cord were higher in the Adjuvant-only (median=0.49 μg/g, IQR=0.31-1.04) and Vaccine groups (median=0.39 μg/g, IQR=0.33-0.62) compared with the Control group (median=0.08 μg/g, IQR=0.01-0.44).

In this study, Al was primarily located in the gray matter in sections from both, lumbar spinal cord and parietal lobe. Interestingly, in the parietal lobe Al was found in close proximity to blood vessels and it was likely intracellular. Despite the scarce number of deposits found at this location, the presence of this vascular-associated Al particles could support the hypothesis of the hematogenous dissemination of Al-loaded macrophages from the regional lymph node via the efferent lymphatics and the thoracic duct (Khan *et al.*, 2013). In the lumbar spinal cord, most Al deposits were found likely associated with glial cells, something that has been previously described in translocation studies of intramuscularly inoculated ABAs in mice (Khan *et al.*, 2013; Crépeaux *et al.*, 2017). The prevalence of Al-associated glial-like cells reinforce the idea of an Al input to the CNS via

Al-loaded macrophages (Khan *et al.*, 2013). Indeed, previous studies in mice have demonstrated Al particles within astroglial and microglial cells (Khan *et al.*, 2013). Moreover, Al-oxyhydroxide has been shown to increase microglial cell density in mice (Crépeaux *et al.*, 2017).

All lumogallion positive deposits found in the present study were dense micron-sized aggregates of Al that were predominantly cell-associated and located in the gray matter. Such characteristics were similar to those described in murine studies of ABAs distribution (Khan *et al.*, 2013). However, these results contrast with other studies in which Al of unknown origin and composition was also found in human CNS as diffuse and plaque-like deposits (Mold *et al.*, 2018; Mold, Cottle and Exley, 2019). Our results may suggest that different routes of entry and different chemical compositions of Al components, may play a major role in the distribution and histopathological morphology of Al in the CNS.

A limitation of the study could be the number of CNS samples analyzed as Al detection in tissue sections by fluorescence microscopy represented a limited sampling of the CNS. Another limitation of the study could be the accelerated vaccination schedule. Although the recommended protocols for each individual product were always fulfilled, this experimental vaccination schedule was designed to detect clinicopathological changes in experimental animals within 15 months. The outcome of the same amount of Al divided in small doses for a longer period of time might differ from the results of the present study.

In any case, our results suggest that sheep selectively accumulate subcutaneously injected Al in the lumbar spinal cord. This selective accumulation of Al may be due to morphological and physiological differences between the blood-brain barrier (BBB) and the blood-spinal cord barrier (BSCB) (Bartanusz *et al.*, 2011). In normal conditions, BSCB present decreased expression of tight junction proteins (ZO-1 and occludin) and adherence junction proteins (VE-cadherin and β-catenin) and it is more permeable to pro-inflammatory cytokines as IFN-γ and TNF-α than BBB (Pan, Banks and Kastin, 1997; Bartanusz *et al.*, 2011). Moreover, studies in mice show that IFN-γ passage through the BBB is saturated at low doses whereas BSCB remains non-saturated in the lumbosacral region at the same doses (Bartanusz *et al.*, 2011). This increased permeability in the lumbar spinal cord has been proposed to play a role in certain processes such as experimental immune

encephalomyelitis (Pan, Banks and Kastin, 1997). This permeability could also account for different input of Al into the CNS either by a direct mechanism, favoring the leukocyte trafficking, or indirectly, accelerating a local neuroinflammatory status which has already been related with higher Al input into the CNS (Pan, Banks and Kastin, 1997; Khan *et al.*, 2013). Alternatively, a weak BSCB at the lumbar area may be contributing to this selective accumulation as already demonstrated in mice with a leaky BBB (Khan *et al.*, 2013).

Selective accumulation of Al in the lumbar spinal cord could be linked to the lesions observed in the chronic phase of the ovine ASIA syndrome, where neurodegenerative changes were mostly observed at the lumbar spinal cord (Luján *et al.*, 2013). Moreover, these Al deposits might contribute to the development of neuropathological problems as Al is a potential neurotoxic molecule (Maya *et al.*, 2016). In this sense, the only finding after a complete histopathological analysis of the CNS of these animals was an increase in the number of dark neurons in the lumbar spinal cord of animals in the Adjuvant-only group (Chapter 1).

CONCLUSIONS

Al analytical measurements and fluorescent Al deposits indicate that, in sheep, the subcutaneous and intensive injection of Al adjuvants increase the levels of Al in the lumbar spinal cord.

APPENDICES

- **Appendix Ch2-1:** Aluminum (Al) deposits found in the lumbar spinal cord by fluorescent lumogallion staining in each animal.
- **Appendix Ch2-2:** Aluminum (Al) deposits found in the parietal lobe by fluorescent lumogallion staining in each animal.

Appendix Ch2-1: Aluminum (Al) deposits found in the lumbar spinal cord by fluorescent lumogallion staining in each animal. Al localization was assessed as Cell-associated deposits (C) or Non cell-associated deposits (NC) in both Gray matter and White matter. Total deposits (T) are the addition of C and NC deposits.

Control			**Adjuvant-only**			**Vaccine**		
Animal	Gray Matter	White Matter	Animal	Gray Matter	White Mater	Animal	Gray Matter	White Matter
131	C: 0 NC: 0 T: 0	C: 0 NC: 1 T: 1	111	C: 4 NC: 1 T: 5	C: 0 NC: 0 T: 0	121	C: 2 NC: 2 T: 4	C: 0 NC: 0 T: 0
132	C: 1 NC: 0 T: 1	C: 0 NC: 0 T: 0	112	C: 1 NC: 1 T: 2	C: 0 NC: 0 T: 0	122	C: 3 NC: 1 T: 4	C: 0 NC: 0 T: 0
133	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	113	C: 2 NC: 0 T: 2	C: 0 NC: 0 T: 0	123	C: 1 NC: 0 T: 1	C: 0 NC: 0 T: 0
134	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	114	C: 1 NC: 1 T: 2	C: 0 NC: 0 T: 0	124	C: 7 NC: 0 T: 7	C: 0 NC: 0 T: 0
135	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	115	C: 5 NC: 1 T: 6	C: 0 NC: 0 T: 0	125	C: 3 NC: 0 T: 3	C: 0 NC: 0 T: 0
136	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	116	C: 2 NC: 0 T: 2	C: 0 NC: 1 T: 1	126	C: 2 NC: 0 T: 2	C: 0 NC: 0 T: 0
137	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	117	C: 1 NC: 0 T: 1	C: 0 NC: 0 T: 0	127	C: 0 NC: 0 T: 0	C: 0 NC: 1 T: 1
TOTAL	C: 1 NC: 0 T: 1	C: 0 NC: 1 T: 1	TOTAL	C: 16 NC: 4 T: 20	C: 0 NC: 1 T: 1	TOTAL	C: 18 NC: 3 T: 21	C: 0 NC: 1 T: 1

Appendix Ch2-2: Aluminum (Al) deposits found in the parietal lobe by fluorescent lumogallion staining in each animal. Al localization was assessed as Cell-associated deposits (C) or Non Cell-associated deposits (NC) in both Gray matter and White matter. Total deposits (T) are the addition of C and NC deposits.

Control			**Adjuvant-only**			**Vaccine**		
Animal	Gray Matter	White matter	Animal	Gray Matter	White Mater	Animal	Gray Matter	White Matter
131	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	111	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	121	C: 1 NC: 0 T: 1	C: 0 NC: 0 T: 0
132	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	112	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	122	C: 1 NC: 0 T: 1	C: 0 NC: 0 T: 0
133	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	113	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	123	C: 1 NC: 0 T: 1	C: 0 NC: 0 T: 0
134	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	114	C: 1 NC: 0 T: 1	C: 0 NC: 1 T: 1	124	C: 1 NC: 0 T: 1	C: 0 NC: 0 T: 0
135	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	115	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	125	C: 2 NC: 0 T: 2	C: 0 NC: 0 T: 0
136	C: 1 NC: 0 T: 1	C: 0 NC: 0 T: 0	116	C: 0 NC: 0 T: 0	C: 0 NC: 1 T: 1	126	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0
137	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	117	C: 0 NC: 0 T: 0	C: 0 NC: 0 T: 0	127	C: 2 NC: 0 T: 2	C: 0 NC: 0 T: 0
TOTAL	C: 1 NC: 0 T: 1	C: 0 NC: 0 T: 0	TOTAL	C: 1 NC: 0 T: 1	C: 0 NC: 1 T: 1	TOTAL	C: 8 NC: 0 T: 8	C: 0 NC: 0 T: 0

CHAPTER 3

Inflammatory and immune signaling pathways at injection-site granulomas, regional lymph node and spleen of lambs repetitively inoculated with aluminum-oxyhydroxide containing vaccines or aluminum-oxyhydroxide alone

ABSTRACT

Al-based vaccines are of common use in human and veterinary medicine conferring protection to a wide range of infectious diseases by inducing a T_h2-biased or humoral responses based on antibody production. However, precise immunological mechanisms governing ABAs are to be revealed. Moreover, protective responses produced by Al-based vaccines may fail in protecting against intracellular pathogens due to low induction of cellular-mediated immunity. Persistent injection-site granulomas are a constant feature after inoculation with ABAs in several animal species including sheep. The aim of this chapter was the characterization of the inflammatory and immune signaling pathways induced at injection-site granulomas, draining lymph nodes and spleen of lambs after repetitive inoculation of ABAs or Al-based vaccines. Immunological activation was more evident in secondary lymphoid organs (i.e., lymph node and spleen) and involved the expression of TLRs, pro-inflammatory cytokines, costimulatory molecules and antiviral proteins. Few differences were found in granulomas likely due to the unavailability of proper control tissue. However, significant reduction in IL-10 expression and the presence of IL-6 may indicate a constant immune activation in granulomas vaccinated animals. More studies are needed to dissect antigen-derived and ABAs-derived effects in order to ensure safety of vaccines in every genetic background.

INTRODUCTION

ABAs have been used in human and veterinary vaccines (Lindblad, 2004; Plotkin *et al.*, 2018) for more than 70 years, successfully contributing to the control of several infectious diseases. However, precise mechanisms of action of these adjuvants are just partially elucidated. ABAs may exert their immunomodulatory effects via the so-called "depot effect", delaying antigen clearance from the injection site (Glenny, Buttle and Stevens, 1931) and allowing recruitment and maturation of APCs (Lu and HogenEsch, 2013) that enhance their MHC class II molecule expression (Mannhalter *et al.*, 1985; Ulanova *et al.*, 2001; Morefield *et al.*, 2005). The main immunological drawback of ABAs is their weak induction of cell-mediated and cytotoxic T cell responses and the bias to induce IgE-mediated immunity potentially originating allergic reactions to vaccine components (Gupta *et al.*, 1995; Gupta, 1998). ABAs increase T_h2 humoral responses without enhancing T_h1 immunity (Brewer *et al.*, 1996; Sokolovska, Hem and HogenEsch, 2007; Cain *et al.*, 2013), making ABAs less indicated for vaccines against intracellular pathogens (HogenEsch, 2013).

Direct stimulation of the innate immunity has been demonstrated as ABAs induce strong pro-inflammatory stimuli at the injection site, likely mediated by local IL-1β, IL-18 and IL-33 production (Li, Nookala and Re, 2007; Li *et al.*, 2008). The role of NLRP3-dependent inflammasome activation in this scenario remains controversial. While some studies in mice demonstrate inflammasome and IL-1β production after ABAs inoculation as essential to induce efficient immune responses (Eisenbarth *et al.*, 2008; Li *et al.*, 2008; HogenEsch, 2013), other studies have shown that NLRP3 or caspase-1 deficiency have no impact on antibody production (Franchi and Núñez, 2008; Kool *et al.*, 2008) or just partial impact in T-cell differentiation and IgE synthesis (Kool *et al.*, 2008). These differences may be associated to the genetic background of mice strains, the immunization schedule and the type and dose of ABAs employed (Marrack, McKee and Munks, 2009). Interestingly, IL-1β is weakly induced in draining lymph nodes following subcutaneous injection of ABAs (Sagara et al 1990). Unfortunately, injection sites were not analyzed. Therefore, additional studies addressing the signaling pathways of ABAs in different locations are crucial to fully understand mechanisms of action underlying the effects of these adjuvants.

Subcutaneous inoculation of ABAs induces the formation of sterile, long-lasting granulomas at the injection in a variety of animal species (Marcato, 1990; Valtulini *et al.*, 2005; Chamaza, 2012), including humans (Gherardi *et al.*, 2001) and sheep (Asín *et al.*, 2019). Injection-site granulomas and may allow slow antigen release to the regional lymph node, as free particles or transported by APC (HogenEsch, 2013). Indeed, macrophages in granulomas may also translocate ABAs to the regional lymph node in sheep (Asín *et al.*, 2019), where initiation of the immune response occurs with a main role for dendritic cells. During migration, APCs process the antigen and undergo maturation that enables presentation and activation of T cells by providing antigen and costimulatory signals. Immunostimulatory activity of ABAs involve APC maturation by increasing expression of MHC-II, several co-stimulatory molecules and cytokines such as IL-6 (HogenEsch, 2013). This activation helps to establish a good immune response against vaccine antigens, which are poorly immunogenic by themselves.

Transcriptomic studies in ovine PBMCs revealed upregulation of NF-κB signaling pathway when inoculated with ABAs or Al-based vaccines, and the downregulation of cytokine-cytokine receptor pathways when inoculated with ABAs but not with Al-based vaccines. Moreover, NLRP3 activation was shown to be dispensable for immune activation following inoculation with Al-based vaccines (Varela-Martínez *et al.*, 2018). Moreover, this whole transcriptome analysis revealed a strong induction of a series of poorly characterized genes related to pro-inflammatory response that could shed light into ABAs mechanistic pathways in injection-sites and secondary immune organs.

In accordance with the Objective 3 of the PhD Thesis, the aim of this chapter was to explore the specific molecular expression pattern triggered by ABAs in granulomas, regional lymph node and spleen in lambs by measuring mRNA relative expression of pattern recognition receptors (PRR), pro-inflammatory and anti-inflammatory cytokines, IFN pathway, and costimulatory molecules. Results could contribute to increase the knowledge on the immune activation pathways induced by ABAs at the inoculation site but also in distant lymphoid organs.

MATERIAL AND METHODS

Experimental design

Animals analyzed were those of Flock 1 (Global Material and Methods, Experiment 1). Briefly, twenty-one, three-month-old, neutered male lambs were divided into three treatment groups (Vaccine, Adjuvant-only and Control; n=7 each) and housed at the experimental farm of the University of Zaragoza. Vaccine group was inoculated with commercial vaccines; Adjuvant-only group received the equivalent dose of Al-oxyhydroxide (Alhydrogel®, CZ Veterinaria); and Control group was injected with PBS. Details of the vaccines used are described in Table M&M-2.

Animals were subjected to an accelerated vaccination schedule for 15 months. The first and the last injection were applied 475 and 5 days prior to euthanasia, respectively. Animals received 19 subcutaneous inoculations, in an effort to parallel the amount of Al received by sheep during their productive lifespan (an average of seven years). Inoculation schedule is described in Figure M&M-1. Vaccine and Adjuvant-only groups received a total of 81.29 mg of Al.

Tissue sampling and RNA extraction

Euthanasia was performed by intravenous injection of an overdose of barbiturate solution (Dolethal®, Vetoquinol). Tissue sampling was performed in the spleen, regional lymph node (i.e., right prescapular lymph node) and a randomly-selected subcutaneous Al-induced injection-site granuloma. About 0.5 cm^3 of these tissues were collected in guanidinium thiocyanate (TRIzol™ Reagent, Thermofisher) and stored at -80°C until use.

About 10 mg of each tissue sample was immerged in 1mL of TRIzol™ Reagent and homogenized in a Micro-Dismembrator U (Sartorius) using steel beads. RNA extraction was performed following a previously described method (De Pablo-Maiso *et al.*, 2017) and detailed in Appendix Ch3-1. Briefly, RNA and DNA were extracted with chloroform and precipitated with isopropanol. RNA and DNA were treated with TURBO DNase-I (Thermofisher) to remove DNA. Remaining RNA was purified with phenol acid, extracted

with chloroform, precipitated with ethanol, ammonium acetate and glycerol and eluted in dietilpirocarbonate-treated water (DEPC H_20).

RNA quantity and purity were assessed with NanoDrop One/One^c Spectrophotometer (ThermoScientific). Two numeric parameters concerning RNA integrity were estimated (A260/280 and A260/A230 ratio). RNA samples with 260/280 ratio>1.8 were used. Finally, 1000 ng of RNA per sample were retrotranscribed to cDNA with PrimeScript RT Reagent Kit (TAKARA) using oligo-dT primers.

Gene expression quantification

Expression of mRNA of target molecules were analyzed by RT-qPCR in 96-well reaction plates using AriaMx Real-time PCR System (Agilent) under the following conditions: start cycle of 95°C for 3 min, 45 cycles of denaturation at 95°C for 5 sec and annealing at 60°C for 10 sec, and a final melting curve. Each qPCR was performed in a 25 µl final volume reaction using: diluted cDNA samples (1:10), dsDNA-binding dye (SYBR Green Master Mix, BioRad) and forward and reverse primers (Metabion international AG). Appropriate positive (standard) and negative (no template) controls were included. Additionally, 1µL of the original RNA sample was analyzed by qPCR to confirm the absence of DNA after the extraction.

Four housekeeping genes (i.e., β-Actin, GAPDH, G6PDH and YWHAZ) were tested in spleen, lymph node and granuloma. Stability of each housekeeping gene was analyzed using geNormv3-1 and NormFinder software as previously described (Vandesompele *et al.*, 2002; Andersen, Jensen and Ørntoft, 2004). The most stable and robust housekeeping gene was selected in each organ. Targeted genes were PRR involved in recognition of PAMPs (TLR 1-10) and DAMPs (DC-Sign, RIG-I, CD163), pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, CCL2), anti-inflammatory and regulatory cytokines (IL-10, FOXP3), interferon pathway and antiviral proteins (IFN-β, IFN-γ, A3Z1), co-stimulatory molecules for antigen presentation (CD28, CD80, CD86, CTLA4), T cell function (IL-2) and other genes found to be dysregulated in lambs after ABAs inoculation (i.e., EGR2, GPRC, HGF, PLAU, PTX3, ND6, SKAP2) were analyzed. Genes and primers used are detailed in Appendix Ch3-2.

Statistical analysis

Relative quantification of mRNA expression levels (fold-change) were calculated by the $2^{-\Delta\Delta Ct}$ method, which is based on the expression of the target gene in comparison with the expression of the housekeeping gene (ΔCt) and then comparing the cDNA levels found in each treatment group (ΔΔCt) (Livak and Schmittgen, 2001). Fold-change results were standardized by log2 transformation and represented as previously reported (Varela-Martínez *et al.*, 2018).

All statistical analyses were performed using IBM SPSS 19.0 for Windows (IBM Corp). Expression levels of molecules were analyzed by Shapiro-Wilk test to assess normality of data. When data followed a normal distribution, individual comparisons (i.e., Vaccine vs Control, Adjuvant-only vs Control, Vaccine vs Adjuvant-only) were performed via Student's t-test. In non-parametric quantitative variables, individual comparisons were performed with Mann–Whitney U test. Statistical significance was considered when p value < 0.05. Statistical tendency was considered when p value ≤ 0.1.

RESULTS

In a lapse between days to weeks following subcutaneous inoculation of Al-containing products in sheep, sterile granulomas were developed at injection sites. Based on clinical examination and hematological analyses, animals remained healthy along the entire experiment.

mRNA expression in Al-induced injection-site granuloma

Twenty-five molecules were successfully evaluated in granuloma samples (Figure Ch3-1). β-actin was the most stable housekeeping gene. Comparisons with control group were not performed since granulomas were absent and RNA extraction performed in the subcutaneous tissue of these animals were unsuccessful. PRR of PAMPs and DAMPS showed no differences between Adjuvant-only and Vaccine groups, except for a slight decrease in TLR1 in the Vaccine group. Pro-inflammatory cytokine IL-6, but not IL-1β was moderately upregulated in Vaccine group in contrast to Adjuvant-only group. On the other hand, IL-10 levels showed a moderate decrease in Vaccine-group. Interferon expression showed no differences in IFN-β, whereas IFN-γ were under the limit of detection. Molecules related to adaptive immunity showed no differences between Vaccine and Adjuvant-only group. Regarding other genes, a moderate increase was observed in PTX3 in Vaccine group in contrast with Adjuvant-only group.

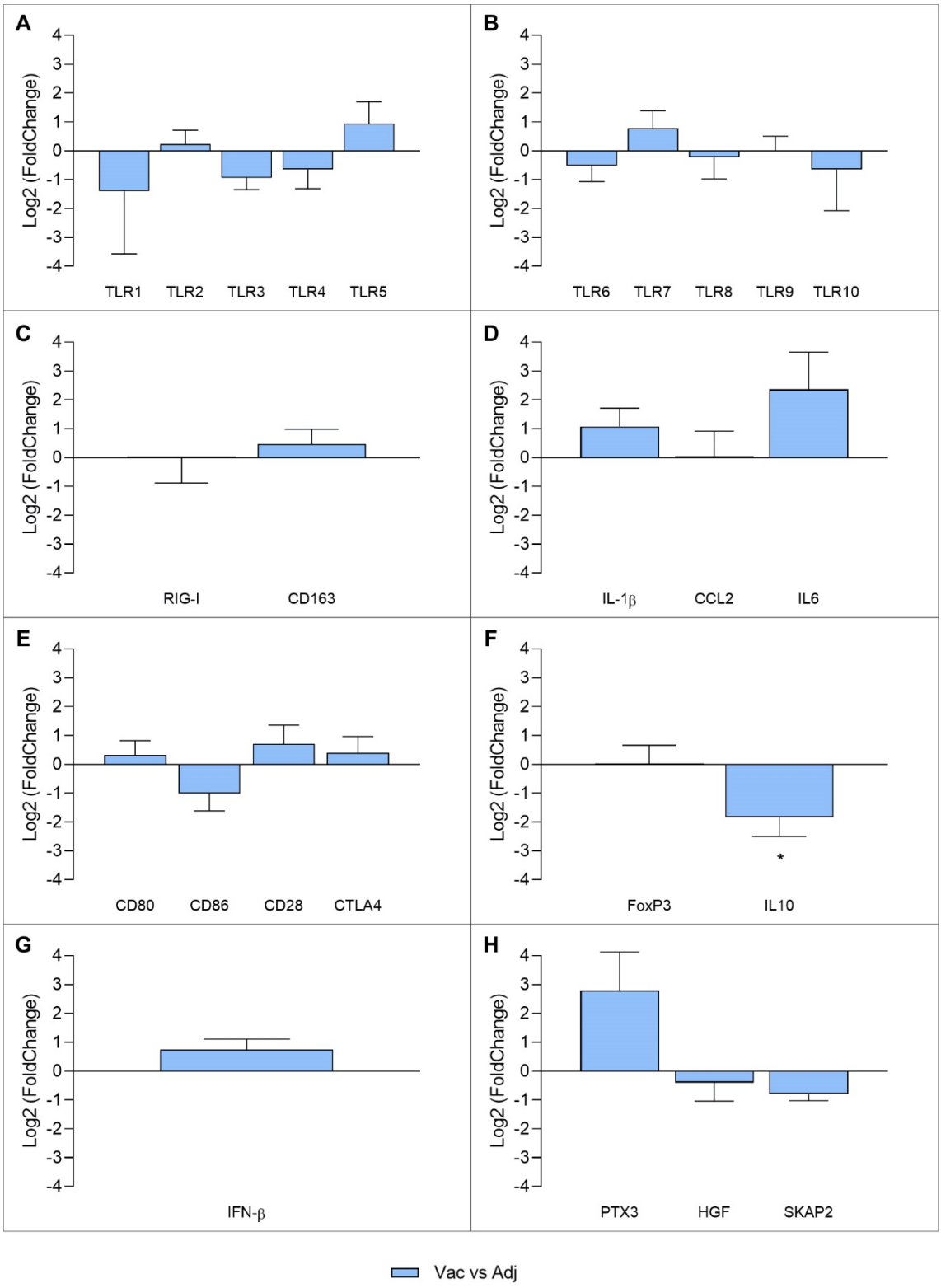

Figure Ch3- 1: Relative expression of mRNA levels of inflammatory and immune signaling pathways in Al-induced injection-site granulomas of animals of the Vaccine and Adjuvant-only groups. **(A-B)** Toll-like receptors. **(C)** Other Pattern recognition receptors. **(D)** Pro-inflammatory cytokines. **(E)** Co-stimulatory molecules for antigen presentation. **(F)** Anti-inflammatory and regulatory cytokines. **(G)** Interferon pathway and antiviral genes. **(H)** Genes overexpressed in previous studies of the same animals.

mRNA expression in regional lymph node

Thirty-four molecules were successfully evaluated in the right prescapular lymph node (Figure Ch3-2), which drained the area where most inoculations were administered and therefore, the granulomas were present. β -actin was the most stable housekeeping gene as in the case of granulomas. TLR4 was significantly upregulated in Vaccine group when compared with Adjuvant-only and Control groups, whereas TLR6 was overexpressed in both, Adjuvant-only and Vaccine group (Figure Ch3-2). Among pro-inflammatory cytokines, a significant increase of TNF-α in Vaccine group and a significant decreased IFN-γ in Adjuvant-only group were observed. Molecules related to adaptive immunity, showed non-significant downregulation of IL-2, mainly evinced in Vaccine group, and no differences in co-stimulatory molecules. Other genes as EGR2, GPRC, HGF and SKAP2 were moderately to markedly downregulated in Vaccine group but also in Adjuvant-only group.

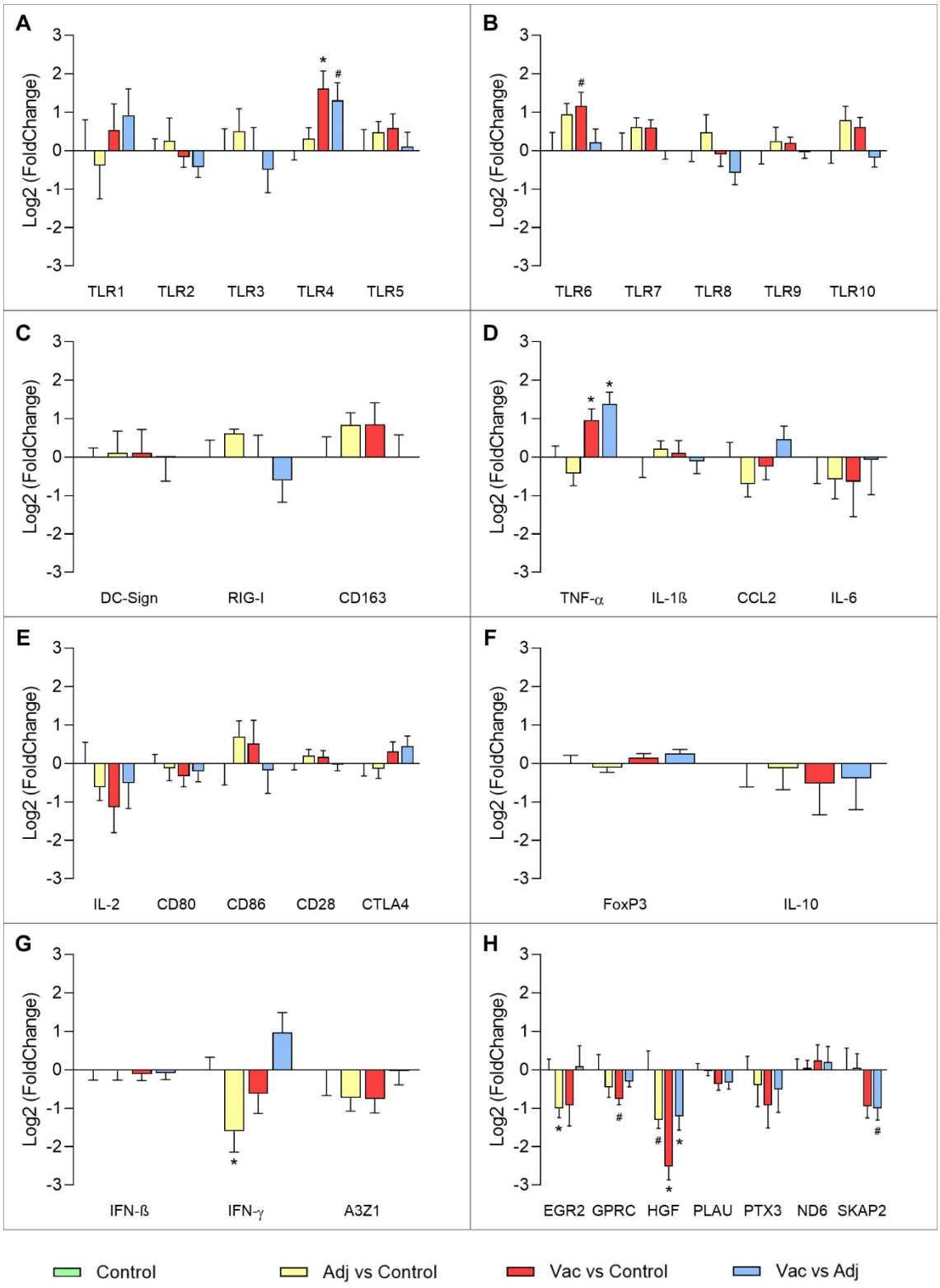

Figure Ch3- 2: Relative expression of mRNA levels of inflammatory and immune signaling pathways in the regional lymph node of animals of the Vaccine, Adjuvant-only and Control groups. **(A-B)** Toll-like receptors. **(C)** Other Pattern recognition receptors. **(D)** Pro-inflammatory cytokines. **(E)** Co-stimulatory molecules for antigen presentation. **(F)** Anti-inflammatory and regulatory cytokines. **(G)** Interferon pathway and antiviral genes. **(H)** Genes overexpressed in previous studies of the same animals. *p<0.05. $^{\#}p$<0.1.

mRNA expression in spleen

Twenty-eight molecules were successfully evaluated in the spleen (Figure Ch3-3). GAPDH was the most stable housekeeping gene in this case. TLR3 was highly increased in Vaccine and Adjuvant-only groups while TLR6 and TLR7 were significantly overexpressed in Vaccine group. Pro-inflammatory cytokines showed significant upregulation of the chemokine CCL2 in Adjuvant-only group, whereas this increase was not evident in Vaccine group. IL-6 was significantly reduced in Adjuvant-only but not in Vaccine group. Interferon pathway was upregulated in Vaccine and Adjuvant-only groups with increased expression of IFN-γ levels and genes encoding antiviral proteins such as A3Z1. CD80 and CTLA-4 molecules, involved in antigen presentation were significantly downregulated in the Adjuvant-only group but increased in Vaccine-group. Moderate upregulation of HGF was observed in Adjuvant-only group.

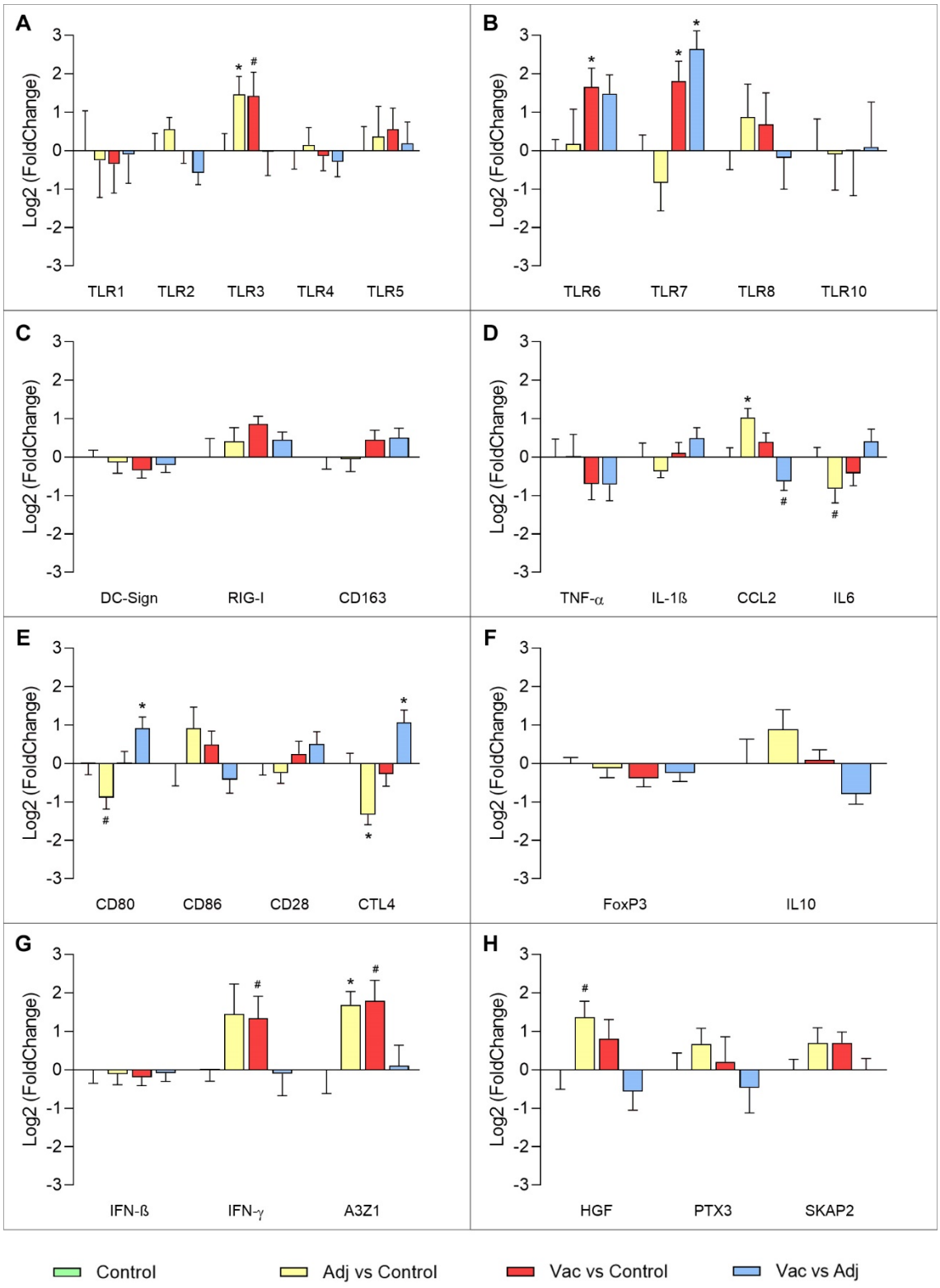

Figure Ch3-3: Relative expression of mRNA levels of inflammatory and immune signaling pathways in the spleen of animals of the Vaccine, Adjuvant-only and Control groups. **(A-B)** Toll-like receptors. **(C)** Other Pattern recognition receptors. **(D)** Pro-inflammatory cytokines. **(E)** Co-stimulatory molecules for antigen presentation. **(F)** Anti-inflammatory and regulatory cytokines. **(G)** Interferon pathway and antiviral genes. **(H)** Genes overexpressed in previous studies of the same animals. *p<0.05. #*p*<0.1.

DISCUSSION

This chapter analyzes the *in vivo* mechanism of action of ABAs by deciphering immune stimuli triggered after repetitive inoculation with ABAs and Al-containing vaccines in lambs. Despite intense research during the last decade on immune mediators induced by ABAs, the mechanisms exerted by ABAs at the injection-site and the signaling pathways induced at secondary immune organs, remain unclear and deeply unexplored. Activation of macrophages and DCs by engaging DAMPs as urate crystals is a widely accepted mechanism for Al-induced injection-site inflammatory response; however the down-streaming events are still to be elucidated (Shardlow, Mold and Exley, 2018).

Granulomas analyzed in the present experiment had a chronicity of at least 95 days, although persistence of up to 15-month has been described in sheep (Asín *et al.*, 2019). The role of this local inflammatory reaction in animals and humans is highly controversial. ABAs may ensure slow antigen release and constant immune activation by recruiting APCs to the injection site. Some studies highlight the key role of these chronic inflammatory reactions in the strong antibody responses elicited by Al-based vaccines (Marrack, McKee and Munks, 2009). In guinea pigs, injection-site nodules excised 7 weeks after vaccination can be employed to efficiently immunize a second guinea pig (Harrison, 1935). Thus as initially stated by Glenny, depot effect by which ABAs allow slow antigen delivery and continued immune activation could rely on granuloma formation (Glenny, Buttle and Stevens, 1931). However, other studies performed in rabbits indicate that only marginal amounts of antigen are present at the site of vaccination after two weeks (White, Coons and Connolly, 1955). In this sense, some authors showed that complete removal of the injection site shortly after inoculation has no effect in the immune response elicited in mice (Hutchison *et al.*, 2012).

In this study, injection-site granulomas showed similar expression of PAMPs and DAMPs receptors (i.e., TLR, RIG-I and CD163), interferon pathway and molecules associated with adaptive immunity in Vaccine and Adjuvant-only groups. These findings support the hypothesis that Al-induced granulomas exert no long-term immunological effect after inoculation. In this sense, chronic immune activation without the appropriate regulation

mechanisms may establish a persistent deleterious pro-inflammatory status in long-lasting granulomas (Kumar, Abbas and Aster, 2021). IL6 but not IL-1β showed a slight increase in Vaccine group. IL-6 expression is likely driven by TLR arrangement induced by vaccine antigen since TLR engagement by ABAs is unlikely (HogenEsch, 2002; Tanaka, Narazaki and Kishimoto, 2014). Accordingly, the anti-inflammatory cytokine IL-10 was downregulated in animals of the Vaccine group. Both, IL-6 increase and IL-10 decrease correlate with the higher histopathological severity degree of granulomas previously found in vaccinated animals (Asín *et al.*, 2019). Additionally, pentraxin3 (PTX3) a protein produced in response to primary pro-inflammatory stimuli and involved in regulation of inflammation and tissue remodeling (Doni *et al.*, 2019) was upregulated in Vaccine-group paralleling the results obtained in PBMC from vaccinated animals (Varela-Martínez *et al.*, 2018).

Secondary lymphoid organs (i.e., regional lymph node and spleen) evinced a more active immune response. Draining lymph node showed a moderate pro-inflammatory profile in Vaccine group. TLR4 and TLR6 and downstream cytokines such as TNF-α were elevated in vaccinated animals. Accordingly, previous transcriptomic studies reported higher TLR4 levels in PBMCs from vaccinated animals and a mild decrease in animals from Adjuvant-only group (Varela-Martínez *et al.*, 2018). Adjuvant-only group showed marked downregulation of IFN-γ expression. Interestingly, TLR activation in Vaccine group seems to have partially counterbalanced the depressor effect of ABAs in the interferon pathway as lesser decrease in IFN-γ levels were observed in Vaccine-group compared to Adjuvant-only group. Moreover, these results align with the T_h2-biased response of ABAs and the detrimental effect over T_h1 responses with decreased IFN-γ production (Brewer *et al.*, 1999). Indeed, Al translocation and marked accumulation in the regional lymph node of these animals has been demonstrated in previous studies of our group (Asín *et al.*, 2019). *In vitro* studies point towards the inhibition of the T_h1 responses via an IL-10 dependent mechanism (Oleszycka *et al.*, 2018), which was elevated in injection-site granulomas. Moderate decrease in IL-2 and slight differences in co-stimulatory molecules also point towards the absence of an efficient adaptive immune response in the regional lymph node. Marked downregulation of EGR2, HGF, GPRC and SKAP2 were recorded in both, Vaccine and Adjuvant-only groups, paralleling the results obtained in PBMCs from animals of Adjuvant-only group, with the exception of GPRC. These genes have been linked to innate

responses in human and mice but their significance is still unclear in the ovine species, although they seem to play a role in ABAs-induced pathways (Varela-Martínez *et al.*, 2018).

Spleen showed the most activated pattern of innate and adaptive immune system molecules among tissues studied. Differences observed between animals treated with ABAs and Al-based vaccines exemplifies the role of vaccine antigens in distant immune organs. Higher expression of TLR3, TLR6 and TLR7 in Vaccine group suggest their upregulation in response to an increase engagement with vaccine antigens. Indeed, TLR3 and TLR7 are described to engage different RNA species, including RNA from damaged cells, while TLR6 is focused on DNA and modified peptides present on Gram+ bacteria (Kawasaki and Kawai, 2014). TLR engagement naturally stimulate innate immune responses, likely reflected in our study by increased IFN-γ. Interferon stimulated genes (ISGs) as A3Z1 are induced by IFN-γ with different antiviral and pro-inflammatory activities (De Pablo-Maiso *et al.*, 2017; Kumar, Abbas and Aster, 2021). Accordingly, A3Z1 expression in Vaccine group was increased. Strikingly, Adjuvant and Vaccine animals showed similar induction of IFN- γ and A3Z1, suggesting the activation of ISGs pathway in distant immune organs in which low levels of Al are expected to be found (Khan *et al.*, 2013). Analytical measurements of Al levels in the spleen could help to clarify this point.

Additionally, spleen evidenced lower costimulatory molecules expression in Adjuvant-only group, mainly CD80 and CTLA4, which are associated with T-cell activation and deactivation, respectively (Kumar, Abbas and Aster, 2021). The low expression of CD80 was also found in PBMCs in animals inoculated with ABAs and point towards the immunomodulatory effect of ABAs in T-cell function (Varela-Martínez *et al.*, 2018). In this sense, marked differences between Vaccine and Adjuvant-only groups in co-stimulatory molecules illustrate the effect of antigens present in the inoculum, suggesting efficient antigen presentation in vaccinated animals. Interestingly, Adjuvant group showed an increased TLR3 expression which was not followed by expression of pro-inflammatory cytokines except for an increase in the chemokine CCL2 in Adjuvant-only group. CCL2 expression has been previously related to Al inoculation in mice and it plays an essential role in Al translocation to distant tissues, including CNS (Khan *et al.*, 2013). Whether

Al-loaded macrophages contribute to this activation profile by reaching the spleen, as they do in CNS, is currently unknown.

Immune activation found in lymph node and spleen can be the result of both, the chronic effect elicited by cumulative inoculations over 15 months of experiment or the acute effect of the last vaccine, which was applied 5 days before the euthanasia. In any case, these results evinced the effective immunization provided by Al-containing inoculations, being limited in the regional lymph node but optimal in the spleen, mainly in vaccinated animals. However, we failed to detect an efficient immune response in Al-induced granulomas although the unavailability of an equivalent tissue in control animals clearly hampered further comparisons and statistical analyses. However, since the time of sampling was not necessarily matched with the time of immune activation in these granulomas, faint immune stimulation reflected by IL-6 and IL-10 expression profiles may suggest a sort of smoking gun in terms of acute inflammation.

From an immunological point of view, pro-inflammatory mediators as IL-1β, CCL2, CCL11, histamine and IL-5 are released at the injection site shortly after inoculation of ABAs (Sharp *et al.*, 2009) leading to recruitment of innate immune cells as neutrophils, eosinophils, immature dendritic cells, monocytes and macrophages (McKee *et al.*, 2009). Phagocytosis is enhanced after monocyte arrival, and differentiation into macrophages is massive after 7 days and nearly absent 3 weeks after immunization (McKee *et al.*, 2009; Lu and HogenEsch, 2013). In the present experiment, granulomas were obtained at a later stage due to the experimental design, highly reducing the chances to detect these short-time immune effects. However, IL-10 reduction found in this study and previous results on PBMC (Varela-Martínez *et al.*, 2018) argue in favor of a non-specific chronic active profile in persistent granulomas induced by ABAs. Further characterization of the local immune response induced in granulomas will contribute to further understand the immunological mechanisms driving initial immune activation, dissecting antigen from adjuvants effects.

CONCLUSIONS

This work summarizes the mRNA expression of genes involved in PAMPs and DAMPs recognition, pro-inflammatory and anti-inflammatory responses, innate and adaptive signaling pathways in lambs subjected to repetitive inoculations with saline solution (Control group), Al-oxyhydroxide adjuvants (Adjuvant-only group) or Al-oxyhydroxide-based vaccines (Vaccine group). Immune responses elicited by ABAs differ in secondary immune organs, with moderate pro-inflammatory signals in regional lymph node and moderate activation of interferon cascade and adaptive immunity pathways in the spleen. Al-induced injection-site granulomas showed a pro-inflammatory environment without stimuli of adaptive immunity pathways.

APPENDICES

- **Appendix Ch3-1:** RNA extraction method from homogenized tissue in TRIzol Reagent.
- **Appendix Ch3-2:** Primers used for quantification of gene expression via RealTime-PCR (qPCR)

Appendix Ch3-1: RNA extraction method from homogenized tissue in TRIzol Reagent.

Reagents:

- DEPC water (at room temperature)
- Acid Phenol (at 4ºC)
- TRIzol (at 4ºC)
- Chloroform (at 4ºC)
- Isopropanol (at room temperature)
- Ethanol 75% (at 4ºC)
- Ethanol 100% (at 4ºC)
- Ammonium acetate 5M (at 4ºC)
- DNase
- RNAzap

Recommendations:

- All reagents are exclusively used for RNA extractions.
- Do not introduce a pipette inside stock bottles. Use always aliquots.
- Clean all pipettes and surfaces with RNAzap
- Always wear gloves.
- Use filtered tips.
- Use RNAse free tubes (Do not need to be autoclaved).

Method:

0. Before starting, precool the centrifuge at 4ºC.
1. Add 100-200uL of PBS per 1ml TRIzol (containing the homogenized tissue). Shake tube gently by hand for 15 sec.
2. Incubate for 5 min at room temperature.
3. Add 100µl of chloroform per 1ml TRIzol used. Shake tube gently by hand for 15 sec.
4. Incubate for 3 min at room temperature.
5. Centrifuge the sample for 10 min at 14.000rpm at 4ºC. Meanwhile, prepare 200µl of chloroform in tubes.
6. Recover the aquose phase (upper phase) and place into the tube containing chloroform. Mix gently by hand for 15seg.
7. Incubate for 5 min at room temperature.
8. Centrifuge the sample for 5 min at 14.000rpm at 4ºC. Meanwhile, prepare 500µl of 100% isopropanol in tubes.
9. Recover the aquose phase and place into the tube containing isopropanol. Mix by inversion.

10. Incubate at room temperature for 15 min.
11. Centrifuge for 15 min at 14.000 rpm at 4ºC.
12. Remove the supernatant leaving only the DNA-RNA pellet. Do not overdry the pellet because then it is difficult to dissolve.
13. Dissolve DNA-RNA pellet in 85µl of DEPC water.
14. Stop point. Store the dissolved DNA-RNA at -80ºC.
15. Turn on Termoblock at 37ºC. Prepare DNAse I mix: 10µl Buffer 10x + 5µl DNAse I (TURBO).
16. Add 15µl of DNAse I mix to 85 µl RNA
17. Incubate 40 min at 37ºC in Termoblock.
18. Add 200 µl of DEPC water.
19. Add 300µl of acid phenol (take lower phase in acid phenol bottle) to RNA tubes.
20. Mix by vortex.
21. Incubate at room temperature for 15 min.
22. Centrifuge 10 min at 14.000 rpm at 4ºC. Meanwhile, prepare 300µl of chloroform in tubes.
23. Recover the aquose phase (upper phase) and place into the tube containing chloroform. Mix gently by hand for 15 seg.
24. Centrifuge 15 min at 14.000 rpm at 4ºC. Meanwhile, prepare 200µl of Ammonium acetate 5M, 1ml of ethanol 100% and 4µL Glycogen.
25. Recover the aquose phase (upper phase) and place into the tube containing Ammonium acetate 5M, 1ml of ethanol 100% and 4µL Glycogen.
26. Incubate at -20ºC for at least 30 min.
27. Centrifuge 20 min at 14000 rpm at 4ºC.
28. Remove the supernatant leaving only the RNA pellet.
29. Wash the pellet with 1000µl of ethanol 75%
30. Incubate at room temperature for 3 min.
31. Centrifuge 10 min at 4ºC at 14000 rpm at 4ºC. Use more time if pellet do not stuck at the bottom.
32. Remove supernatant leaving only the RNA pellet. Do not overdry the pellet.
33. Dissolve RNA in 25µl of DEPC water.
34. Conserve RNAs stocks at -80ºC.

Appendix Ch3-2: Primers used for quantification of gene expression via RealTime-PCR (qPCR)

Gene (mRNA)	Name	Sequence	Pos.	Amplicon size
B-Actin	b-actine Fw	CTCACGGAGCGTGGCTACA	Fw	107
	b-actine Rv	GCCATCTCCTGCTCGAAGTC	Rv	
GAPDH	GAPDHFw	CTTGGCAGGTTTCTCCAGG	Fw	187
	GAPDHRv	CGGGAAGCTGTGGCGTGATG	Rv	
G6PDH	G6PDHFw	TGACCTATGGCAACCGATACAA	Fw	76
	G6PDHRv	CCGCAAAAGACATCCAGGAT	Rv	
YWHAZ	YWHAZFw	TGTAGGAGCCCGTAGGTCATCT	Fw	102
	YWHAZRv	TTCTCTCTGTATTCTCGAGCAATCT	Rv	
TLR1	TLR1qPCRFw	CCCACAGGAAAGAAATTCCA	Fw	208
	TLR1qPCRRv	GGAGGATCGTGATGAAGGAA	Rv	
TLR2	TLR2qPCRFw	ACGACGCCTTTGTGTCCTAC	Fw	192
	TLR2qPCRRv	CCGAAAGCACAAAGATGGTT	Rv	
TLR3	TLR3qPCRFw	GAGGCAGGTGTCCTTGAACT	Fw	329
	TLR3qPCRRv	GCTGAATTTCTGGACCCAAG	Rv	
TLR4	Ov qTLR4 Fw	TGGATTTATCCAGATGCGAAA	Fw	152
	Ov qTLR4 Rv	GGCCACCAGCTTCTGTAAAC	Rv	
TLR5	TLR5qPCRFw	AAAACCACATCGCCAACATC	Fw	191
	TLR5qPCRRv	CATCAGATGGAACTGGGACA	Rv	
TLR6	TLR6qPCRFw	CAAAGCAGGGAACAATCCAT	Fw	206
	TLR6qPCRRv	CCACAATGGTGACAATCAGC	Rv	
TLR7	TLR7qPCRFw	ACTCCTTGGGGCTAGATGGT	Fw	180
	TLR7qPCRRv	GCTGGAGAGATGCCTGCTAT	Rv	
TLR8	TLR8qPCRFw	TCCACATCCCAGACTTTCTACGA	Fw	150
	TLR8qPCRRv	GGTCCCAATCCCTTTCCTCTA	Rv	
TLR9	TLR9qPCRFw	CTCGTATCCCTGTCGCTGAG	Fw	210
	TLR9qPCRRv	CACCTCCGTGAGGTTGTTGT	Rv	
TLR10	TLR10qPCRFw	TCTGCCTGGGTGAAGTATGA	Fw	190
	TLR10qPCRRv	AATGGCACCATTCAGTCTGG	Rv	
DC-SIGN	QDC Fw	GGTTCCGGAGTCTGACTGAAG	Fw	74
	QDC 74 Rv	GGTCAGGCGCTGTAGGATCTC	Rv	
RIG-I	qRIG-I Fw	GCTGACGGCCTCAGTTGGT	Fw	84
	qRIG-I Rv	TCGAGAGAAGCACACAGTCTGC	Rv	
CD163	CD163 qFw	GGCAGTGCAGACATCACGAAT	Fw	63
	CD163qRv	TCACACCAGCGTCTTCGTTATG	Rv	
TNF-α	TNF-α Fw	GGTGCCTCAGCCTCTTCTC	Fw	135
	TNF-α Rv	GAACCAGAGGCCTGTTGAAG	Rv	

IL-1β	Ov qIL-1β Fw	GAAGCTGAGGAGCCGTGCCTACGAACA	Fw	185
	Ov qIL-1β Rv	CCAGCACCAGGGATTTTTGCTCTCTGTCC	Rv	
CCL2	CCL2Fw	GCTGTGATTTTCAAGACCATCCT	Fw	72
	CCL2Rv	GGCGTCCTGGACCCATTT	Rv	
IL-6	IL6 Fw	ACCTGGACTTCCTCCAGAAC	Fw	162
	IL6 Rv	TTGAGGACTGCATCTTCTCC	Rv	
CD80	CD80 Fw	CTGTGATTACAACACGACCACTGA	Fw	129
	CD80 Rv	ATGGTGCGGTTCTCGTATTCA	Rv	
CD86	CD86 183F	GCCAAGAGAAGCCCACTAACG	Fw	149
	CD86 331R	CAGTCCTTGGGACCTTCTATGATG	Rv	
CD28	CD28 Fw2	AACAGGATGCATCAGAGCGAGTA	Fw	84
	CD28 Rv 2	TGGCGCATAGGGCTGGTA	Rv	
CTLA4	CTLA4 Fw2	CCATGGACACTGGGCTCTATGT	Fw	121
	CTLA4 Rv2	TCAGAATCCGGGCATGGTT	Rv	
FoxP3	QFP3 Fw	AAGCACTGCCAGGCAGACC	Fw	61
	QFP3 Rv	TCTGGAGCAGACACTGCGC	Rv	
IL-10	IL-10 FW	CGGCGCTGTCATCGTTTT	Fw	83
	IL-10 RV	TCTTGGAGCATATTGAAGACTCTCTTC	Rv	
IFN-β	IFN-b Fw qPCR	GATGCCGTATTGGTCATGTA	Fw	126
	IFN-b Rv qPCR	CATCTGCCCATAGAGTTCCT	Rv	
IFN-γ	IFN Fw	GAACGGCAGCTCTGAGAAAC	Fw	203
	IFN Rv	GCAGGCAGGAGAACCATTAC	Rv	
A3Z1	A3Z1 qFw	TCCGTTCTTGGAATCTGGAC	Fw	151
	A3Z1 qRv	GTATAGATGCGGGAGGCAAA	Rv	
EGR2	Ov qEGR2 FW	CACGTCGGTGACCATTT	Fw	86
	Ov qEGR2 RV	TGTTGATCATGCCATCTCC	Rv	
GPRC	Ov qGPRC5C FW	AGTGCCAACTCCACCCT	Fw	86
	Ov qGPRC5C RV	GGGACTGAGCCTTCCTTG	Rv	
HGF	Ov qHGF FW	TCAAGTGCAAGGACCTAAGA	Fw	93
	Ov qHGF RV	CAACTCGGATGTTTGGATCAG	Rv	
PLAU	Ov qPLAU Fw	CCAGGAGTGCATGGTGCAG	Fw	143
	Ov qPLAU Rv	GCTGGCTCTCGATGGTGGT	Rv	
PTX3	Ov qPTX3 Fw	AGCAATGCATATCTCTGTGATTCTGT	Fw	99
	Ov qPTX3 Rv	TTGTCCAAATTCACATACATGAGCT	Rv	
ND6	Ov qND6 Fw	AGCGAGGAGGCTATGGGAAT	Fw	101
	Ov qND6 Rv	TCCATAATCACTACAACACCAATCAAT	Rv	
SKAP2	Ov qSKAP2 FW	ACCACACCACAGGAGATAAA	Fw	90
	Ov qSKAP2 RV	ATGACAGTTCATCAGAAAGAGC	Rv	

CHAPTER 4

Aluminum-induced granulomas impact on serological response and virus kinetics in sheep naturally-infected by small ruminant lentiviruses

ABSTRACT

SRLV are retroviruses with a defined tropism for the mononuclear-phagocytic system. Al-oxyhydroxide is the most commonly used adjuvant in veterinary medicine and induce the formation of injection-site granulomas with abundant macrophages. The aim of this chapter was to study the effect of Al-induced post-vaccination granulomas on SRLV pathogenesis. In a first step, male lambs were divided in three treatment groups (Vaccine, Adjuvant-only and Control) of 13 animals each and raised in SRLV-positive commercial sheep farms. Animals were clinically monitored along the study and molecular, serological and pathological studies were performed. New seropositive animals were more abundant in lambs from Vaccine and Adjuvant-only groups compared to Control group. Secondly, adult female sheep naturally infected by SRLV and affected by bilateral arthritis were divided in four treatment groups (Vaccine, Vaccine-extra, Adjuvant-only and Control). In arthritic sheep, radiographic and thermographic analyses showed an accelerated, significant progression of articular lesions in Vaccine and Adjuvant-only groups. These animals presented injection-site granulomas that evinced granular, intracytoplasmic SRLV labelling in macrophages. These SRLV-containing granulomas are likely responsible of the increased antibody titers in Vaccine, Vaccine-extra and Adjuvant-only groups along the experiment. SRLV viral DNA copies markedly increased in blood of Control animals, whereas Vaccine, Vaccine-extra and Adjuvant-only groups showed a significant decrease. In conclusion, serological response as well as virological and pathological features may be affected in animals inoculated with ABAs, underscoring the importance of Al-induced granulomas in the pathogenesis of macrophage-tropic viruses.

INTRODUCTION

SRLV cause highly prevalent chronic infections in sheep and goats. SRLV are linked to important deleterious effects on animal production as a direct consequence of the disease in the lung, brain, mammary gland and joints (Minguijón *et al.*, 2015). Additionally, production losses have been also described in asymptomatic animals (Dohoo *et al.*, 1987; Martínez-Navalón *et al.*, 2013; Echeverría *et al.*, 2020). SRLV are retroviruses with a defined tropism for the mononuclear-phagocytic system. Interestingly, SRLV infection is highly productive in M2-like and tissue differentiated macrophages, but restricted in monocytes and classically activated M1-macrophages (Crespo *et al.*, 2011; Blacklaws, 2012). Four main genotypes (A, B, C and E) and more than 35 subgroups with significant antigenic heterogeneity have been already characterized within SRLV (Michiels, Adjadj and De Regge, 2020). In the articular form, the main clinical sign is unilateral or bilateral carpal arthritis which in sheep it is mainly caused by genotype B2 strains (Glaria *et al.*, 2009; Pérez *et al.*, 2015).

Vaccines are key players in controlling infectious diseases from animals and humans (Canouï and Launay, 2019). Most ovine vaccines are based on inactivated pathogens or recombinant proteins that are often poorly immunogenic as they need adjuvants to strengthen the immune response (Lacasta *et al.*, 2015; Apostólico *et al.*, 2016; Bastola *et al.*, 2017). Aluminum-oxyhydroxide is the most widespread adjuvant in veterinary medicine. In sheep, the development of injection-site reactions consisting on long-lasting subcutaneous granulomas is a constant response after the inoculation of ABAs either alone or in a vaccine (Asín *et al.*, 2019). These granulomas consist on abundant Al-loaded activated macrophages and multinucleated giant cells admixed with variable amounts of lymphocytes and plasma cells, usually surrounded by fibrous connective tissue (Asín *et al.*, 2019).

In the absence of effective vaccines, SRLV and other virus infections are only controlled through early diagnosis, specific isolation measures and removal of seropositive animals from the flock (Minguijón *et al.*, 2015). SRLV infection is mainly revealed by

serological tests although escape mutants, antigenic heterogeneity among subtypes and delayed seroconversion jeopardize sensitive identification of infected animals.

Al-induced granulomas could be a new niche for SRLV replication, which may favor antigenic exposure to the immune system, thereby modifying viral pathogenesis and immunological host responses. In accordance with the Objective 4 of the PhD Thesis, the aim of this chapter was to study the effect of Al-induced post-vaccination granulomas on SRLV pathogenesis.

EXPERIMENT 1:

EVOLUTION OF SRLV ANTIBODY TITERS IN LAMBS RAISED IN COMMERCIAL LAMBS *IN FIELD* CONDITIONS

MATERIAL AND METHODS

This experiment was initially designed to assess effects on animal production after intense vaccination in commercial lambs. However, it also provided interesting data on the influence of Al-induced subcutaneous granulomas on *in field* natural SRLV infection in the same lambs.

Three-month-old neutered male lambs were born and raised in two independent commercial sheep farms (Flock 2 and Flock 4; described in Global material and methods, Experiment 1) and subjected to an intensive inoculation schedule along 15 months. Animals were divided in three treatment groups (Vaccine, Adjuvant-only and Control) in each Flock. Vaccine groups were inoculated with commercial vaccines, Adjuvant-only groups received Al-oxyhydroxide (Alhydrogel ®, CZ Veterinaria) and Control groups were injected with PBS. In total, each treatment group consisted on 13 lambs. In each commercial sheep farm, there were the same number of animals of each treatment group.

In vivo studies

All lambs were examined before the experiment to assess their optimal health status. For the purpose of this Experiment, blood samples taken at 0 and 454±8 dpi were used. Samples were obtained using 6 mL EDTA tubes (BD Vacutainer®) for cell blood count and PBMCs isolation and 6 mL Clot Activator tubes (BD Vacutainer®) for serological analysis. Whole blood was centrifuged and 2 mL of serum were stored at -20°C for further antibody analysis by different ELISA tests. PBMCs were isolated on a Ficoll-Hypaque gradient (δ=1.077; Lymphoprep Axis-Shield).

Virus and antibody quantification in blood

Blood serum was screened against SRLV antibodies using two commercially available ELISA kits, to obtain qualitative results: Elitest-MVV (Hyphen-Biomed, France) and Eradikit™ SRLV Screening kit (IN3 diagnostic, Italy). Elitest-MVV is based on p25 recombinant protein and a transmembrane synthetic peptide both derived from genotype A (Saman *et al.*, 1999) whereas Eradikit™ SRLV Screening kit is based on a mixture of Gag and Env antigens belonging to SRLV viral genotypes A, B and E (Reina *et al.*, 2009). Moreover, to quantify antibody titers, a home-made peptide ELISA was performed at the beginning and the end of the experiment using previously described synthetic peptides. As the SRLV genetic diversity was expected to be high, a combination of 98M (VDMPQSYIEKQKRNK), 126M1 (ELDCWHYHQYCVTST) and 126M2 (ELDCWHYQHYCVTS) peptides was chosen to cover both, genotype A and B SRLV strains as previously described (Sanjosé *et al.*, 2015). Peptides were obtained from Thermo Scientific, diluted in carbonate buffer (pH 9.6) at 1 mg/mL and stored at -20°C until use. Briefly, 96-well microplates were coated with a mixture of the three synthetic peptides (75 ng of 98M, 75 ng of 126M1 and 150ng of 126M2). Plates were dried overnight at 37°C and then were washed with PBS supplemented with 0.1% Tween® 20 and blocked with bovine casein 2.5% (Sigma-Aldrich) for 1 h at 37°C. Plasma samples were diluted 1:20, 1:40 and 1:80 in dilution buffer (PBS containing 1.25% bovine casein) and plates were incubated for 1 h at 37°C. Peroxidase-conjugated anti-ruminant IgG (Sigma) was added 1:8000 in dilution buffer, and plates incubated for 1 h at 37°C. One hundred microliters per well of 3,3',5,5'-

Tetramethylbenzidine (TMB, BD Biosciences) were added as the substrate and the optical density measured at 450 nm after 30 min.

Genomic DNA from PBMCs was extracted by using E.Z.N.A Blood DNA Mini Kit (Omega bio-tek). Proviral load quantification was performed by qPCR using primers and fluorogenic probes specifically designed for strain Ov496 (representative of genotype B2) [40] and strain Ev1 (representative of the genotype A) as well as a SybrGreen-based qPCR using primers previously described (Appendix Ch4-1) (González *et al.*, 2005).

Statistical analyses

Assessment of the association between groups in qualitative variables (i.e., number of new seropositive animals), was carried out using Likelihood ratio test.

Quantitative variables (i.e. antibody titers) were represented in two complementary ways: first as mean values with standard error means; second as percentage of increase (Δ) between the end of the experiment and the beginning of the experiment.

Quantitative variables (i.e. antibody titers) were tested for normality distribution of data by Shapiro-Wilk´s test. Unpaired comparisons between groups were performed at each sampling date by using: ANOVA test followed by Duncan's multiple range test as a *post-hoc* as they were normally distributed.

RESULTS

Virus and antibody quantification

Results from both commercial serological tests and flocks showed differences between groups. At the end of the experiment, Flock 2 showed higher number of SRLV seropositive animals in the Vaccine group (4 out of 7), and Adjuvant-only group (3 out of 7) than in the Control group (1 out of 7) as detailed in Table Ch4-1. Flock 4 showed fewer differences between Vaccine (2 out of 6), Adjuvant-only (1 out of 6) and Control groups (1 out of 6). Differences in the number of new seropositive animals between groups showed a statistical tendency in Flock 2 but they were statistically non-significant in Flock 4 or all

animals together (Table Ch4-1). When both Flocks were analyzed together, a non-statically significant increase was observed in the number of new seropositive animals in the Vaccine group, when comparing with the Adjuvant-only and Control groups.

Table Ch4-1: Serological SRLV results obtained with commercial ELISA tests in lambs from Vaccine, Adjuvant-only and Control groups included in Flock 2 and Flock 4. Positive animals at the beginning (0 days after the first inoculation, dpi) and the end (454±8 dpi) of the experiment and new seropositive animals along the experiment.

	GROUP	ELISA (Positive Animals / Total Animals)				New Seropositive Animals (Positive Animals at 454±8dpi / Negative Animals at 0dpi)		
		0 dpi		454±8 dpi				
		N	%	N	%	N	%	*p*-value
Flock 2	Vaccine	1/7	14%	4/7	57%	3/6	50%	$0.098^{LR\#}$
	Adjuvant-only	1/7	14%	3/7	43%	2/6	33%	
	Control	2/7*	29%	1/7	14%	0/5	0%	
Flock 4	Vaccine	1/6	17%	2/6	33%	1/5	20%	0.504^{LR}
	Adjuvant-only	2/6‡	33%	1/6	17%	0/4	0%	
	Control	0/6	0%	1/6	17%	1/6	17%	
Both Flocks	Vaccine	2/13	15%	6/13	46%	4/11	36%	0.247^{LR}
	Adjuvant-only	3/13‡	23%	4/13	31%	2/10	20%	
	Control	2/13*	15%	2/13	15%	1/11	9%	

*One animal of the Control group was positive at 0dpi and seronegative at 454±8 dpi (Presumed colostral antibodies)

‡One animal of the Adjuvant-only group was positive at 0dpi and seronegative at 454±8 dpi (Presumed colostral antibodies)

LR Likelihood ratio test

# $p<0.1$

Home-made ELISA results paralleled those obtained with commercial ELISAs. At the beginning of the experiment, mean absorbance was low in the three treatment groups. At the end of the experiment, Vaccine group and Adjuvant-only group showed higher mean absorbance compared to Control group (Figure Ch4-1A). This difference was higher in Vaccine-group than in Adjuvant-only group. The reactivity increase was moderate in Vaccine group, mild in Adjuvant-only group and minimal to absent in the Control group (Figure Ch4-1B).However, none of these differences were statistically significant (p=0.541). Molecular analysis of the viral load was not possible since the qPCR used render no consistent results.

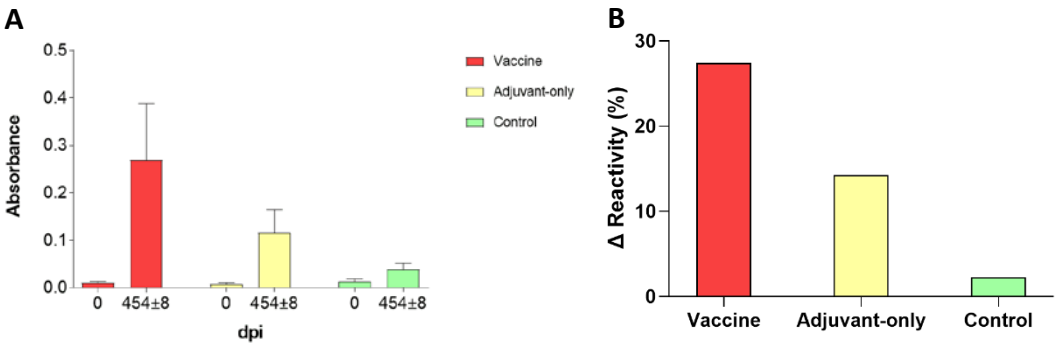

Figure Ch4-1: Serological SRLV results obtained with home-made ELISA tests in lambs from Vaccine, Adjuvant-only and Control groups included in Flock 2 and Flock 4. **(A)** Mean absorbance at the beginning and the end of the experiment. **(B)** Reactivity percentage variation between the beginning and the end of the experiment.

EXPERIMENT 2:

EVOLUTION OF SRLV-INDUCED ARTHRITIS, ANTIBODY TITERS AND VIRAL LOAD IN NATURALLY-INFECTED ADULT SHEEP AFTER ALUMINUM INOCULATION

MATERIAL AND METHODS

Fifteen adult (>4 year-old) female commercial Rasa Aragonesa sheep naturally infected by SRLV and showing bilateral arthritis were selected from different flocks of Aragon (Spain). Sheep were lodged at the experimental farm of the University of Zaragoza and divided into 3 treatment groups: Vaccine group (n=5) was inoculated with commercial vaccines, Adjuvant-only group (n=5) received Al-oxyhydroxide (Alhydrogel ®, CZ Veterinaria) and Control group (n=5) was injected with PBS. Six animals (one sheep in the Vaccine group, two sheep in the Adjuvant-only group and three sheep in the Control group) were excluded after the quarantine period for being either pregnant or diagnosed with concomitant diseases. . Therefore, the final number of animals in each group was: Vaccine group (n=4; animals V-1, V-2, V-3 and V-4); Adjuvant-only group (n=3; animals A-5, A-6 and A-7); Control group (n=2; animals C-8 and C-9). Vaccination schedule of these animals prior to the study was unknown. Inoculation schedule performed during the experiment and vaccines used are detailed in Figure M&M-2 and Table M&M-3, respectively. Animals

received 8 subcutaneous inoculations at different time points and were euthanized at 75 dpi. Vaccine and Adjuvant-only groups received a total of 43.36 mg of Al per animal. Al content in PBS was always under the limit of detection of the technique: 0.074 µg/mL.

Additionally, a Vaccine-extra group (n=2; animals V-10 and V-11) was included at the end of the first experiment to clarify and complement the molecular results obtained. These two sheep were subjected to the same injection protocol but they were euthanized at 40 dpi, therefore receiving only four inoculations in total.

In vivo studies

A general clinical examination was performed at each inoculation date, including systematic articular examination focusing on carpal joints. Blood sampling was performed at the beginning and the end of the experiment using 6 mL EDTA tubes (BD Vacutainer®) for cell blood count, serological analysis and peripheral blood mononuclear cells (PBMCs) isolation in all experimental groups.

All sheep were examined before the experiment to determine the severity of the arthritis. Dorso-palmar and latero-medial radiographies of all carpi were performed following standard procedures. Radiographies were taken the day before the first injection and at 70 dpi in Vaccine, Adjuvant-only and Control groups. Three experienced clinicians scored radiographies to assess severity degree in a blind and independent manner following a severity grading system adapted from Mokbel et al. (Mokbel *et al.*, 2011). Grading system of carpal osteoarthritis ranged between 0 (no radiographic lesion) and 6 (maximum lesion). Radiographic grading system and example images are detailed in Appendix Ch4-2. Surface temperature of the carpal joints was recorded with a thermal camera (FLIR® Systems, USA) at 0, 7, 14, 21, 28, 49 and 69 dpi and analyzed with FLIR Tools software 5.13 (Appendix Ch4-3) in Vaccine, Adjuvant-only and Control groups. Environmental temperatures of all days were collected from the Spanish Agency of Meteorology (AEMET©) and they are showed in Appendix Ch4-4. Carpal photographs over a graphic squared template were taken at 0, 28 and 69 dpi and progression of carpal diameter was analyzed with Adobe Acrobat DC (Appendix Ch4-3).

Pathology and microbiology

Euthanasia and postmortem studies were performed at the end of the experiment, 75 days after the first inoculation and 14 days after the last one. All tissues were fixed in 10% neutral-buffered formalin for 48-72h. Additionally, granulomas found in each animal of the Vaccine-Extra group were fixed in a Zinc solution for 36 h to carry out IHC against SRLV capsid antigen (Benavides *et al.*, 2006). Moreover, articular cotton swabs from all carpi were tested by qPCR against several arthritis-associated pathogens in sheep (SRLV, *Mycoplasma agalactiae, Chlamydia abortus, Streptococcus dysgalactiae* and *Erysipelothrix rushiopathiae*) as well as microbiologic culture for other bacteria.

Samples of granulomas belonging to Vaccine-Extra group, were routinely processed for paraffin embedding and 4 µm sections were stained with either standard HE, lumogallion or IHC. Lumogallion is a specific staining for Al that was carried out only in granulomas as previously described (Mirza *et al.*, 2016; Asín *et al.*, 2019). Samples for IHC were subjected to pre-treatment for antigen retrieval (30 minutes in a solution of 10mM citric acid pH 6 immerged in a water bath at 95ºC); endogenous peroxidase inhibition and non-specific binding sites blockade (20 min in normal horse serum diluted 1:200 in PBS). Tissue samples were incubated with specific monoclonal antibody against p28 capsid protein of SRLV (CAEP5A1, VMRD) overnight at 4ºC, and finally labelled at room temperature for 30 minutes with anti-mouse EnVision HRP System (DAKO, Agilent Technologies) and revealed with 3-3'-diaminobenzidine. Granulomas from SRLV-free animals obtained from previous studies were used as control tissues.

Virus and antibody quantification in blood

Blood plasma was screened against SRLV antibodies using two commercially available ELISA kits: Elitest-MVV (Hyphen-Biomed, France) and Eradikit™ SRLV Screening kit (IN3 diagnostic, Italy). Both assays were performed to confirm infection status at the beginning and the end of the experiment. Additionally, a home-made peptide ELISA was performed at the beginning and the end of the experiment as described in Experiment 1.

Genomic DNA was extracted from PBMCs by using E.Z.N.A Blood DNA Mini Kit (Omega bio-tek). Proviral load quantification was performed by qPCR using primers and a fluorogenic probe (Appendix Ch4-1) specifically designed for strain Ov496 (genotype B2) (Pinczowski *et al.*, 2017), the main local viral strain that induces lentiviral arthritis (Pérez *et al.*, 2015).

Statistical analyses

Each carpus was studied independently and Cronbach's alpha was used to check internal consistency between the three observers that scored carpal radiographies. In this test, alpha values over 0.9 stand for an excellent consistency, whereas alpha values under 0.6 imply poor inter-observer reliability (Daniel and Cross, 2013).

Antibody titers and SRLV DNA viral loads were represented in two complementary ways: first as mean values with standard error means; second as percentage of increase (Δ) between the end of the experiment and the beginning of the experiment.

Quantitative variables (i.e. antibody titer, viral load, carpal temperature and carpal diameter) were tested for normality distribution of data by Shapiro-Wilk´s test. Unpaired comparisons between groups were performed at each sampling date by using ANOVA test followed by Duncan's multiple range test as a *post-hoc* for normally distributed variables and Kruskal-Wallis test followed by Dunn's test as a *post-hoc* for non-normal and ordinal (i.e. radiographic severity of arthritis) variables. Paired comparisons within each group between the sampling dates along the experiment were performed using Student´s t test for paired samples or Wilcoxon test depending on normal distribution of data (Daniel and Cross, 2013).

RESULTS

Clinicopathological changes

Clinical examination and cell blood count revealed no significant differences between the treatment groups. Microbiological analysis of the right carpus of sheep A-7 (Adjuvant-only group) was positive for *Chlamydia abortus* and subsequently eliminated

from the study. Remaining carpal joints (n=17) were negative for all tested pathogens except for SRLV (Appendix Ch4-5). Arthritis evolution was blindly evaluated by three independent observers using radiographies at -1 and 70 dpi and the inter-observer consistency was excellent (Cronbach's α = 0.966). Right carpus of sheep A-6 and C-8, belonging to Adjuvant-only and Control groups respectively, were already classified with the maximum degree of severity at the beginning of the experiment, thus they were excluded from this part of the study, as no radiographic progression of the arthritis was measurable.

A variable increase in carpal radiographic severity degree in all groups was observed. Interestingly, radiographies showed an accelerated evolution of the arthritis in the Vaccine group (Increase mean: 0.75 severity degree) compared with the Adjuvant-only (Increase mean: 0.5 severity degree) and Control groups (Increase mean: 0.33 severity degree). Consistently, statistically significant differences were only found in the Vaccine group (p=0.034) between the beginning and the end of the experiment with 5 out of 8 carpi presenting an increased degree of lesion severity (Figure Ch4-2A).

Thermography studies were affected by mean environmental temperatures that varied sharply along the study. However, these temperatures were similar at 0 and 69 dpi (10.3 and 10.9°C, respectively) (Appendix Ch4-3) and therefore, only thermographies from these two sampling dates were analyzed. There was a significant increase in the carpal temperature of Vaccine (Increase mean: 5.05°C; p=0.012) and Adjuvant-only (Increase mean: 2.7°C; p=0.042) groups and a non-significant increment in the Control group (Increase mean: 2.4°C; p=0.068) (Figure Ch4-2B). All groups presented a statistically significant increase in carpal diameter between 0 and 69 dpi (Vaccine p<0.001; Adjuvant-only p=0.015; Control p=0.015). Additionally, carpal diameter significantly increased between 0 and 28 dpi in the Vaccine group (p=0.02) and between 28 and 69 dpi in the Adjuvant-only group (p=0.015; Figure Ch4-2C).

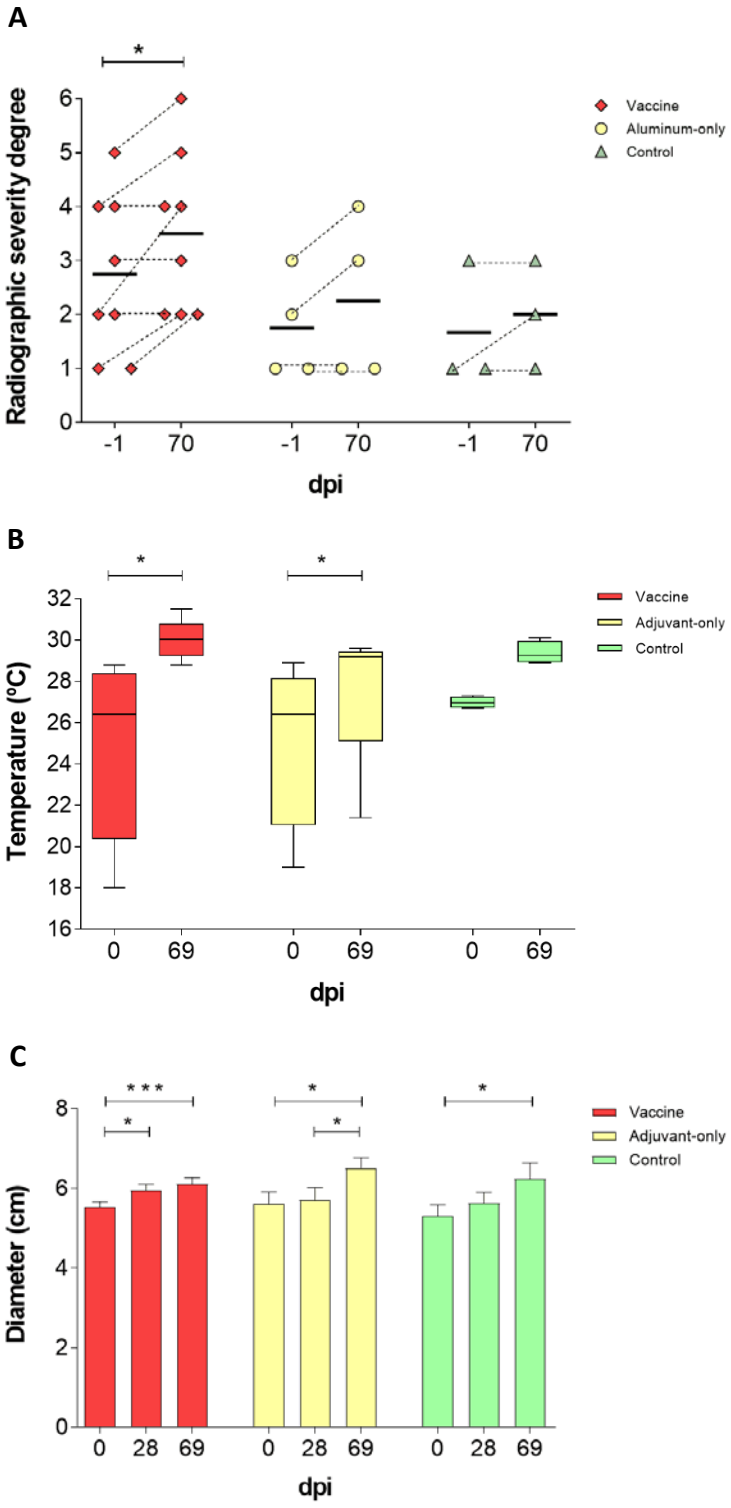

Figure Ch4-2: Arthritis evolution in Vaccine, Adjuvant-only and Control groups from SRLV naturally-infected adult sheep. (A) Analysis of carpal radiographies. Radiographic severity degree was scored from 0 to 6 at minus 1 day after the first inoculation (dpi), and at the end of the experiment (70 dpi). A significant radiographic worsening (**p*=0.034) was only observed in the Vaccine group. (B) Thermographic analysis. Progression of carpal temperature between 0 and 69 dpi. A significant increase of carpal temperature is observed in the Vaccine (**p*<0.012) and Adjuvant-only groups (**p*<0.042). (C) Carpal diameter measurements. Evolution of carpal diameters at 0, 28 and 69 dpi. All groups show significantly increase carpal diameter (**p*<0.05; ****p*<0.001).

During post-mortem examination, 31 subcutaneous granulomas out of 32 inoculations were recovered (96.7%) from animals of the Vaccine group. In the Vaccine-extra group, 8 subcutaneous granulomas out of 8 inoculations (100%) were found. In the Adjuvant-only group, 19 granulomas out of 24 inoculations were recovered (79.2%). Interestingly, one of the Control animals (C-8) exhibited a single low sized granuloma, most likely due to a prior in-field vaccination. In the Vaccine and Vaccine-extra group, subcutaneous granulomas showed moderate differences in size and shape among them, being bigger when injection had been recently inoculated (Appendix Ch4-6). Slight differences between the different vaccines used were also noted. Granulomas were slightly bigger in the Vaccine and Vaccine-extra group in contrast to Adjuvant-only group. Histologically, granulomas were basically identical to those previously described (Asín *et al.*, 2019) and consisted on abundant epithelioid macrophages surrounding a central necrotic area and admixed with moderate amounts of lymphocytes and plasma cells and lesser multinucleated giant cells (Appendix Ch4-7A and Ch4-7B). Presence of Al within macrophages was confirmed by lumogallion staining in all evaluated granulomas (Appendix Ch4-7C). Al was found in small intracytoplasmic aggregates within cells (Appendix Ch4-7D).

Presence of SRLV in post-vaccination granulomas was confirmed by IHC. Granular, intracytoplasmic positive immunolabelling was found in macrophages and multinucleated giant cells, whereas no labelling was observed in fibrous capsule, lymphocytes and/or plasma cells (Appendix Ch4-8). Granulomas from SRLV negative animals consistently showed no positive staining (Appendix Ch4-8).

Arthritis was confirmed by pathological means in all animals of the Vaccine, Vaccine-extra and Adjuvant-only and Control groups (Appendix Ch4-9). Grossly, carpi were markedly swollen and showed severe diffuse synovial proliferation, eburnation of articular cartilage and abundant osteophytes (Appendix Ch4-10). Histopathological analyses showed villous-like synovial growths with sloughing of synoviocytes. The stroma was infiltrated by abundant lymphocytes and plasma cells with lesser numbers of macrophages and neutrophils (Appendix Ch4-11). Right carpus of sheep V-4 and left carpus of sheep C-8 could not be microscopically analyzed as no synovial tissue was present in the samples. Right

carpus of sheep A-7 was excluded from the analysis for being positive to *Chlamydia abortus*. A summary of SRLV-associated lesions, subcutaneous granulomas and other pathological changes found in these animals is presented in Appendix Ch4-9.

Virus and antibody quantification

Results from both commercial serological tests showed no differences along the experiment, since all animals were always positive, irrespectively of the time point analyzed (Table Ch4-2).

Table Ch4-2: Commercial ELISA tests results in naturally SRLV-infected sheep in Vaccine, Vaccine-extra, Adjuvant-only and Control groups. Numbers show positive animals at the beginning and the end of the experiment.

Groups	Beginning of experiment		End of experiment	
	N	%	N	%
Vaccine	4/4	100%	4/4	100%
Vaccine-extra	2/2	100%	2/2	100%
Adjuvant-only	3/3	100%	3/3	100%
Control	2/2	100%	2/2	100%

Kinetics of the humoral response studied by home-made end-point dilution ELISA indicated increased absorbance in the Vaccine and Adjuvant-only groups along the experiment while the Control group showed almost no variation (Figure Ch4-3). Accordingly, the reactivity increase was moderate in Adjuvant-only group, mild in Vaccine group and minimal to absent in the Control group. However, none of these differences were statistically significant. Results obtained in Vaccine-extra group paralleled those obtained in the Vaccine group.

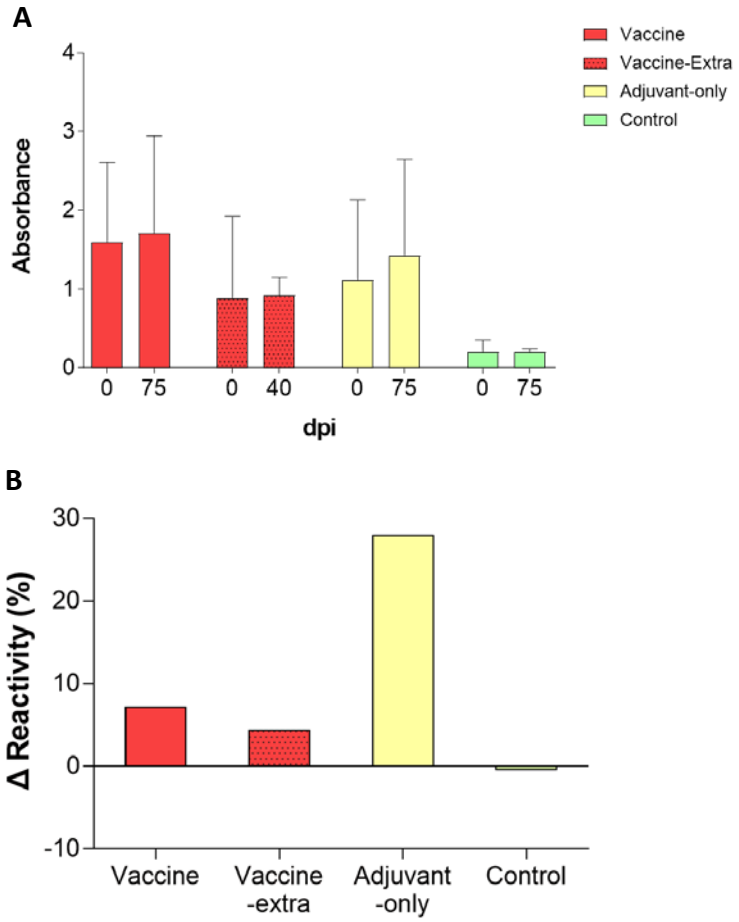

Figure Ch4-3: Serological changes in naturally SRLV-infected sheep in Vaccine, Vaccine-extra, Adjuvant-only and Control groups. **(A)** Home-made ELISA. Mean absorbance at the beginning and the end of the experiment. **(B)** Home-made ELISA. Reactivity percentage variation between the beginning and the end of the experiment.

Real time quantification of SRLV DNA in circulating PBMCs showed an increased viral DNA copies in animals of the Control group at 75 dpi, when compared with 0 dpi. In contrast, Vaccine and Adjuvant-only groups presented reduced viral DNA copies at 75 dpi compared with 0 dpi and also showed a reduced viral DNA load compared to the Control group (Figure Ch4-4). These differences were marked in terms of raw values but they were statistically non-significant. Results obtained in Vaccine-extra group paralleled those obtained in the Vaccine group.

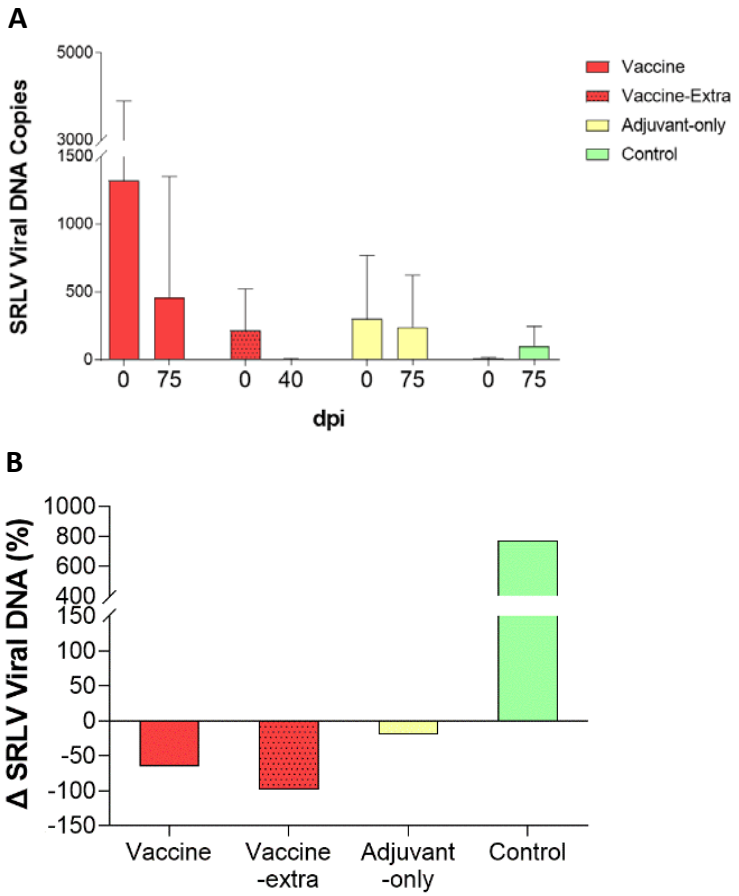

Figure Ch4-4: Viral load changes in naturally SRLV-infected sheep in Vaccine, Vaccine-extra, Adjuvant-only and Control groups. **(A)** qPCR. SRLV viral DNA copies variation between the beginning and the end of the experiment (0 and 75 dpi). **(B)** qPCR. Percentage of viral DNA variation between the beginning and the end of the experiment (0 and 75 dpi).

DISCUSSION

This chapter studies the interaction between lentiviruses and Al-induced injection-site granulomas, a relationship that can open a new, unknown research field. Actually, this relationship provides an ideal model for globally understanding SRLV-host interaction: almost all sheep flocks in Spain are infected by SRLV and certainly all sheep flocks receive Al-containing vaccines, a fact that is unavoidably followed by granuloma formation (Minguijón *et al.*, 2015; Asín *et al.*, 2019). As a consequence, almost all sheep in natural conditions will have the chance for this interaction to take place. Activated macrophages present in Al-induced granulomas could have a role in the pathogenesis of SRLV in naturally-infected sheep, as this interaction could alter SRLV body biodistribution and perhaps also modify the host immune response against SRLV infection. SRLV infection

in sheep causes a multi-organic inflammatory process that includes chronic arthritis. This articular syndrome is the only one caused by SRLV that can be diagnosed and monitored *in vivo* in a straightforward manner in naturally-infected sheep, thus providing an excellent model to evaluate arthritis evolution over time.

Our results in the groups of lambs raised in two commercial SRLV-positive flocks (Flock 2 and Flock 4) indicate a trend to higher seroconversion rates in lambs from the Vaccine group when compared to the Control group. Most likely, the infection was transmitted from adult, infected animals to the lambs by aerosols or direct contact, the most common infection routes in SRLV (Minguijón *et al.*, 2015). This trend was more evident in Flock 2, likely due to different management systems (intensive production system in Flock 2 versus extensive production in Flock 4), since SRLV infection is more easily transmitted in intensive reared flocks due to a closer contact between animals (APHIS, 2003). Interestingly, increased flock seroprevalence was observed in Spanish and French sheep flocks after compulsory vaccination against bluetongue virus (M. Vila, personal communication; Valas *et al.*, 2011). This in field observation could be explained by an increased availability of activated macrophages accumulating in granulomas, thus facilitating local SRLV replication. However, our results may also suggest that after repeated inoculation with Al oxyhydroxide-containing products, the chemotactic signals induced by Al at the inoculation site could recruit SRLV infected monocytes in SRLV-seronegative but infected animals. These monocytes would evolve into activated macrophages forming granulomas and facilitate SRLV replication. In both cases, replication in granulomas may expose greater quantities of viral antigens, followed by activation of the immune system against the virus, increased antibody titers in individuals and a modification of the flock global seroprevalence. (Marrack, McKee and Munks, 2009; Lu and HogenEsch, 2013). Results obtained in SRLV-naturally infected sheep showing clinical arthritis (Experiment 2) and injected with Al-containing products, align with the results obtained in lambs (Experiment 1): they also showed higher antibody titers in Vaccine and Adjuvant-only groups in comparison with the Control group, although this difference was not significant, likely due to low sample size.

Radiographic and thermographic data revealed a worsening in the arthritic condition in sheep from the Vaccine and Adjuvant-only groups. Indeed, analysis of radiographies showed that all groups increased their carpal radiographic severity degree but the increase was more important and statistically significant in the Vaccine group. There was also an increase of carpal temperature, which was statistically significant only for Vaccine and Adjuvant-only groups. These data demonstrate an accelerated progression of carpal articular lesions in animals from the Vaccine and Adjuvant-only groups. Acceleration of the arthritic process in the Vaccine and Adjuvant-only groups could be explained by the systemic pro-inflammatory stimulus triggered by Al-oxyhydroxide adjuvants in sheep, which is higher in animals inoculated with whole vaccines than in those inoculated only with Al-oxyhydroxide adjuvants (Varela-Martínez *et al.*, 2018). Moreover, increased levels of pro-inflammatory cytokines, such as TNFα, have been linked to lentiviral arthritis progression in goats (Lechner *et al.*, 1997; Murphy *et al.*, 2006). Increased antibody titers in Al-oxyhydroxide inoculated animals may also contribute to the increased arthritis progression, as high antibody titers in synovial fluid and blood have been linked to increased infection and disease progression *in vivo* (Knowles *et al.*, 1990; González *et al.*, 2005). The arthritis progression observed in the Control group was an expected evolution of the natural SRLV infection and it could reflect an augmented viral load due to viral replication. Interestingly, similar results were observed in experimentally vaccinated and SRLV-infected goats in contrast with control animals inoculated with medium (McGuire *et al.*, 1986).

At necropsy, almost one granuloma per injection was detected. Indeed, 96.7%, 100% and 79.2% of the expected granulomas were recovered in the Vaccine, Vaccine-extra and Adjuvant-only groups, respectively. A similar result was obtained in a previous study (Asín *et al.*, 2019), in which higher numbers of granulomas were recovered in vaccinated animals compared with sheep injected with Al-oxyhydroxide only. Interestingly, sheep C-8 (Control group) showed an Al-containing subcutaneous granuloma, likely as a result of previous vaccination, since these granulomas can persist for at least 15 months (Asín *et al.*, 2019). Granulomas were microscopically characterized and identical to those previously described, including the presence of Al (lumogallion) and viral protein (IHC) within the cytoplasm of epithelioid, activated macrophages and multinucleated giant cells (Asín *et al.*,

2019). The IHC signal was observed within intracytoplasmic granules with a pattern very similar to that of Al-containing phagolysosomes (Asín *et al.*, 2019). Perhaps the vacuoles induced by Al phagocytosis provide a suitable atmosphere for viral replication. Additionally, the cytoplasmic location and granular appearance of immunohistochemically-labelled SRLV in the granuloma closely resembled the intramacrophagic distribution of SRLV in other sheep target tissues (Luján, Begara and Watt, 1994; Angelopoulou, Brellou and Vlemmas, 2006).

Results obtained by qPCR in arthritic sheep were not statistically significant but indicated that viral load in PBMC might decrease during the experiment in Vaccine, Vaccine-extra and Adjuvant-only groups in contrast to Control animals. Viral load increase is strongly related to disease development in SRLV-infected animals (Zhang *et al.*, 2000). Migration of uninfected or SRLV-infected circulating monocytes to granulomas might offer the most likely explanation to this apparent reduction of viral DNA in blood. It is well known that migration of blood monocytes is the most common mechanism for macrophage accumulation in interstitial tissues (Kumar, Abbas and Aster, 2021) and Al-containing macrophages in post-vaccination granulomas are derived from circulating monocytes in mice (Khan *et al.*, 2013). An alternative explanation could indicate that the increased antibody production against SRLV may be responsible for the reduced viral load, suggesting that antibodies may control, at least temporarily, virus circulation in PBMCs (Barquero *et al.*, 2011). An estimation of viral load in target organs may clarify this point.

Granuloma formation is not exclusive of Al-inoculation as other exogenous substances (i.e. oil-formulated adjuvants) and infectious agents (i.e Mycobacteria complex) are also related to granuloma development. Interestingly, *Mycobacterium avium spp. paratuberculosis* induce granulomatous enteritis characterized by abundant macrophages that can have M1-like or M2-like pattern of differentiation depending on the stage of the disease (Fernández *et al.*, 2016), being M1 differentiation able to restrict SRLV replication *in vitro* (Crespo *et al.*, 2011). Evaluating the interaction between slow infections such as SRLV and pathogens that induce other types of granulomatous inflammation could be of particular interest in future studies.

The main limitation of this study is the small number of arthritic animals in the Control group (n=2), which jeopardizes optimal statistical analyses. Unfortunately, several control animals had to be excluded during quarantine for several reasons, as explained above. Another limitation is that each arthritic animal showed different lesion severity, antibody titers and viral load at the beginning of the experiment, which makes difficult to compare data that can only be obtained at the end of the experiment (i.e. macroscopic and microscopic lesions in carpi, lungs and udder).

CONCLUSIONS

Our results indicate that SRLV infection, pathogenesis and clinical outcome could be altered after subcutaneous administration of Al-oxyhydroxide containing products. Remarkably, Al-induced injection-site granulomas provide an ideal environment for both SRLV replication and antigen presentation, thereby modifying serological responses as well as SRLV-associated lesions. Al-induced granulomas and viruses with tropism for the mononuclear-phagocytic system are widely present in nature. Therefore, it is important to know whether findings described in this study are exclusive to the SRLV infection in sheep, or can be observed in other macrophage-tropic infections in other species.

APPENDICES

- **Appendix Ch4-1:** Characteristics of primers used in this work for quantification of Small Ruminant Lentiviruses DNA.
- **Appendix Ch4-2:** Examples of carpal joint osteoarthritis under radiographic analysis in sheep naturally infected by small ruminant lentiviruses.
- **Appendix Ch4-3:** Carpal thermography and carpal diameter measurement in sheep V-2.
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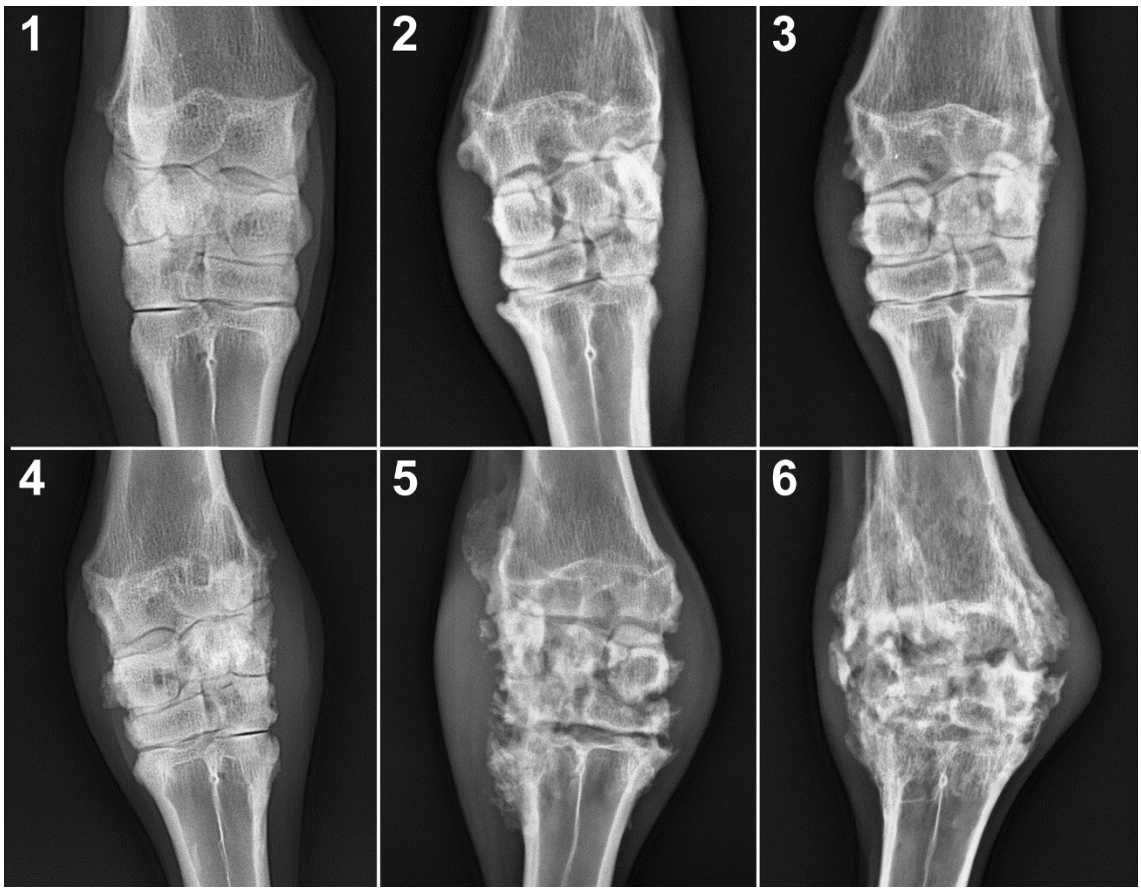
- **Appendix Ch4-7:** Histopathology in aluminum (Al)-induced injection-site granulomas in Vaccine extra group.
- **Appendix Ch4-8:** Immunohistochemistry against SRLV in aluminum (Al)-induced injection-site granulomas of the Vaccine extra group.
- **Appendix Ch4-9:** Pathological findings in sheep of Vaccine, Vaccine-extra, Adjuvant-only and Control groups.
- **Appendix Ch4-10:** Macroscopic images of carpal joints affected by SRLV-induced arthritis.
- **Appendix Ch4-11:** Microscopic images of carpal joints affected by SRLV-induced arthritis.

Appendix Ch4-1: Characteristics of primers used in this work for quantification of Small Ruminant Lentiviruses DNA.

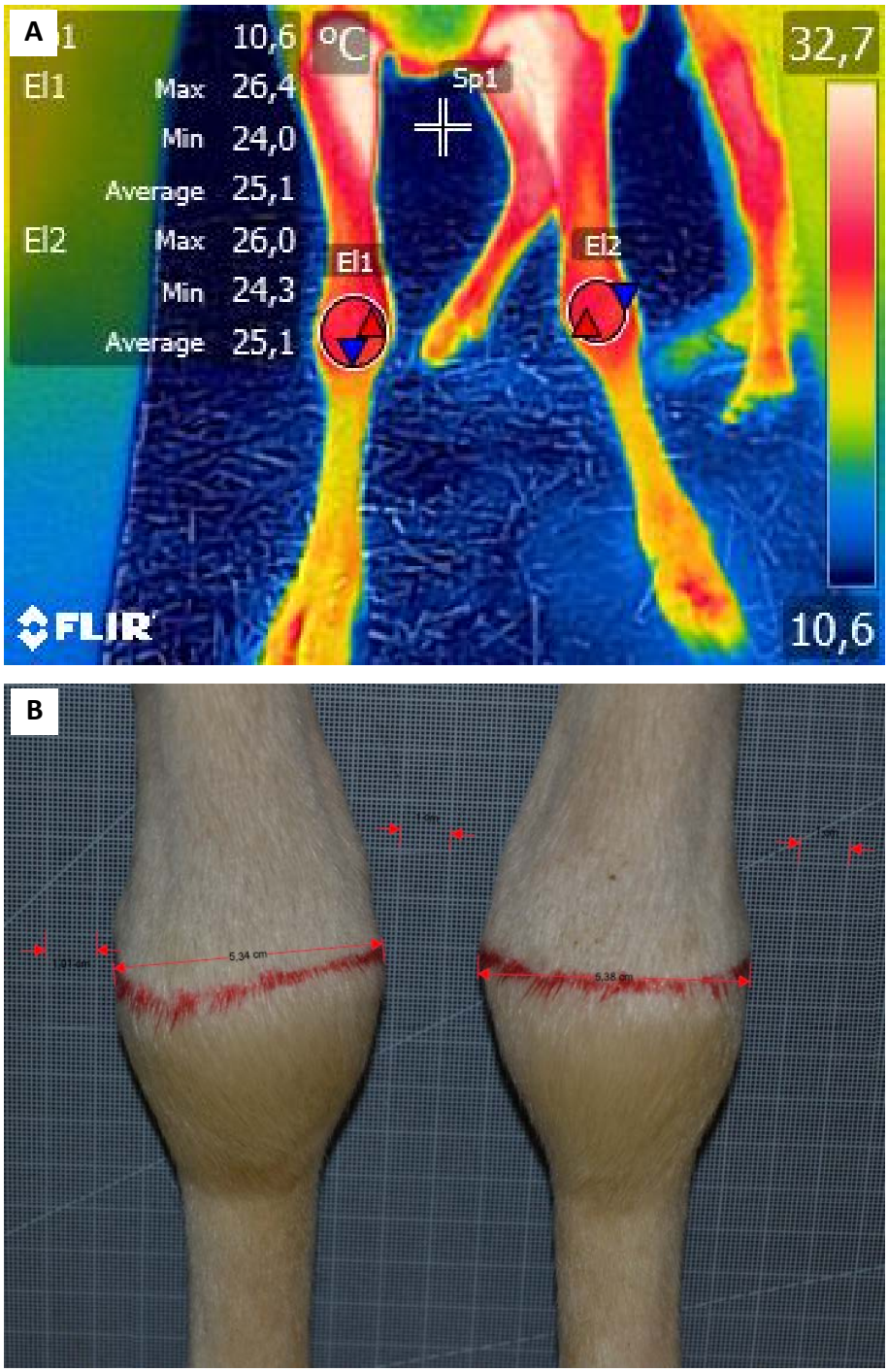
Genotype	Designation	Location	Sequence (5´-3´)	Orientation	Ref.
B2	qPCR-496 Fw	GAG	GGCCAGGAAATCCTATGTTAGT	Fw	[1]
	qPCR-496 Rv		CGTGACTGGTTCTGCATCTAT	Rv	
	probe 496		6-FAM-CTTTGCAGCAAGGCTGCTAGA-TAMRA		
B2	qPCR 496 Fw	LTR	TGCTGCTTGCACTTCRGAGTT	Fw	[2]
	qPCR 496 Rv		GGCAGTAAGGCAATCACTCCTT	Rv	
A	qPCR Ev1 Fw	ENV	CTCCTTGCAGGCCACAATG	Fw	[3]
	qPCR Ev1 Rv		GCTGCTTGCACTGTCTCGG	Rv	
	probe Ev1		6-FAM-TGCCTTATGTGTAGTCAGC-TAMRA		

[1](Pinczowski *et al.*, 2017) [2](Crespo *et al.*, 2011) [3](González *et al.*, 2005)

Appendix Ch4-2: Examples of carpal joint osteoarthritis under radiographic analysis in sheep naturally infected by small ruminant lentiviruses. Carpal radiographs were scored from 0 to 6 with higher scores indicating more severe articular damage according to Mokbel et al., 2011. **0:** No lesion. **1:** Mild joint space narrowing or mild remodeling of articular margins (without subchondral bone lysis). **2:** Mild joint space narrowing and mild remodeling of articular margins (without subchondral bone lysis). **3:** Moderate joint space narrowing and moderate remodeling of articular margins (with or without subchondral bone lysis). **4:** Severe joint space narrowing and moderate remodeling of articular margins (with or without subchondral bone lysis). **5:** Severe joint space narrowing and severe remodeling of articular margins (with subchondral bone lysis). **6:** Severe joint space narrowing and massive bone remodeling of articular margins (with subchondral bone lysis). In this score, it is not possible to identify individual carpal bones.



Appendix Ch4-3: Carpal thermography and carpal diameter measurement in sheep V-2 at 0 days after the first inoculation (0 dpi). **(A)** Carpal thermography in the right (EI1) and left (EI2) carpus. **(B)** Carpal diameter.



Appendix Ch4-4: Environmental temperatures at 0, 7, 14, 21, 28, 49 and 69 days post first inoculation (dpi), corresponding to the dates of thermographic analyses. Environmental data were collected from the Spanish Agency of Meteorology (AEMET©) from the closest weather station (ID: 9434P; Valdespartera, Zaragoza) to the experimental farm where animals were housed.

	0 dpi	**7 dpi**	**14 dpi**	**21 dpi**	**28 dpi**	**49 dpi**	**69 dpi**
T. mean*	10.3	7.6	2.9	9.6	3.2	0.7	10.9
T. min‡	6.4	4.4	1.6	5.9	1.8	-2.1	6.9
Time T. min (hh:mm)	23:20	04:50	06:50	01:00	23:00	08:00	23:59
T. max†	14.2	10.9	4.2	13.3	4.7	3.5	14.9
Time T. max (hh:mm)	14:20	14:30	14:00	14:00	00:30	14:40	13:00

*T. mean: mean temperature for the day

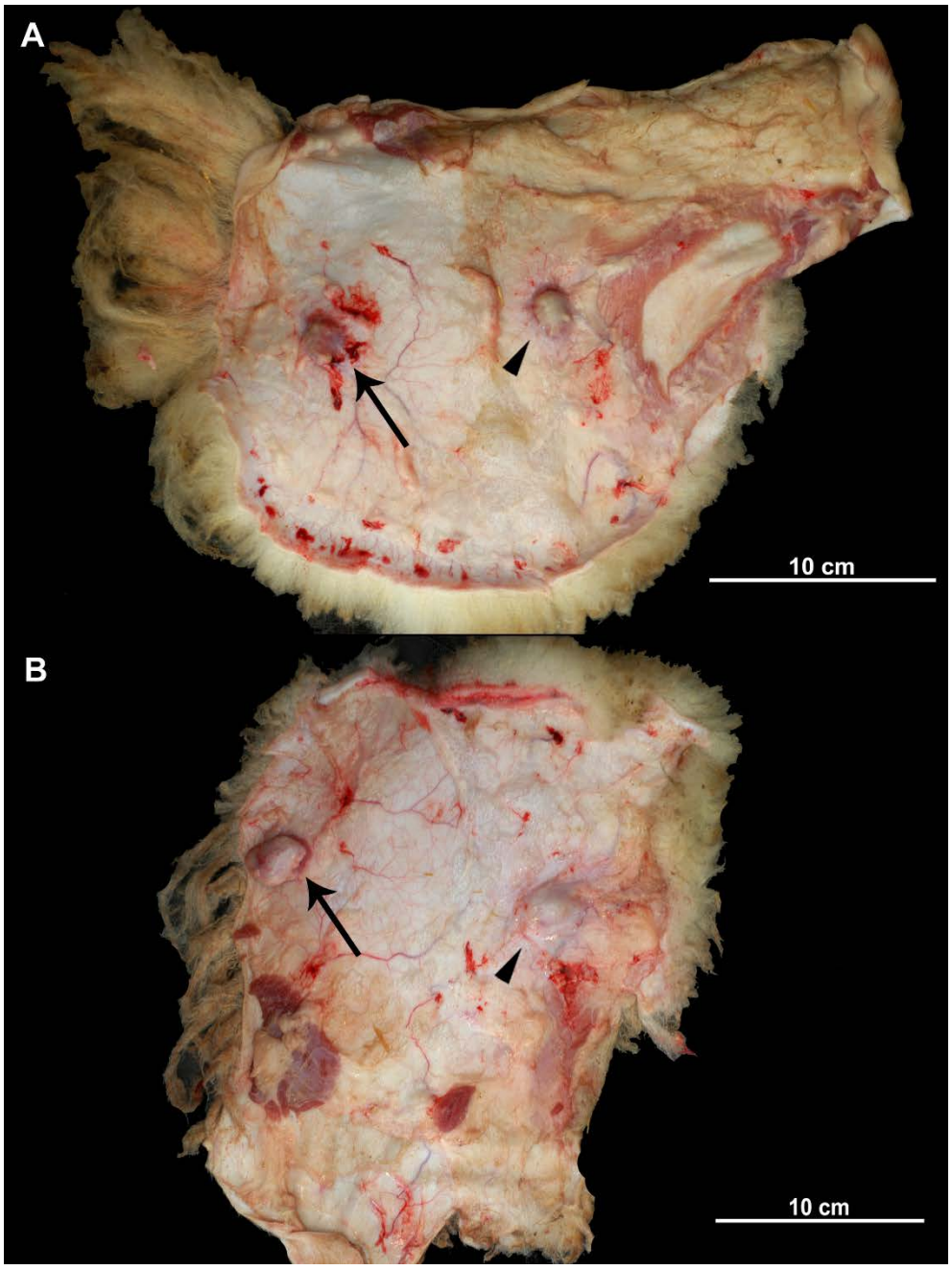
‡T. min: minimum temperature for the day

†T. max: maximum temperature for the day

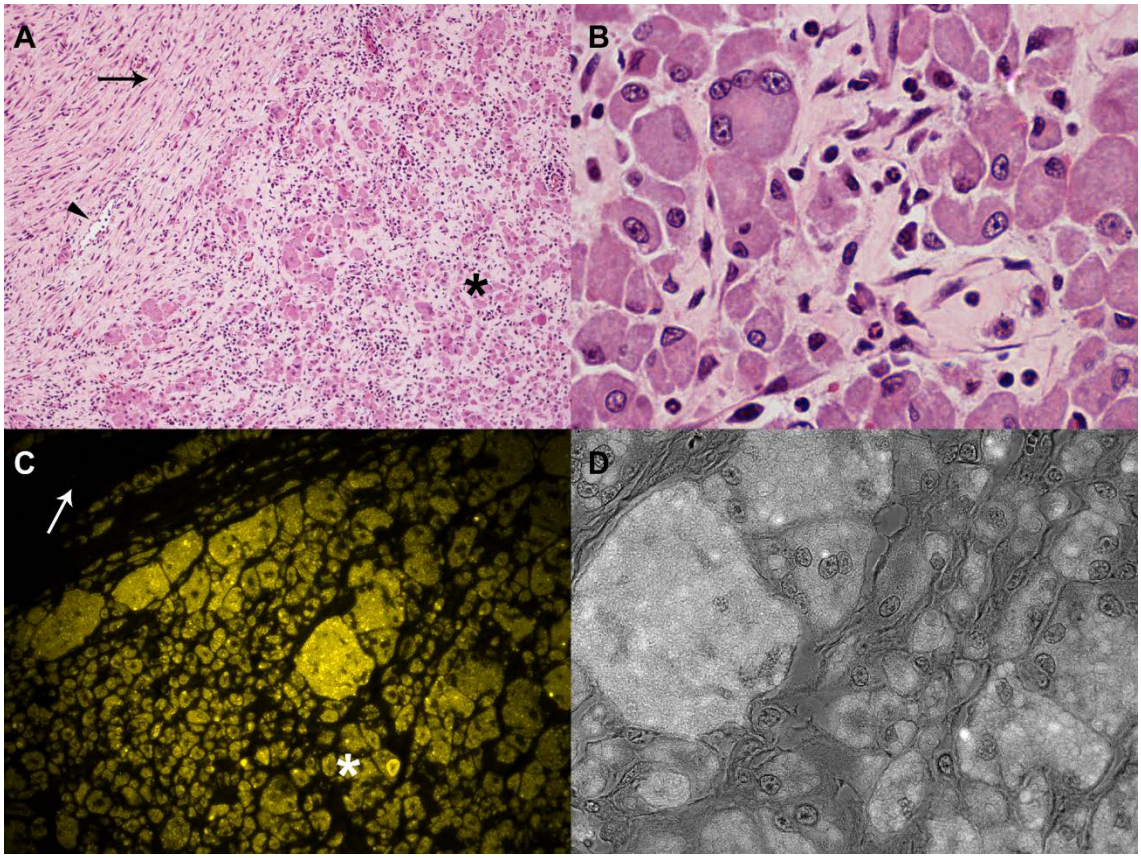
Appendix Ch4-5: Microbiological analysis of carpal joints. Cotton swabs of both, right carpus (R) and left carpus (L) from Vaccine (V), Adjuvant-only (A) and Control (C) sheep were tested by quantitative PCR against common articular pathogens of sheep.

Pathogen	V-1		V-2		V-3		V-4		A-5		A-6		A-7		C-8		C-9	
	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L
Mycoplasma agalactiae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chlamydia abortus	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Streptococcus dysgalactiae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Erysipelothrix rhusiopathiae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Small Ruminant Lentiviruses	+	+	-	+	-	-	-	-	-	-	+	+	-	-	+	-	-	-

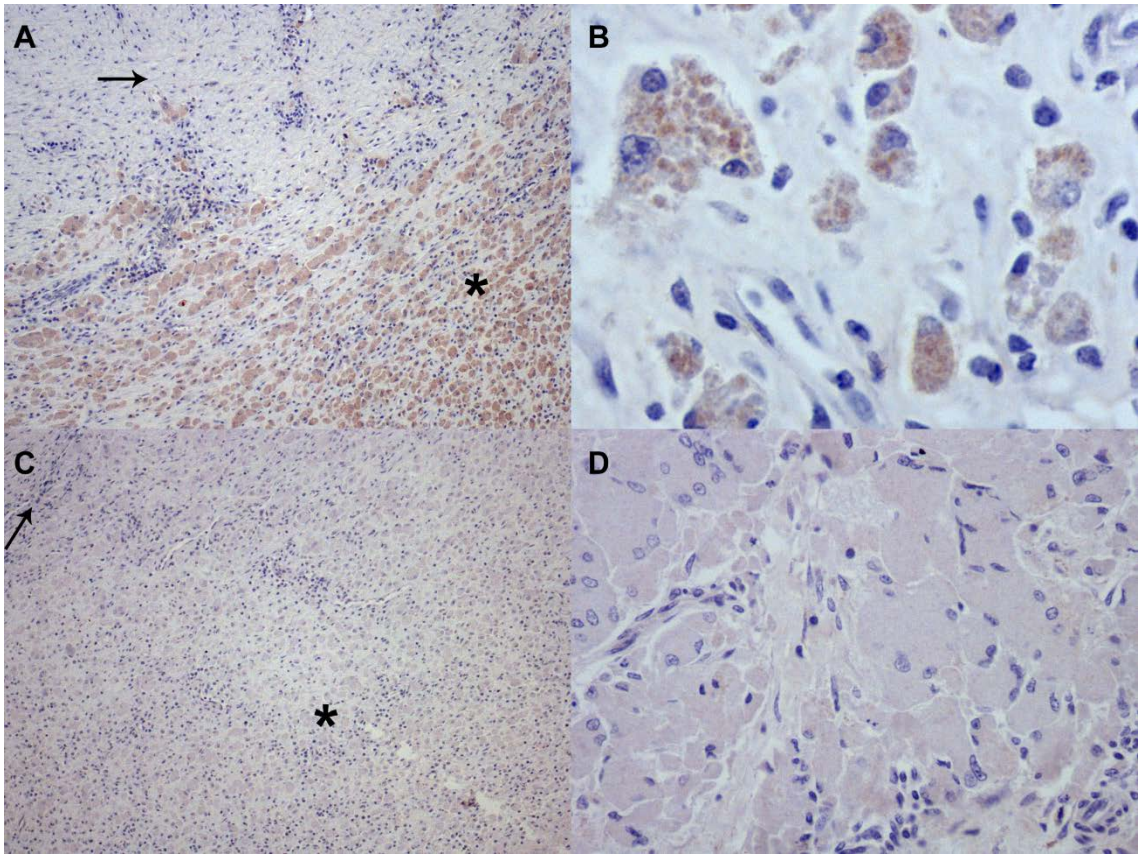
Appendix Ch4-6: Subcutaneous tissue (picture shows the inner aspect of the skin). Sheep V-10. Multiple post-vaccination granulomas. **(A)** Left flank. Post-vaccination granuloma induced at 0dpi (arrowhead) and 21dpi (arrow). **(B)** Right flank. Post-vaccination granuloma induced at 0dpi (arrowhead) and 21dpi (arrow). Animal euthanized at 40dpi.



Appendix Ch4-7: Histopathology in aluminum (Al)-induced injection-site granulomas of the Vaccine-extra group**. (A)** Abundant epithelioid macrophages (asterisk) admixed with moderate amounts of lymphocytes and plasma cells and surrounded by a fibrous capsule. HE stain. **(B)** Higher magnification of epithelioid macrophages and multinucleated giant cells showing abundant granular and eosinophilic cytoplasm. HE stain. **(C)** Abundant epithelioid macrophages and multinucleated giant cells depicting yellow-orange fluorescence aluminum deposits (asterisk) and lack of fluorescence in the surrounding fibrous capsule (arrow). Lumogallion stain. Fluorescence channel. Color picture. **(D)** Intracytoplasmic granular deposits within epithelioid macrophages and multinucleated giant cells depicting bright white fluorescence. Lumogallion stain. Fluorescence channel and bright field overlay. Black-and-White picture.



Appendix Ch4-8: Immunohistochemistry against SRLV in aluminum (Al)-induced injection-site granulomas of the Vaccine-extra group**. (A-B)** SRLV positive sheep. **(A)** Positive immunolabelling of epithelioid macrophages (asterisk) within the granuloma. Note the negative result in the connective tissue (arrow). **(B)** Detail of positive macrophages and multinucleated giant cells showing granular, intracytoplasmic labelling. **(C-D)** SRLV negative sheep used as negative control. There is absence of immunolabelling of epitheliod macrophages (asterisk) and surrounding connective tissue (arrow).



Appendix Ch4-9: Pathological findings in sheep of Vaccine, Vaccine-extra, Adjuvant-only and Control groups. Lesions associated with Small Ruminants Lentiviruses (SRLV) were globally scored attending to histological criteria as: absent (-), mild (+), moderate (++) or severe (+++). Subcutaneous granulomas and other lesions detected during necropsies and histopathological evaluation are summarized.

		SRLV-Associated lesions					Granulomas		Other lesions
Group	Animal	Lung[†]	Brain[‡]	Left Carpus*	Right Carpus*	Udder[#]	Right Flank	Left Flank	
Vaccine	V-1	-	-	+++	++	+	4	3	Caseous lymphadenitis Verminous pneumonia. Hepatic calcifications Abomasal ostertagiasis
	V-2	++	-	+++	+	+	4	4	Abomasal ostertagiasis Chronic bronchopneumonia
	V-3	+	-	++	++	-	4	4	Abomasal ostertagiasis Caseous lymphadenitis
	V-4	+	-	+	ND[δ]	++	4	4	Caseous lymphadenitis
Vaccine-Extra	V-10	ND	ND	ND	ND	ND	2	2	
	V-11	ND	ND	ND	ND	ND	2	2	Interstitial nephritis
Adjuvant-only	A-5	-	-	+++	+	-	4	2	
	A-6	++	-	+	+++	+++	4	4	Hepatic dicrocoeliasis
	A-7	+	-	+++	ND	++	0	5	Verminous pneumonia Abomasal ostertagiasis
Control	C-8	++	-	ND	+++	+	1	0	
	C-9	+++	-	++	++	++	0	0	Verminous pneumonia

Histopathological features evaluated:

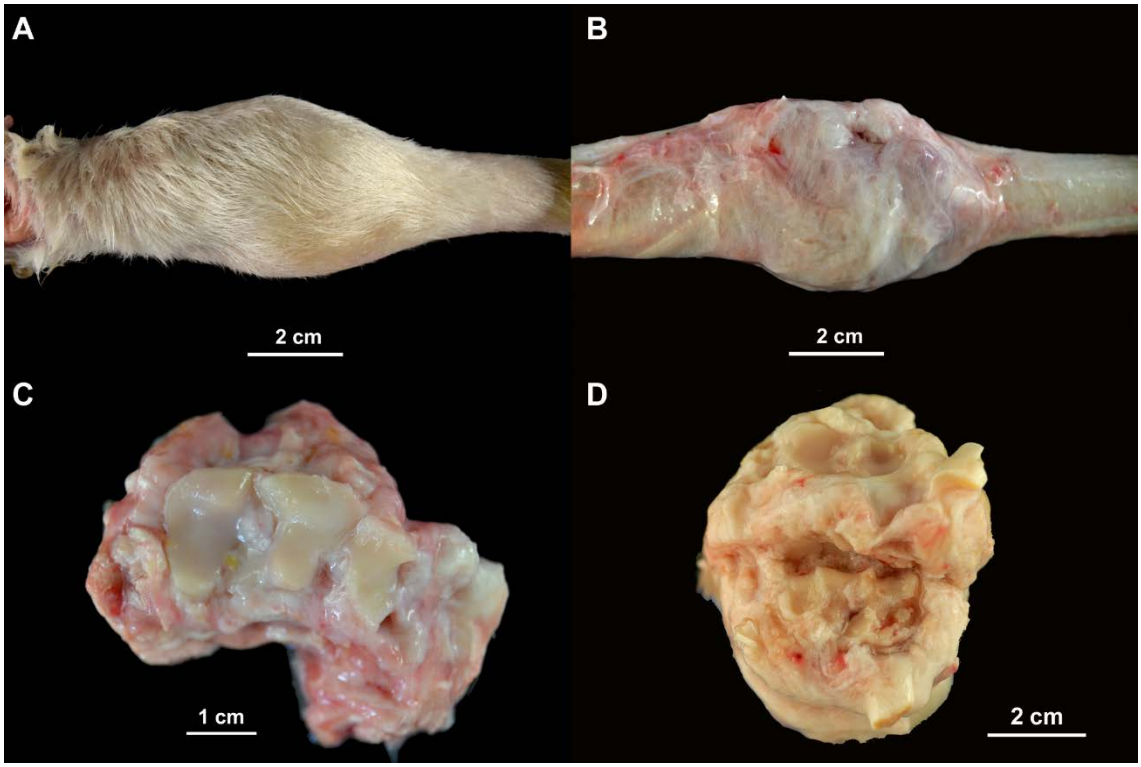
[†] **Lung**: Alveolar septa thickening; Bronchiolar-associated lymphoid tissue hyperplasia; Peribronchitis and peribronchiolitis; Bronchial smooth muscle hypertrophy; Perivascular fibrosis.

[‡] **Brain:** Gliosis; Satellitosis; Perivascular cuffing; Spongiosis.

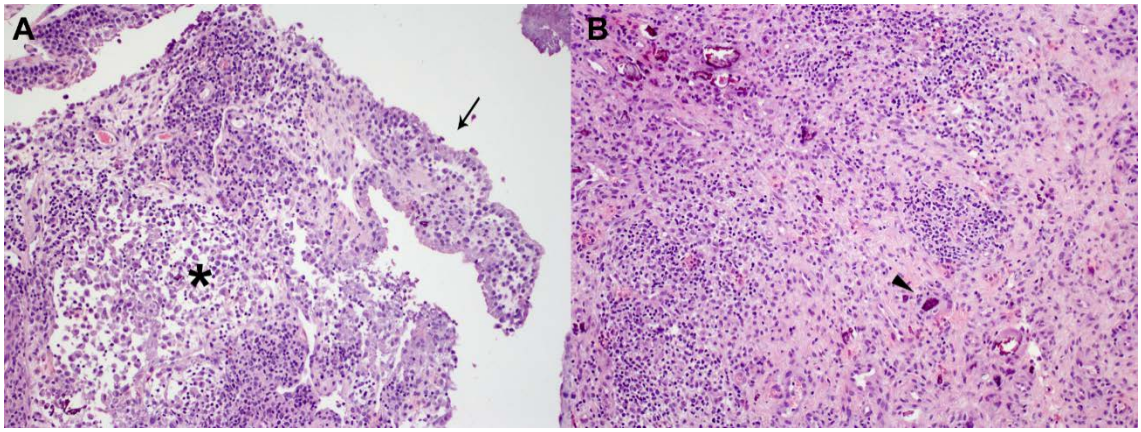
***Carpus:** Synovial membrane thickening; Synoviocyte hyperplasia; Fibrosis; Inflammatory infiltrate

[#] **Udder:** Acinar atrophy and degeneration; Inflammatory infiltrate; Lymphoid follicles formation; Interlobular fibrosis.[δ]ND: Not determined

Appendix Ch4-10: Macroscopic images of carpal joints affected by SRLV-induced arthritis**. (A-B)** Sheep V-3, Right carpus. Markedly swollen joint with moderate edema and periarticular fibrosis. **(C)** Sheep V-3, Right carpus. Proliferative arthritis with eburnation of articular cartilage and osteophytes formation. **(D)** Sheep V-2, Right carpus. Proliferative arthritis with fibrin-like material in the synovial fluid and osteophyte formation.



Appendix Ch4-11: Microscopic images of carpal joints affected by SRLV-induced arthritis. **(A)** Villous-like synovial proliferation (arrow) with erosion and sloughing of synoviocytes. Stroma expanded by abundant lymphocytes and plasma cells and moderate numbers of macrophages and neutrophils (asterisk). H&E. **(B)** Stroma expanded by fibroblast proliferation and collagen fibers admixed with areas of mineralization (arrowhead) and abundant lymphocytes, plasma cells and macrophages.



CHAPTER 5

Worldwide prevalence of Small Ruminant Lentiviruses in sheep: A systematic review and meta-analysis

ABSTRACT

SRLV are highly prevalent retroviruses with significant genetic diversity and antigenic heterogeneity that cause a progressive wasting disease of sheep called Visna/maedi. This chapter provides a systematic review and meta-analysis of the last 40 years (1981-2020) of scientific publications on SRLV individual and flock prevalence. 58 publications and 314 studies were included. Most articles used a single diagnostic test to estimate prevalence (77.6%) whereas articles using 3 or more test were scarce (6.9%). Serological tests are more frequently used than direct methods and ELISA has progressively replaced AGID over the last decades. SRLV infection in sheep is widespread across the world, with Europe showing the highest individual prevalence (40.9%) and being the geographical area in which most studies have been performed. Africa, Asia and North America show values in a range between 16.7% to 21.8% at individual level. South and Central America show the lowest individual SRLV prevalence (1.7%). There was a strong positive correlation between individual and flock prevalence (ρ=0.728; $p \leq 0.001$). Despite the global importance of small ruminants, the coverage of knowledge on SRLV prevalence is patchy and inconsistent. There is lack of a gold standard method and a defined sampling strategy among countries and continents.

INTRODUCTION

Visna/maedi is a progressive wasting disease of sheep that causes important deleterious effects in animal production and limits animal trade worldwide (Blacklaws, 2012; Minguijón *et al.*, 2015; Juste *et al.*, 2020). This condition is caused by SRLV, a group of single-stranded RNA viruses with high mutation and recombination potential (Pritchard and McConnel, 2007). Indeed, four main genotypes (A, B, C and E) and more than 35 subgroups have been already characterized (Michiels, Adjadj and De Regge, 2020). This phylogenetic diversity implies high genetic and antigenic diversity, which hinder serologic and molecular diagnosis (Ramírez *et al.*, 2013).

SRLV have tropism for the mononuclear-phagocyte system and induce slow, chronic and persistent inflammation in four main target organs: lung, joints, nervous system and mammary gland, inducing different clinical forms (i.e., pulmonary, articular, nervous and mammary). Interestingly, the occurrence of each clinical form as well as the severity of the lesions depend on viral factors as well as the host immune response (Blacklaws, 2012; Minguijón *et al.*, 2015; Pinczowski *et al.*, 2017; Gayo *et al.*, 2018). The most common issue after SRLV infection is increased replacement rates due to decreased animal condition and production (Minguijón *et al.*, 2015).

There are no treatments or commercial vaccines for Visna/maedi. Thus, accurate diagnosis is the cornerstone for setting up an optimal control program of the infection and reduce its prevalence. Multiple diagnostic techniques can be used to detect SRLV infection. Indirect methods (AGID and ELISA) have been proposed as the most appropriate to detect infected animals, ELISA having higher sensitivity and lower specificity than AGID (De Andrés *et al.*, 2005). Direct methods to detect SRLV (PCR, indirect immunofluorescence, *in situ* hybridization) are also efficient diagnostic techniques (Larruskain and Jugo, 2013). Recent studies have demonstrated the inherent inaccuracy of using a single diagnostic test (Echeverría *et al.*, 2020), which is likely related to the wide SRLV antigenic diversity. However, host response can also play a role since animals from the same herd infected with the same SRLV exhibit significant differences in the susceptibility to infection and viral replication (Crespo *et al.*, 2016). Furthermore, delay in seroconversion can be very variable among individuals (Carrozza

et al., 2009). Although initial descriptions of SRLV infection are from the fifties of the 20th century (Sigurdsson, Grímsson and Pálsson, 1952), only during the last forty years there is a growing body of publications assessing individual and flock SRLV prevalence around the world. However, a comprehensive compilation of the diagnostic methods used and their prevalence results in each continents is lacking.

In accordance with the Objective 5 of the PhD Thesis, the aim of this chapter is to estimate and compare the prevalence of SRLV in the world by performing a systematic review and meta-analysis of the articles published during the last 40 years (1981-2020) complemented by comprehensive description of diagnostic test used.

MATERIAL AND METHODS

Literature search and recording of information

Literature of the last 40 years (1981-2020, both included) dealing with SRLV prevalence in sheep was collected following PRISMA guidelines (Moher *et al.*, 2009; Hutton, Catalá-López and Moher, 2016). A flow diagram describing the selection process of references is detailed in Appendix Ch5-1. Different databases were checked including PubMed, WOS and Scopus. Keywords included: maedi, maedi-visna, maedi/visna, small ruminant lentivirus, SRLV and/or prevalence. Criteria for eligibility were: i) detailed information on SRLV prevalence in countries or regions within countries; ii) abstract written or translated in English or Spanish; iii) publication between 1981 and 2020, both years included. The reference year of each prevalence study was the date in which the study was performed. Exceptionally, for publications in which this date was not available, the date of article publication was used. Criteria for exclusion were: i) total number of sampled animals not detailed; ii) studies focused on diseased sheep. Data items systematically collected are detailed in Appendix Ch5-2.

Analyses

Qualitative epidemiological variables obtained from publications (i.e., presence or absence of data on animal prevalence, flock prevalence, population size) and

information on diagnostic techniques (i.e., presence or absence of data on sensitivity and specificity, number and type of tests used) were analyzed using contingency tables and represented as absolute and relative frequencies. Additionally, the type of diagnostic tests used for SRLV prevalence determinations was described over the four decades that this meta-analysis includes. A test was considered as diagnostic when applied to all samples collected or a randomly selected group of them. Graphs were performed with Prism 8.0.2 (GraphPad Software).

The five main statistical parameters used in this meta-analysis are detailed in Appendix Ch5-3. Apparent prevalence (percentage of positive animals) was used as most publications did not detail specificity and sensitivity of diagnostic tests and consequently true prevalence (percentage of infected animals) could not be estimated. When the number of positive reactors to the test was not provided in the study, it was calculated with the prevalence and the sample size. When multiple diagnostic tests were performed, animals were considered positive if they were positive to at least one diagnostic test. Data obtained from publications were subdivided in two main groups: individual and flock prevalence. Moreover, prevalence data were grouped by continents and countries and values were calculated as the weighted arithmetic mean to attribute each study its relative importance, depending on sample size. The 95% confidence interval for the estimated prevalence values was calculated by using the formula of Wilson et al. (Wilson, 1927). This indicator provides a range of values in which the population prevalence can be found with a 95% degree of confidence. Heterogeneity of studies was quantified with the heterogeneity statistic I^2. This parameter is based on Cochran's Q test of homogeneity and provides useful information in the variability between the studies included in the meta-analysis. Additionally, an historical evolution of the infection by decades was performed. All the above-mentioned analyses were performed with Excel (Microsoft Office Professional Plus 2019), except for heterogeneity statistic I^2, which was calculated with OpenMeta[Analyst] software (Wallace *et al.*, 2012). Maps were performed with GeoNames extension (DSAT, Microsoft) for Excel. Finally, correlation and determination coefficients between animal and flock prevalence were calculated with Spearman's rank test by using IBM SPSS 19.0 for Windows (IBM Corporation).

RESULTS

Analysis of publications and diagnostic tests

A total of 58 publications were included in this meta-analysis (Appendix Ch5-1). All these publications showed individual prevalence studies whereas 65.5% (38/58) also contained flock prevalence studies. A total of 314 prevalence studies were recorded. Information on the total number of animals and flocks in the studied geographical area (global population size) was only provided in 31% (18/58) and 34.2% (13/38) of publications, respectively. Sensitivity and specificity of the diagnostic tests were detailed in 36.2% (21/58) of publications. Most articles used a single diagnostic test (77.6%, 45/58) to calculate prevalence whereas articles using 3 or more tests were scarce (6.9%, 4/58; Figure Ch5-1a). AGID was the most common diagnostic test from 1981 to 2000, showing a decreased importance over the years (Figure Ch5-1b). The use of ELISA showed a marked increase from 1981 to 2000, becoming the most important technique from 2001 to 2020, with constant values over the two decades. PCR has been used as a diagnostic tool for prevalence studies from 2001 to 2020 but there is a scarcity of publications using this technique as the main diagnostic tool. Histology and western blot have been occasionally used as diagnostic tools.

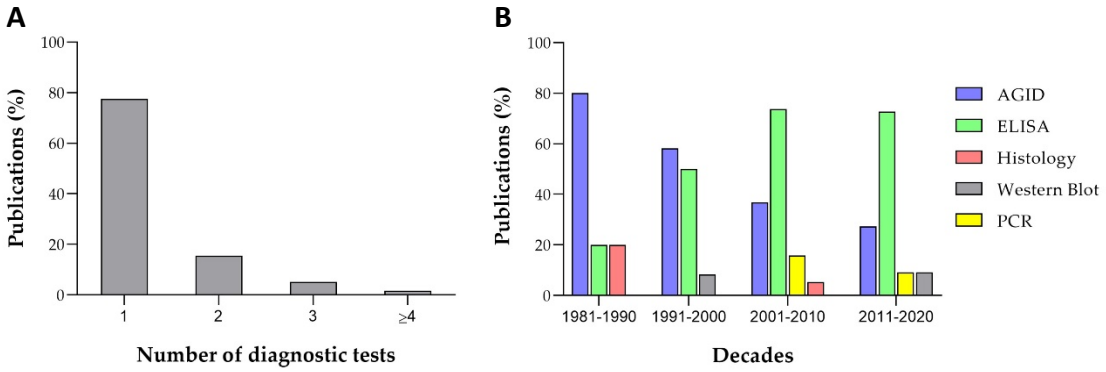


Figure Ch5-1: Analysis of diagnostic tests. (A) Number of diagnostic tests performed in each article. **(B)** Evolution of the diagnostic tests from 1981 to 2020.

Individual SRLV prevalence

Prevalence of SRLV infection in sheep per continent is provided in Table Ch5-1. Europe shows by far the highest value for SRLV prevalence (40.9%) whereas Africa, Asia

and North America show values in a range between 16.7% to 21.8%. South and Central America show the lowest individual SRLV prevalence (1.7%). Interestingly, results for Europe derive from 65 studies of SRLV prevalence in 22 publications with a total of 407509 sheep tested during the last 40 years, whereas Africa only shows 5 studies in 4 publications and a total of 1688 animals studied during the last four decades. All continents showed marked heterogeneity among studies, with prevalence values ranging from 0% to 71.2%. Appendix Ch5-4 provides an evolution of individual SRLV prevalence along decades in each continent.

Infected animals per country are detailed in Figure Ch5-2 and extended information is provided in Appendix Ch5-5. A total of 33 countries provided studies with valid data and were located in: Africa n=3, Asia n=10, Europe n=14, North America n=3 and South & Central America n=3. Countries with the highest individual SRLV prevalence are Lebanon, Greece and Spain. Spain is the country with the highest number of animals studied (308858).

Table Ch5-1: Individual SRLV prevalence in each continent. Data extracted from scientific literature published from 1981 to 2020.

Continent	Studies	N[1]	Prevalence (%)		Range (%)		Heterogeneity	Refs
			Mean	CI 95%[2]	Min	Max	I^2[3] (p value)	
Africa	5	1688	16.65	14.95 - 18.50	1.37	24.80	98.09 (<0.001)	[4]
Asia	47	8309	20.38	19.52 - 21.26	0.00	71.20	98.60 (<0.001)	[5]
Europe	65	407509	40.90	40.75 - 41.05	0.00	66.43	98.98 (<0.001)	[6]
North America	46	124542	21.76	21.53 - 21.99	0.00	52.00	99.61 (<0.001)	[7]
South & Central America	41	46418	1.67	1.56 - 1.79	0.00	30.00	91.52 (<0.001)	[8]
Global	**204**	**588466**	**33.39**	**33.27 - 33.51**	**0.00**	**71.20**	**99.95 (<0.001)**	

[1]N: Number of animals tested. [2]CI 95%: 95% Confidence Interval. [3]I^2: Heterogeneity statistic.

Publications: [4](Belino and Ezeifeka, 1984; Mahin, Chadli and Houwers, 1984; Bouljihad and Leipold, 1994; Ayelet *et al.*, 2001). [5](Preziuso *et al.*, 2010; Azkur, Gazyagci and Aslan, 2011; Giangaspero *et al.*, 2011; Albayrak *et al.*, 2012; Muz *et al.*, 2013; Tolari *et al.*, 2013; Zhang *et al.*, 2013; Norouzi *et al.*, 2015; Didugu *et al.*, 2016; Mosa and Zenad, 2020)(Giangaspero *et al.*, 1997; Mahmood *et al.*, 2012; Tabet *et al.*, 2017). [6](Rémond and Larenaudie, 1982; Caporale *et al.*, 1983; León and Prats, 1996; Schaller P, 2000; Sihvonen *et al.*, 2000; Espi-Felgueroso *et al.*, 2001; Berriatua *et al.*, 2003; Straub, 2004; Karanikolaou *et al.*, 2005; Alba *et al.*, 2008; Pérez *et al.*, 2010; Huttner, Seelmann and Feldhusen, 2010; Ritchie, Davies and Smith, 2012; Lago *et al.*, 2012; Junkuszew *et al.*, 2016; Olech, Osiński and Kuźmak, 2017; Michiels *et al.*, 2018; Savic *et al.*, 2020; Cana *et al.*, 2020; Echeverría *et al.*, 2020)(Hönger, Leitold and Schuller, 1990; Barták *et al.*, 2018). [7](Simard and Morley, 1991; Cutlip *et al.*, 1992; Campbell *et al.*, 1994; Arsenault *et al.*, 2003; APHIS, 2003; Fournier, Campbell and Middleton, 2006; Shuaib *et al.*, 2010; Gerstner *et al.*, 2015; Uzcanga, 2015; Heinrichs *et al.*, 2017). [8](Robles *et al.*, 2003; da Costa *et al.*, 2007; Lombardi *et al.*, 2009; Martinez *et al.*, 2011; Mendonça *et al.*, 2013; Trezeguet *et al.*, 2013; Villagra-Blanco *et al.*, 2015; Alves *et al.*, 2017; Vinha and Silva, 2020)

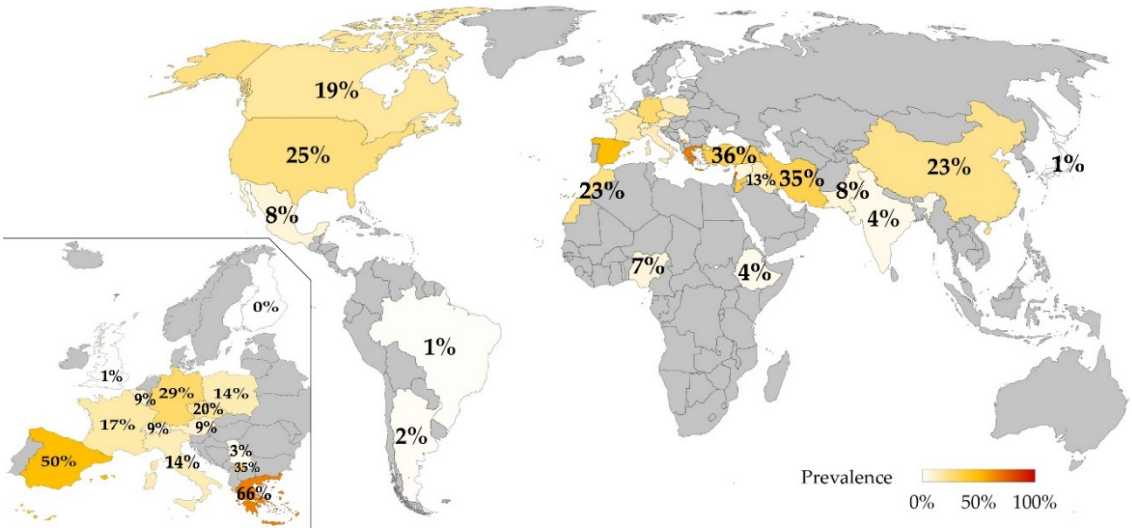


Figure Ch5-2: Individual SRLV prevalence (%) in sheep per country. Data extracted from scientific literature published from 1981 to 2020 and detailed in Appendix Ch5-5. Inset: Higher magnification of Europe.

Flock SRLV prevalence

Prevalence of flock SRLV infection per continent is provided in Table Ch5-2. Asia is the continent showing the highest flock prevalence (66%) whereas Europe and North America are within a range of 44.4 and 48.6%. Africa is the continent showing a lesser percentage of prevalence (7.7%). Flock SRLV prevalence showed marked heterogeneity among studies in all continents. Appendix Ch5-6 provides an evolution of SRLV prevalence along decades in each continent. Distribution of flock SRLV prevalence along decades parallels the temporal evolution of individual prevalence.

Infected flocks per country are detailed in Figure Ch5-3 and extended information is provided in Appendix Ch5-7. A total of 23 countries provided studies with valid data and were located in: Africa n=1; Asia n=7: Europe n=9; North America n=3; South & Central America n=3. Countries with the highest SRLV flock prevalence are Lebanon and China whereas Poland is the country with the highest number of flocks studied (1621).

Table Ch5-2: Flock SRLV prevalence in each continent. Data extracted from scientific literature published from 1981 to 2020.

Continent	Studies	N[1]	Prevalence (%)		Range (%)		Heterogeneity	Refs
			Mean	CI 95%[2]	Min	Max	I^2[3] (p value)	
Africa	1	13	7.69	1.37 - 33.31	7.69	7.69	N/A[4]	[5]
Asia	8	197	65.99	59.12 - 72.24	21.43	100.00	95.58 (<0.001)	[6]
Europe	35	4590	44.38	42.95 - 45.82	0.00	100.00	99.98 (<0.001)	[7]
North America	32	1933	48.58	46.35 - 50.81	0.00	100.00	95.26 (<0.001)	[8]
South & Central America	34	2358	18.87	17.34 - 20.50	0.00	100.00	83.15 (<0.001)	[9]
Global	**110**	**9091**	**39.07**	**38.07. 40.08**	**0**	**100**	**99.94 (<0.001)**	

[1]N: Number of animals tested. [2]CI 95%: 95% Confidence Interval. [3]I^2: Heterogeneity statistic. [4]N/A: Not applicable.

Publications: [5](Mahin, Chadli and Houwers, 1984). [6] (Giangaspero *et al.*, 1997, 2011; Muz *et al.*, 2013; Tolari *et al.*, 2013; Zhang *et al.*, 2013; Norouzi *et al.*, 2015; Tabet *et al.*, 2017). [7] (Rémond and Larenaudie, 1982; Caporale *et al.*, 1983; León and Prats, 1996; Sihvonen *et al.*, 2000; Berriatua *et al.*, 2003; Alba *et al.*, 2008; Pérez *et al.*, 2010; Huttner, Seelmann and Feldhusen, 2010; Ritchie, Davies and Smith, 2012; Lago *et al.*, 2012; Olech, Osiński and Kuźmak, 2017; Barták *et al.*, 2018; Michiels *et al.*, 2018; Cana *et al.*, 2020; Echeverría *et al.*, 2020). [8](Simard and Morley, 1991; Cutlip *et al.*, 1992; Campbell *et al.*, 1994; Arsenault *et al.*, 2003; APHIS, 2003; Shuaib *et al.*, 2010; Gerstner *et al.*, 2015; Uzcanga, 2015; Heinrichs *et al.*, 2017). [9](Robles *et al.*, 2003; da Costa *et al.*, 2007; Lombardi *et al.*, 2009; Mendonça *et al.*, 2013; Trezeguet *et al.*, 2013; Villagra-Blanco *et al.*, 2015; Vinha and Silva, 2020).

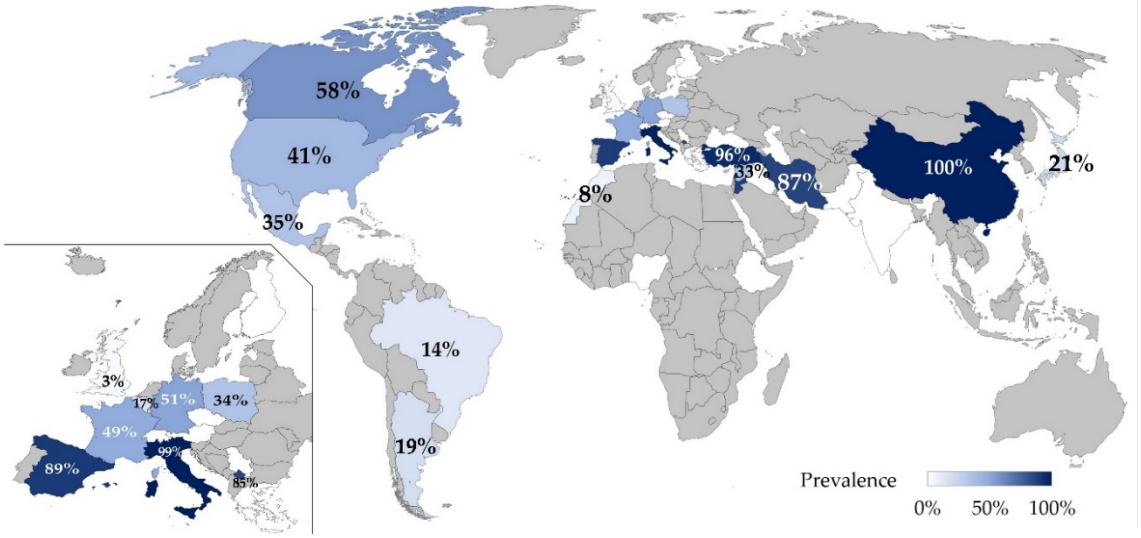


Figure Ch5-3: Flock prevalence (%) of SRLV in sheep by country. Data extracted from scientific literature published from 1981 to 2020 and detailed in Appendix Ch5-7. Inset: Higher magnification of Europe.

Correlation between individual and flock SRLV prevalence

Individual and flock prevalence was obtained from 118 studies. There was a strong positive correlation between individual and flock prevalence (ρ=0.728; p≤0.001). Linear regression (y=2.174x) demonstrated that each increase in individual prevalence induced at least 2-fold increase in flock prevalence. Indeed, in some cases, flock prevalence reached 60% when individual prevalence was below 30% (Figure Ch5-4). Determination coefficient was 0.530.

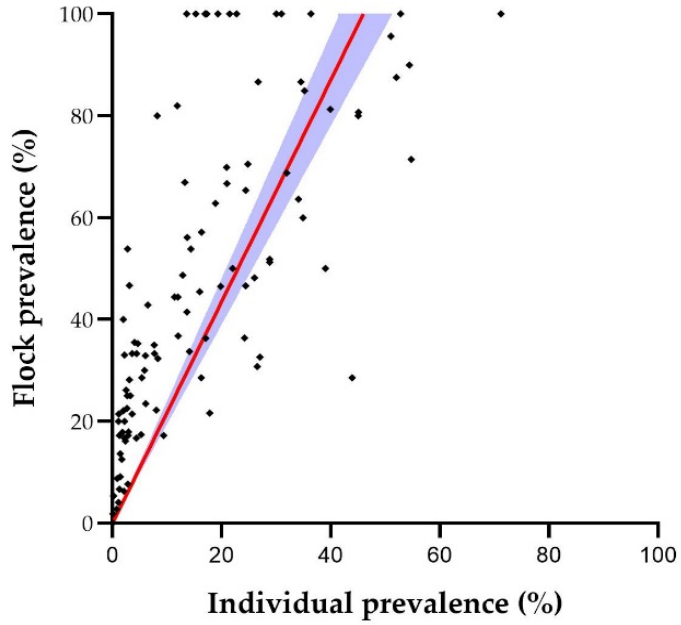


Figure Ch5-4: Correlation between individual and flock SRLV prevalence. Dots: data of each individual study (n=118). Red line: Linear regression. Blue area: 95% Confidence interval of the linear regression.

Publications: (Rémond and Larenaudie, 1982; Caporale *et al.*, 1983; Mahin, Chadli and Houwers, 1984; Simard and Morley, 1991; Cutlip *et al.*, 1992; Campbell *et al.*, 1994; León and Prats, 1996; Giangaspero *et al.*, 1997, 2011; Sihvonen *et al.*, 2000; Arsenault *et al.*, 2003; Berriatua *et al.*, 2003; Robles *et al.*, 2003; APHIS, 2003; da Costa *et al.*, 2007; Alba *et al.*, 2008; Lombardi *et al.*, 2009; Huttner, Seelmann and Feldhusen, 2010; Shuaib *et al.*, 2010; Lago *et al.*, 2012; Ritchie, Davies and Smith, 2012; Mendonça *et al.*, 2013; Muz *et al.*, 2013; Tolari *et al.*, 2013; Trezeguet *et al.*, 2013; Zhang *et al.*, 2013; Gerstner *et al.*, 2015; Norouzi *et al.*, 2015; Uzcanga, 2015; Villagra-Blanco *et al.*, 2015; Junkuszew *et al.*, 2016; Heinrichs *et al.*, 2017; Tabet *et al.*, 2017; Michiels *et al.*, 2018; Vinha and Silva, 2020; Cana *et al.*, 2020; Echeverría *et al.*, 2020).

DISCUSSION

This meta-analysis based on published research presents the distribution and prevalence of SRLV considering individual animals and flocks in the world over the last 40 years. Results indicate a widespread SRLV infection in all continents and underline the importance of SRLV in sheep throughout the world. SRLV is heterogeneously distributed, with marked variations not only between continents but also between regions in the same continent. This heterogeneity between studies reflect the multiple factor that influence SRLV prevalence.

Europe is the continent with most information on prevalence and distribution of infection as 1/3 of publications included in the study and 2/3 of animals analyzed belong to this continent. Recent phylogenetic studies suggest that SRLV-genotype A, historically associated with Visna/maedi in sheep, may have arisen in a territory within the current borders of Turkey and spread across the world with human migratory movements (Muz *et al.*, 2013; Carrozza *et al.*, 2018). First reports of lesions compatible with those caused by SRLV were likely reported in The Netherlands (Houwers, 1990) and the description of the disease together with the infection took place in Iceland (Pálsson, 1990). This could explain the marked interest of European countries in the study of this infection. North America and Asia also show a notable SRLV prevalence and a growing number of published studies in both continents. There are striking differences among countries in the number of animals tested against SRLV, Spain being the country with higher number of studied animals (n=308858) and Pakistan the country with lower tested sheep (n=93). Interpretation of prevalence studies with low sample size should be performed cautiously.

SRLV prevalence data depend on the different routes for viral spread. Horizontal (aerosols and direct contact) transmission is the main route for SRLV propagation and it is likely the route responsible for most of the SRLV clinical cases (Minguijón *et al.*, 2015). This route is influenced by numerous environmental, demographic and management factors (APHIS, 2003; Minguijón *et al.*, 2015). Additionally, vertical lactogenic transmission also plays an important epidemiological role, with up to 16% of lambs born from seropositive ewes being infected during their first day of life (Álvarez *et al.*, 2005).

The high individual SRLV prevalence found in Europe is likely associated with high density of ovine populations and intensive management. For instance, Greece and Spain show the highest prevalence values in Europe and both countries are within the top ten countries in milk production (Kilgour *et al.*, 2008), which is usually performed in intensive management systems and implies continuous close contact within animals. However, Italy is also among these top ten countries but its prevalence is not that high. Average flock size in dairy sheep are similar in Greece, Spain and Italy (140 to 161 ewes/farm) (Pulina *et al.*, 2018) thus this factor cannot explain the Italian moderate SRLV prevalence values. Of note, Europe is the third continent in the ranking of flock prevalence in spite of being the first in individual prevalence. This decrease might be associated to the several control and eradication programs performed in European flocks during the last decades.

Iran, Turkey, China, Jordan and Lebanon are the Asiatic countries with the highest SRLV individual prevalence. Indeed, Iran, Turkey and China are major producers of meat and milk (Kilgour *et al.*, 2008; Morris, 2017), further highlighting the relevance of the production type and management system (Global sheep distribution, 2010; FAO, 2021; Gilbert *et al.*, 2018). Based on the FAO database, Asia is the continent with the highest ovine population of the world and this fact could be determinant in SRLV transmission between flocks, as Asia is the continent with the highest flock SRLV prevalence. Interestingly, most ovine breeding stocks are located in China and India and individual SRLV prevalence in these countries is strikingly different, pointing out that the total number of animals in a given geographical area is not a relevant factor for intra-flock SRLV transmission. Differences between individual prevalence values of these two countries could be likely explained by differences in management systems, being mainly semi-intensive to intensive in the former and extensive to nomadic in the latter (Singh *et al.*, 2005; Kilgour *et al.*, 2008). A similar explanation could be applied to the differences found between individual prevalence values in African countries, where Morocco shows much higher values than Ethiopia and Nigeria, for instance. About 75% of sheep in Ethiopia are kept on small-scale mixed farms, with an average number of 5.3 sheep per farm, usually raised in privately owned land (Holden and Shiferaw, 2000; Mekoya *et al.*, 2000; Kilgour *et al.*, 2008). The Animal and Plant Health Inspection Service

of the United States Department of Agriculture provides an outstanding explanation on the influence of cultural, geographical and management factors in SRLV prevalence in North America (APHIS, 2003) showing the significant role of transport (i.e., crowding of animals) and the knowledge and concern of farmers about the disease. Finally, low values of individual SRLV prevalence in South America are likely associated with the low number of animals in this region together with management factors, mostly extensive rearing (Global sheep distribution, 2010; FAO, 2021; Gilbert et al., 2018).

Results of this meta-analysis greatly depend on the generation of prevalence data and their publication in scientific repositories. Indeed, a discrepancy between OIE reports and the information available in the scientific literature on SRLV prevalence was noted. Based on the information provided by the OIE (World Animal Health Information Database, 2021), there are 28 additional countries[8] with SRLV infection in sheep that are absent of our study because no public publications from those countries were found, or they did not fulfilled the inclusion criteria. On the contrary, from 2005 to 2019, China, Costa Rica, Iran, Morocco and Pakistan have never reported SRLV infection to the OIE and India, Lebanon, Czech Republic, Syria and Turkey appear as "disease absent" despite available scientific descriptions from all these countries (Mahin, Chadli and Houwers, 1984; Bouljihad and Leipold, 1994; Giangaspero *et al.*, 1997; Preziuso *et al.*, 2010; Azkur, Gazyagci and Aslan, 2011; Albayrak *et al.*, 2012; Mahmood *et al.*, 2012; Zhang *et al.*, 2013; Muz *et al.*, 2013; Villagra-Blanco *et al.*, 2015; Norouzi *et al.*, 2015; Didugu *et al.*, 2016; Tabet *et al.*, 2017; Barták *et al.*, 2018). First reports on SRLV infection in Brazil according to OIE are from 2017, whereas scientific publications already reported the disease in 2007 (da Costa *et al.*, 2007). Therefore, publicly available scientific literature might not reflect the real situation in different countries; for instance, a prevalence of 0.7% is the only data available from the UK (Ritchie, Davies and Smith, 2012) but the infection seems to be much more widespread, reaching a high number of flocks and individual animals. Indeed, recent studies in Scottish flocks with a novel multiplex ELISA (MVD-Enferplex GSMD multiplex ELISA Kit, MV Diagnostics, Edinburgh, UK) revealed an

[8] Andorra, Bosnia and Herzegovina, Bulgaria, Chile, Colombia, Comoros, Croatia, Cyprus, Denmark, Estonia, North Macedonia, Hungary, Israel, Latvia, Liechtenstein, Luxembourg, Malta, Mexico, Mongolia, Netherlands, Norway, Autonomous Palestinian Territories, Portugal, Romania, Russia, Slovakia, Slovenia, Sweden.

individual prevalence of 11.7% (sample size: 2659 animals) and SRLV infection present in 15 out of 17 studied flocks (N. Watt, unpublished data, 2020). Accordingly, flocks with more than 10 years within the Scottish Visna/maedi control scheme showed spontaneous outbreaks of disease with up to 90% of animals infected (Synge and Ritchie, 2010; Ritchie and Hosie, 2014)

As expected, results clearly indicate that flock prevalence is linked to individual prevalence. Indeed, flock prevalence generally doubles individual prevalence. Interestingly, some infected areas show a 100% prevalence in flocks while the infection is just about 20% in animals, suggesting that the multiplying factor could be higher than two-fold under certain conditions. Increased prevalence is related to any activity that may imply prolonged close contact between animals favoring horizontal transmission such as intensive production system (APHIS, 2003; Minguijón *et al.*, 2015), transportation or sharing milking machines (Barquero *et al.*, 2013; Savic *et al.*, 2020). Flock size might also play a role as higher numbers of animals per flock relate to higher prevalence (Cutlip *et al.*, 1992; Gerstner *et al.*, 2015).

SRLV diagnostic methods in sheep have substantially changed during the last decades but serologic methods have always been the most used techniques in prevalence studies. AGID was the most common technique in the 80's and 90's of the last century as it was the recommended test by the OIE for regulatory purposes (De Andrés *et al.*, 2005). However, from the beginning of the 21st century, several ELISA tests have replaced AGID as the most reliable method. Interestingly, most of the publications included in this meta-analysis used only a single diagnostic test to estimate the SRLV prevalence. The use of a single test has proved to underestimate the number of infected animals, impairing proper segregation of infected and non-infected individuals, which leads to a slower control of the infection and hinders accurate analysis of productive and clinicopathological parameters (Muz *et al.*, 2013; Echeverría *et al.*, 2020). In the current situation of uncertainty regarding circulating SRLV strains, the most reliable/efficient strategy to identify infected animals would involve performing at least two diagnostic tests in parallel. The election of the most suitable diagnostic tests should be carefully considered based on the most prevalent circulating strain/s in a geographical area. The first test should be ELISA-based as they are highly sensitive and

specific, thus suitable for high-throughput testing (De Andrés *et al.*, 2005). However, sensitivity and specificity of any ELISA diagnostic test should not be considered universal due to the scarcity of cross-reacting antibodies among different SRLV genotypes (Leginagoikoa *et al.*, 2006; Glaria *et al.*, 2012). Serologic approaches may have disadvantages as they cannot detect animals with low antibody titers, which can remain as carriers and potentially cause disease outbreaks (Saman *et al.*, 1999). The second test should be complementary to the first one and targeted towards specific animal populations. For instance, most of the publications analyzed here excluded animals younger than 6 months as colostral antibodies can interfere with serologic testing. This could imply overlooking an important group of animals that are pivotal for SRLV transmission (Álvarez *et al.*, 2005; Leginagoika *et al.*, 2006).. Direct methods such as PCR could mitigate ELISA drawbacks by detecting the viral load peak found in infected lambs during the neonatal period (Álvarez *et al.*, 2006; Shuaib *et al.*, 2010). Therefore, using a direct technique as a second diagnostic test can help to detect recently-infected animals without a detectable serologic response (de Andrés *et al.*, 2013; Muz *et al.*, 2013). A combination of ELISA and PCR has already been proposed as a synergic way to accurately diagnose SRLV infection, as it provides excellent results and improves the accuracy of the diagnosis in both, acute and chronic infections (Belino and Ezeifeka, 1984; Carrozza *et al.*, 2009; Giangaspero *et al.*, 2011; Mosa and Zenad, 2020). Until recently, a commercial diagnostic PCR was not available commercially despite several publications setting up different protocols (Chassalevris *et al.*, 2020; Echeverría *et al.*, 2020). Therefore, this strategy can increase diagnosis sensitivity and potentially imply success in SRLV control strategies. However, it could simultaneously increase costs and, potentially, reduce diagnosis specificity. In any case, this strategy has proven to be useful as it has demonstrated the infection in flocks that were previously declared as uninfected and it has improved the sensitivity of the diagnosis in countries such as Spain (Echeverría *et al.*, 2020; Ramírez *et al.*, 2021), UK (Ritchie and Hosie, 2014) and Switzerland (Cardinaux *et al.*, 2013).

This analysis is based on multiple publications with important differences in the study design and efforts to group publications based on animal information (breed, sex or age) were fruitless. Despite this heterogeneity between SRLV prevalence, the present

meta-analysis provides a unique and valuable approach to worldwide SRLV distribution. Moreover, review of individual publications can help to dissect the influence of these individual parameters. For instance, breed is clearly a predisposing factor in multiple geographic areas (Cutlip *et al.*, 1992; Ayelet *et al.*, 2001; Muz *et al.*, 2013). In this sense, genetic selection of resistant animals has been proposed as a control method, with TMEM154 as a promising target gene (Molaee, Eltanany and Lühken, 2018). However, a recent study demonstrates that control measures based on a single gene may not be as useful as expected (Ramírez *et al.*, 2021). Age also seems to be related to higher prevalence of infection, with a non-linear increase that reaches the maximum at about four years of age (Cutlip *et al.*, 1992; APHIS, 2003; Gerstner *et al.*, 2015). Influence of sex in prevalence is not that obvious, some studies indicate males being more predisposed than females (Savic *et al.*, 2020) whereas others indicate non-significant differences (Simard and Morley, 1991; Cutlip *et al.*, 1992; Arsenault *et al.*, 2003).

Limitations of this work arise from the diversity of study designs and data expression. Specificity and sensitivity of the test was only specified in 36.2% of publications and we decided to deal with apparent prevalence to avoid disregarding most of the selected publications. Technical information of the diagnostic test employed should be stated whenever possible to ease further data analysis (Field *et al.*, 2014; PRISMA Statement Flow Diagram, 2020). Further studies investigating the sensitivities and specificities of the test that were not provided in the studied publications will be useful and interesting so that further analysis of these data based on real prevalence values could be performed. Unfortunately, recurring prevalence studies in the same geographical area are scarce. A publication bias might exist when SRLV infection is discovered in a certain area, likely leading to an increased number of reports, paralleling the increase of scientific interest. In areas where the disease is enzootic, the interest might not be similar.

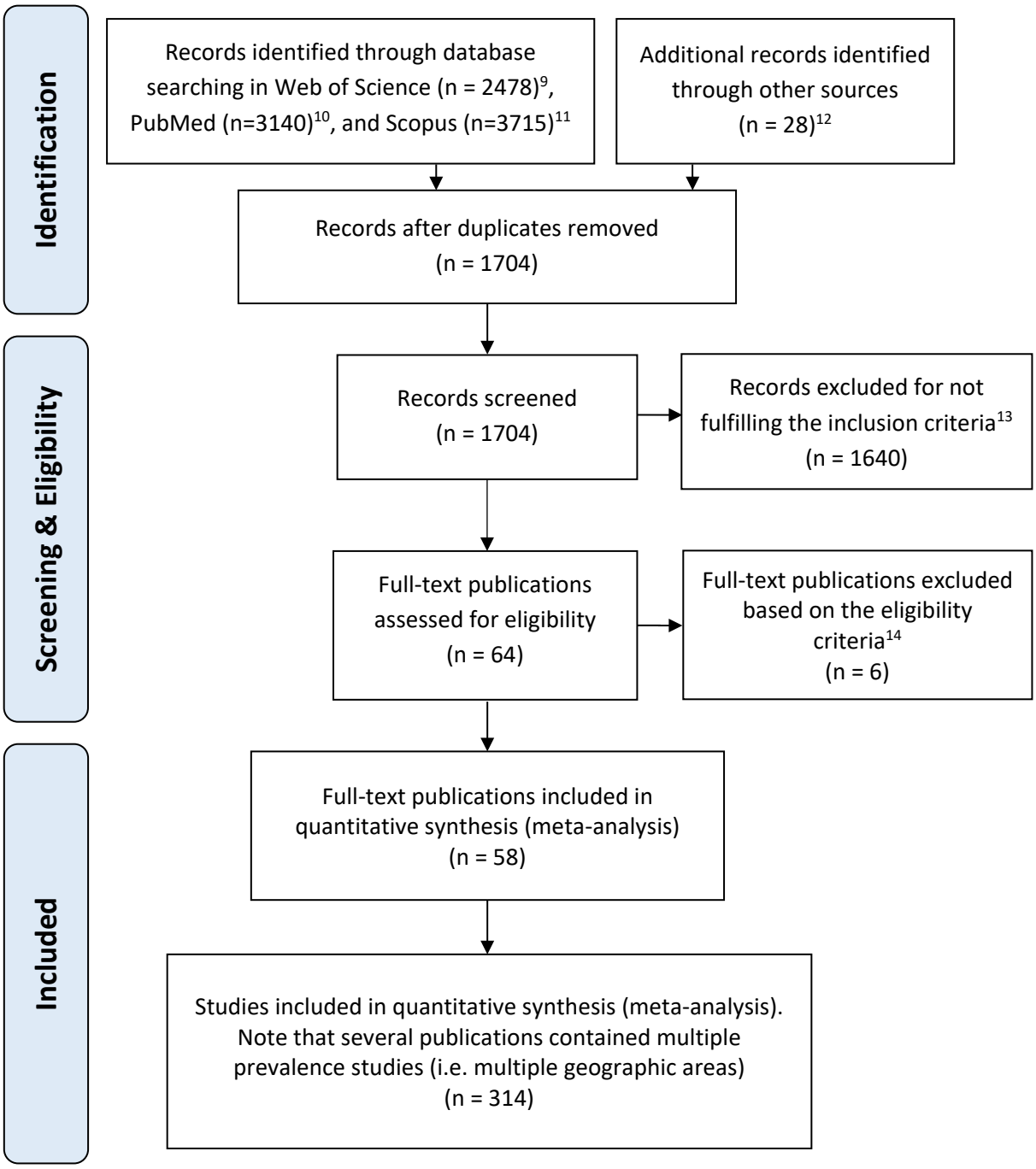
CONCLUSIONS

SRLV infection in sheep is widespread across the world, Europe showing the highest individual prevalence and being the geographical area in which more studies have been performed. Prevalence of infected flocks shows a strong correlation with the individual prevalence. Most studies are based on a single diagnostic test, implying a risk of underestimating the real infection prevalence. Serological tests are more commonly used than direct methods and among them, ELISA has progressively replaced AGID along the last two decades. There is a moderate disagreement between the information reported to the OIE and the scientific literature. This review highlights the need for more systematic and frequent prevalence studies using a consistent testing strategy.

APPENDICES

- **Appendix Ch5-1:** Flow diagram indicating procedure for identification, screening, selection and inclusion of publications in this meta-analysis.
- **Appendix Ch5-2:** Information that was systematically extracted from the publications included in the meta-analysis.
- **Appendix Ch5-3:** Definition of the parameters used in the epidemiological meta-analysis.
- **Appendix Ch5-4:** Individual SRLV prevalence in each continent by decades.
- **Appendix Ch5-5:** Individual SRLV prevalence in each country.
- **Appendix Ch5-6:** Flock SRLV prevalence in each continent by decades.
- **Appendix Ch5- 7:** Flock SRLV prevalence in each country.

Appendix Ch5-1: Flow diagram indicating procedure for identification, screening, selection and inclusion of publications in this meta-analysis. Modified from the PRISMA Statement (PRISMA Statement Flow Diagram, 2020).[91011121314]



[9] **Keywords in Web of Science.** maedi: 1641; maedi prevalence: 196; maedi-visna: 161; maedi-visna prevalence: 161; maedi/visna: 161; SRLV: 40; SRLV prevalence: 40; small ruminant lentivirus: 39; small ruminant lentivirus prevalence: 39

[10] **Keywords in Pubmed.** maedi: 733; maedi prevalence: 156; maedi-visna: 709; maedi-visna prevalence: 152; maedi/visna: 709; SRLV: 189; SRLV prevalence: 73; small ruminant lentivirus: 328; small ruminant lentivirus prevalence: 91

[11] **Keywords in Scopus.** maedi: 1019; maedi prevalence: 189; maedi-visna: 578; maedi-visna prevalence: 150; maedi/visna: 968; SRLV: 281; SRLV prevalence: 85; small ruminant lentivirus: 326; small ruminant lentivirus prevalence: 119

[12] **Other sources.** Conference Proceedings: 9; Google Scholar: 19

[13] **Eligibility criteria:** Information on SRLV prevalence; Abstract available in English; Publication from 1981 to 2020, both included.

[14] **Exclusion criteria:** Total number of sampled animals not detailed; Study focused on diseased sheep.

Appendix Ch5-2: Information that was systematically extracted from the publications included in the meta-analysis.

- Information related to the publication time and location:
    - Country of the study.
    - Continent of the study.
    - Year of the study.
    - Year of the publication.
- Information related to diagnostic procedures:
    - Number of techniques employed in the study.
    - Type of diagnostic technique.
    - Sensitivity and specificity of tests.
- Information related to epidemiological data of the population:
    - Total number of animals in the geographical area of the study.
    - Total number of flocks in the geographical area of the study.
    - Information related to epidemiological data of the sample.
    - Sample size.
    - Number of positive animals and/or flocks (A flock was considered positive when one or more animals were positive to SRLV test).
    - Subgroups of samples within the same publication. (i.e. Prevalence in different regions. These were considered as independent studies).
- Information related to production system factors:
    - Flock size.
    - Type of management system
    - Information related to the animal factors.
    - Breed of sampled animals.
    - Sex of sampled animals.
    - Age of sampled animals.

Appendix Ch5-3: Definition of the parameters used in the epidemiological meta-analysis.

Mathematical equations
$\text{Apparent prevalence} = \left(\frac{\text{Pos}}{\text{n}}\right) \cdot 100$
$\text{Positive animals or flocks} = \frac{\text{P} \cdot \text{n}}{100}$
$\text{Confidence interval} = \frac{2\text{nP} + Z_{\frac{\alpha}{2}}^2 \pm Z_{\frac{\alpha}{2}} \sqrt{4\text{nP}(1-\text{P}) + Z_{\frac{\alpha}{2}}^2}}{2\left(\text{n} + Z_{\frac{\alpha}{2}}^2\right)}$
$\text{Weighted artihmetic mean of prevalences} = \frac{\sum \text{Pi} \cdot \text{ni}}{\sum \text{ni}}$
$\text{Heterogeneity statistic I\^2} = \frac{Q - df}{\text{Q}} \times 100$

Pos: Number of positive animals.
n: Sample size.
P: Prevalence value.
Z value: Varies depending of the % of confidence.
α: Alpha level or difference between 100% and the confidence interval.
Pi: Prevalence value of each study to be averaged.
ni: Sample size of each study to be averaged.
Q: Cochran's homogeneity test statistic.
df: degrees of freedom.

Appendix Ch5-4: Individual SRLV prevalence in each continent by decades. Mean, number of animals tested (N), 95% Confidence Interval (CI 95%) and Range are provided.

Animal SLRV prevalence in each continent from 1981 to 1990.

Continent	Studies	N	Prevalence		Range	
			Mean	CI 95%	Min	Max
Africa	1	72	2.78	0.77 - 9.57	2.78	2.78
Asia	0	0	N/A[1]	N/A	N/A	N/A
Europe	5	24969	16.34	15.88 - 16.80	1.70	26.67
North America	19	57593	19.13	18.81 - 19.45	0.00	39.92
South & Central America	0	0				
Global	**25**	**82634**	**18.27**	**18.01 - 18.54**	**0.00**	**39.92**

[1]N/A: Not applicable.

Animal SLRV prevalence in each continent from 1991 to 2000.

Continent	Studies	N	Prevalence		Range	
			Mean	CI 95%	Min	Max
Africa	2	1267	20.99	18.84 - 23.32	6.74	24.80
Asia	13	2890	6.02	5.21 - 6.95	0.00	12.20
Europe	11	26232	9.10	8.76 - 9.45	0.00	24.00
North America	21	39444	26.49	26.05 - 26.92	2.19	52.00
South & Central America	0	0	N/A[1]	N/A	N/A	N/A
Global	**47**	**69833**	**19.01**	**18.72 - 19.30**	**0.00**	**52.00**

[1]N/A: Not applicable.

Animal SLRV prevalence in each continent from 2001 to 2010.

Continent	Studies	N	Prevalence		Range	
			Mean	CI 95%	Min	Max
Africa	2	349	3.72	2.19 - 6.27	1.37	5.42
Asia	17	3889	28.57	27.17 - 30.01	0.00	68.97
Europe	5	296695	51.25	51.07 - 51.43	24.80	66.43
North America	3	23923	22.21	21.69 - 22.74	1.95	28.82
South & Central America	18	26529	1.09	0.97 - 1.22	0.00	4.42
Global	**45**	**351385**	**45.19**	**45.02 - 45.35**	**0.00**	**68.97**

Animal SLRV prevalence in each continent from 2011 to 2020.

Continent	Studies	N	Prevalence		Range	
			Mean	CI 95%	Min	Max
Africa	0	0	N/A[1]	N/A	N/A	N/A
Asia	17	1530	26.67	24.51 - 28.94	4.29	71.20
Europe	44	59613	13.65	13.38 - 13.93	0.00	54.73
North America	3	3582	8.93	8.04 - 9.91	4.63	15.19
South & Central America	23	19889	2.45	2.24 - 2.67	0.00	30.00
Global	**87**	**84614**	**11.05**	**10.84 - 11.27**	**0.00**	**71.20**

[1]N/A: Not applicable.

Appendix Ch5-5: Individual SRLV prevalence in each country. Mean and 95% Confidence Interval and Range are provided. Data extracted from scientific literature published from 1981 to 2020.

Country	Studies	N[1]	Prevalence (%)		Range (%)		Refs
			Mean	CI 95%[2]	Min	Max	
AFRICA							
Ethiopia	2	349	3.72	2.19 - 6.27	1.37	5.42	[3]
Morocco	2	1072	23.32	20.89 - 25.94	2.78	24.80	[4]
Nigeria	1	267	6.74	4.31 - 10.40	6.74	6.74	[5]
ASIA							
China	12	672	22.77	19.76 - 26.09	5.36	50.00	[6]
India	1	140	4.29	1.98 - 9.03	4.29	4.29	[7]
Iraq	1	210	12.86	8.99 - 18.06	12.86	12.86	[8]
Iran	1	220	34.55	28.58 - 41.05	34.55	34.55	[9]
Japan	4	771	0.78	0.36 - 1.69	0.00	1.24	[10]
Jordan	1	231	36.36	30.43 - 42.74	36.36	36.36	[11]
Lebanon	1	184	71.20	64.27 - 77.25	71.20	71.20	[12]
Pakistan	1	93	7.53	3.69 - 14.73	7.53	7.53	[13]
Syria	13	2890	6.02	5.21 - 6.95	0.00	12.20	[14]
Turkey	12	2898	35.51	33.79 - 37.27	0.00	68.97	[15]
EUROPE							
Austria	1	883	9.40	7.65 - 11.50	9.40	9.40	[16]
Belgium	1	555	9.37	7.22 - 12.08	9.37	9.37	[17]
Czech Republic	1	2801	19.85	18.41 - 21.37	19.85	19.85	[18]
Finland	1	10802	0.00	0.00 - 0.04	0.00	0.00	[19]
France	3	23404	16.68	16.20 - 17.16	1.70	26.67	[20]
Germany	2	2252	28.51	26.68 - 30.41	0.00	28.80	[21]
Greece	1	143	66.43	58.35 - 73.65	66.43	66.43	[22]
Italy	1	682	13.64	11.26 - 16.42	13.64	13.64	[23]
Kosovo	6	10544	35.19	34.28 - 36.10	12.92	45.93	[24]
Poland	16	19253	14.33	13.84 - 14.83	0.16	54.73	[25]
Serbia	14	11709	3.38	3.07 - 3.73	0.00	9.96	[26]
Spain	16	308858	49.84	49.67 - 50.02	1.23	54.41	[27]
Switzerland	1	3866	9.00	8.14 - 9.94	9.00	9.00	[28]
United Kingdom	1	11757	0.74	0.60 - 0.91	0.74	0.74	[29]
NORTH AMERICA							
Canada	29	68019	19.21	18.92 - 19.51	0.00	50.00	[30]
Mexico	1	157	7.64	4.43 - 12.88	7.64	7.64	[31]
United States	16	56366	24.87	24.51 - 25.23	2.19	52.00	[32]
SOUTH AND CENTRAL AMERICA							
Argentina	29	42597	1.69	1.57 - 1.82	0.00	30.00	[33]
Brazil	6	3103	1.32	0.98 - 1.79	0.11	8.20	[34]
Cosa Rica	6	718	1.95	1.16 - 3.25	0.00	4.26	[35]

[1]N: Number of animals tested. [2]CI 95%: 95% Confidence Interval.

Publications: [3](Ayelet *et al.*, 2001) [4](Mahin, Chadli and Houwers, 1984; Bouljihad and Leipold, 1994) [5](Belino and Ezeifeka, 1984) [6](Zhang *et al.*, 2013) [7](Didugu *et al.*, 2016) [8](Mosa and Zenad, 2020) [9](Norouzi *et al.*, 2015) [10](Giangaspero *et al.*, 2011) [11](Tolari *et al.*, 2013) [12](Tabet *et al.*, 2017) [13](Mahmood *et al.*, 2012) [14](Giangaspero *et al.*, 1997) [15](Preziuso *et al.*, 2010; Azkur, Gazyagci and Aslan, 2011; Albayrak *et al.*, 2012; Muz *et al.*, 2013) [16](Hönger, Leitold and Schuller, 1990) [17](Michiels *et al.*, 2018) [18](Barták *et al.*, 2018) [19](Sihvonen *et al.*, 2000) [20](Rémond and Larenaudie, 1982) [21](Straub, 2004; Huttner, Seelmann and Feldhusen, 2010) [22](Karanikolaou *et al.*, 2005) [23](Caporale *et al.*, 1983) [24](Cana *et al.*, 2020) [25](Junkuszew *et al.*, 2016; Olech, Osiński and Kuźmak, 2017) [26](Savic *et al.*, 2020) [27](León and Prats, 1996; Espi-Felgueroso *et al.*, 2001; Berriatua *et al.*, 2003; Alba *et al.*, 2008; Pérez *et al.*, 2010; Lago *et al.*, 2012; Echeverría *et al.*, 2020) [28](Schaller P, 2000) [29](Ritchie, Davies and Smith, 2012) [30](Simard and Morley, 1991; Arsenault *et al.*, 2003; Fournier, Campbell and Middleton, 2006; Shuaib *et al.*, 2010; Heinrichs *et al.*, 2017) [31](Uzcanga, 2015) [32](Cutlip *et al.*, 1992; Campbell *et al.*, 1994; APHIS, 2003; Gerstner *et al.*, 2015) [33](Robles *et al.*, 2003; Trezeguet *et al.*, 2013) [34](da Costa *et al.*, 2007; Lombardi *et al.*, 2009; Martinez *et al.*, 2011; Mendonça *et al.*, 2013; Alves *et al.*, 2017; Vinha and Silva, 2020) [35](Villagra-Blanco *et al.*, 2015)

Appendix Ch5-6: Flock SRLV prevalence in each continent by decades. Mean, number of flock tested (N), 95% Confidence Interval (CI 95%) and Range are provided.

Flock SLRV prevalence in each continent from 1981 to 1990.

Continent	Studies	N	Prevalence		Range	
			Mean	CI 95%	Min	Max
Africa	1	13	7.69	1.37 - 33.31	7.69	7.69
Asia	0	0	N/A[1]	N/A	N/A	N/A
Europe	4	477	58.49	54.02 - 62.83	12.50	98.94
North America	12	675	62.96	59.26 - 66.52	0.00	100.00
South & Central America	0	0	N/A	N/A	N/A	N/A
Global	**17**	**1165**	**60.52**	**57.68 - 63.28**	**0**	**100**

[1]N/A: Not applicable.

Flock SLRV prevalence in each continent from 1991 to 2000.

Continent	Studies	N	Prevalence		Range	
			Mean	CI 95%	Min	Max
Africa	0	0	N/A[1]	N/A	N/A	N/A
Asia	1	73	32.88	23.19 - 44.27	32.88	32.88
Europe	8	445	11.01	8.43 - 14.26	0.00	100.00
North America	15	357	53.22	48.04 - 58.34	20.00	100.00
South & Central America	0	0	N/A	N/A	N/A	N/A
Global	**24**	**875**	**30.06**	**27.11 - 33.18**	**0.00**	**100.00**

[1]N/A: Not applicable.

Flock SLRV prevalence in each continent from 2001 to 2010.

Continent	Studies	N	Prevalence		Range	
			Mean	CI 95%	Min	Max
Africa	0	0	N/A[1]	N/A	N/A	N/A
Asia	3	67	76.12	64.67 - 84.73	21.43	95.65
Europe	4	912	92.65	90.78 - 94.17	51.22	100.00
North America	2	759	34.91	31.61 - 38.37	22.08	36.36
South & Central America	17	1234	12.97	11.21 - 14.96	0.00	25.00
Global	**26**	**2972**	**44.82**	**42.67 - 46.24**	**0.00**	**100.00**

[1]N/A: Not applicable.

Flock SLRV prevalence in each continent from 2011 to 2020.

Continent	Studies	N	Prevalence		Range	
			Mean	CI 95%	Min	Max
Africa	0	0	N/A[1]	N/A	N/A	N/A
Asia	4	57	96.49	88.08 - 99.03	83.33	100.00
Europe	19	2756	31.35	29.64 - 33.11	2.75	100.00
North America	3	142	41.55	33.77 - 49.77	35.00	51.85
South & Central America	17	1124	25.36	22.90 - 27.98	0.00	100.00
Global	**43**	**4079**	**30.96**	**29.56 - 32.40**	**0.00**	**100.00**

[1]N/A: Not applicable.

Appendix Ch5- 7: Flock SRLV prevalence in each country. Mean and 95% Confidence Interval and Range are provided. Data extracted from scientific literature published from 1981 to 2020.

Country	Studies	N[1]	Prevalence (%)		Range (%)		Refs
			Mean	CI 95%[2]	Min	Max	
AFRICA							
Ethiopia	0	0	N/A[3]	N/A	N/A	N/A	N/A
Morocco	1	13	7.69	1.37 - 33.31	7.69	7.69	[4]
Nigeria	0	0	N/A	N/A	N/A	N/A	N/A
ASIA							
China	1	24	100.00	86.20 - 100.00	100.00	100.00	[5]
India	0	0	N/A	N/A	N/A	N/A	N/A
Iraq	0	0	N/A	N/A	N/A	N/A	N/A
Iran	1	30	86.67	70.32 - 94.69	86.67	86.67	[6]
Japan	1	14	21.43	7.57 - 47.59	21.43	21.43	[7]
Jordan	2	17	88.24	65.66 - 96.71	83.33	100.00	[9]
Lebanon	1	16	100.00	80.64 - 100.00	100.00	100.00	[10]
Pakistan	0	0	N/A	N/A	N/A	N/A	N/A
Syria	1	73	32.88	23.19 - 44.27	32.88	32.88	[11]
Turkey	1	23	95.65	79.01 - 99.23	95.65	95.65	[12]
EUROPE							
Austria	0	0	N/A	N/A	N/A	N/A	N/A
Belgium	1	87	17.24	10.74 - 26.52	17.24	17.24	[13]
Czech Republic	0	0	N/A	N/A	N/A	N/A	N/A
Finland	1	340	0.00	0.00 - 1.12	0.00	0.00	[14]
France	3	383	48.56	43.60 - 53.56	12.50	86.67	[15]
Germany	1	41	51.22	36.48 - 65.75	51.22	51.22	[16]
Greece	0	0	N/A	N/A	N/A	N/A	N/A
Italy	1	94	98.94	94.22 - 99.81	98.94	98.94	[17]
Kosovo	1	318	84.91	80.56 - 88.42	84.91	84.91	[18]
Poland	15	1621	34.24	31.97 - 36.58	3.77	71.43	[19]
Serbia	0	0	N/A	N/A	N/A	N/A	N/A
Spain	11	980	89.49	87.41 - 91.26	6.67	100.00	[20]
Switzerland	0	0	N/A	N/A	N/A	N/A	N/A
United Kingdom	1	726	2.75	1.79 - 4.22	2.75	2.75	[21]
NORTH AMERICA							
Canada	15	849	58.30	54.96 - 61.58	0.00	100.00	[22]
Mexico	1	20	35.00	18.12 - 56.71	35.00	35.00	[23]
United States	16	1064	41.07	38.15 - 44.05	20.00	87.50	[24]
SOUTH AND CENTRAL AMERICA							
Argentina	29	2239	18.94	17.37 - 20.61	0.00	100.00	[25]
Brazil	4	104	14.42	8.94 - 22.44	1.85	80.00	[26]
Cosa Rica	1	15	40.00	19.82 - 64.25	40.00	40.00	[27]

[1]N: Number of animals tested. [2]CI 95%: 95% Confidence Interval. [3]N/A: Not Applicable.

Publications: [4](Mahin, Chadli and Houwers, 1984) [5](Zhang *et al.*, 2013) [6](Norouzi *et al.*, 2015) [7](Giangaspero *et al.*, 2011) [9](Tolari *et al.*, 2013) [10](Tabet *et al.*, 2017) [11](Giangaspero *et al.*, 1997) [12](Muz *et al.*, 2013) [13](Michiels *et al.*, 2018) [14](Sihvonen *et al.*, 2000) [15](Rémond and Larenaudie, 1982) [16](Huttner, Seelmann and Feldhusen, 2010) [17](Caporale *et al.*, 1983) [18](Cana *et al.*, 2020) [19](Olech, Osiński and Kuźmak, 2017) [20](León and Prats, 1996; Berriatua *et al.*, 2003; Alba *et al.*, 2008; Pérez *et al.*, 2010; Lago *et al.*, 2012; Echeverría *et al.*, 2020) [21](Ritchie and Hosie, 2014) [22](Simard and Morley, 1991; Arsenault *et al.*, 2003; Shuaib *et al.*, 2010; Heinrichs *et al.*, 2017) [23](Uzcanga, 2015) [24](Cutlip *et al.*, 1992; Campbell *et al.*, 1994; APHIS, 2003; Gerstner *et al.*, 2015) [25](Robles *et al.*, 2003; Trezeguet *et al.*, 2013) [26](da Costa *et al.*, 2007; Lombardi *et al.*, 2009; Mendonça *et al.*, 2013; Vinha and Silva, 2020) [27](Villagra-Blanco *et al.*, 2015).

GLOBAL DISCUSSION AND FUTURE PERSPECTIVES

Veterinary vaccination is a very important tool to control infectious diseases, allowing an undebatable increase in animal welfare and production as well as avoiding transmission of zoonotic pathogens to humans (Roth and Sandbulte, 2021). For most species, vaccine formulations are mainly based on inactivated protein parenterally delivered and administered with Al-adjuvants. Such immunization results in strong antibody production that confer partial or total protection against the pathogen. However, it can also incur in the generation of other immune reactions such as the formation of persistent granulomas.

This PhD work carries out research on the topic of ABAs in sheep and their relation with probably the most extended macrophage-tropic infection of sheep: SRLV. The experimental part of this project was divided in two main experiments designed to evaluate the effect of repetitive inoculations with Al-based vaccines in sheep and to study the interaction between Al-induced injection-site granulomas and the pathogenesis of SRLV. Since each chapter has been individually discussed, this final global dissertation wants to summarize the take-home messages and bring the reader to the conclusions.

Analysis of productive parameters of animals inoculated with ABAs showed moderate growth rate reduction in Vaccine group, likely associated with transient, short-termed fever episodes after inoculations and decreased daily intake. Indeed, differences in ADG were more pronounced in summer, probably related to the high environmental temperatures reached during this period and the associated heat-stress. However, none of the lambs injected with the adjuvant only or with Al-containing vaccines unequivocally developed a wasting syndrome similar to the described after the compulsory vaccination campaigns against bluetongue, i.e., the ASIA syndrome (Luján *et al.*, 2013). ASIA syndrome is a very complex form of disease, with many factors acting at the same time that occurred after the compulsory vaccination against bluetongue. Despite vaccination was the obvious triggering element, our results indicate that other factors are needed. In other words, Al is necessary but not sufficient to reproduce all the productive and clinicopathological features characterizing ASIA syndrome. Stresses in general such as extreme temperatures, lambing out of the natural period or the

repetitive use of vaccines with Al over time are within these factors but their relative importance has not been scientifically determined yet.

According to previous results, repetitive inoculation of Al-oxyhydroxide increases stress biomarkers (i.e., plasma cortisol) and induces a wide spectrum of significant ethological changes (i.e., increased aggressive interactions and stereotypies and decreased affiliative interactions) (Asín, *et al.*, 2020). However, present results indicate that clinical, hematological, and histopathological values revealed minimal abnormalities in the same group of animals, including lack of significant histopathological lesions. In this scenario, it can be concluded that presence of Al in the CNS can be directly related to the ethological changes observed while producing few, if any, clinicopathological changes. Indeed, whole transcriptomic studies in the encephalon of these animals showed that Al-oxyhydroxide dysregulated genes associated with neurological functions and the mitochondrial energy metabolism, both associated to neurological diseases (Varela-Martínez *et al.*, 2020).

Subcutaneous inoculation of Al-oxyhydroxide in sheep invariably leads to the formation of chronic inflammatory nodules composed of abundant Al-loaded macrophages, therefore defining the so-called Al-induced injection-site granulomas. These granulomas are more severe and persist longer in Vaccine groups than Adjuvant-only groups (Asín *et al.*, 2019). Translocation of Al-loaded macrophages from the subcutaneous tissue to the regional lymph node has been already observed (Asín *et al.*, 2019). Additionally, analytical measurements of Al content performed in this work, revealed higher amounts of Al in the lumbar spinal cord, mainly in the Adjuvant-only group but also in the Vaccine group. Lumogallion fluorescence stain allowed the location of the Al particles within the CNS of the same animals. Lumogallion-reactive deposits, more abundant in the lumbar spinal cord than in the parietal lobe may contribute to explain ethological changes and molecular routes linked to neurological damage. Micron-sized Al-deposits were more abundant in the gray matter than in the white matter and they were usually associated with glial-like cells. Increased number of pyknotic and shrunken neurons (dark neurons) found in the lumbar spinal cord of Adjuvant-only animals, may be related to the increased Al levels recorded in this

location. Thus, Al accumulation in the spinal cord is likely related to some of the motoneuronal problems and weakness observed in the chronic phase of ASIA syndrome.

Transcriptomic analysis in circulating PBMCs from Al-inoculated animals revealed marked upregulation of the *NF-κB* signaling pathway in Vaccine and Adjuvant-only groups and higher induction of the immune response in Vaccine group in contrast with Adjuvant-only group (Varela-Martínez *et al.*, 2018). Expression of genes associated with inflammatory signaling pathways in Al-induced granulomas, regional lymph node and spleen of these animals correlate with findings in circulating PBMCs. Indeed, chronic granulomas and regional lymph node showed moderate activation of pro-inflammatory pathways and spleen showed moderate upregulation of genes associated with innate and adaptive immune responses. *In vivo* studies in different animal species are crucial to shed light into the mechanism of action ABAs (Ghimire, 2015).

SRLV cause a serious and widespread viral infection in sheep with a marked tropism for the mononuclear-phagocytic system. By inducing and infecting M2 anti-inflammatory macrophages (Crespo *et al.*, 2011), SRLV modify the immune response to a T_h2-biased profile (Blacklaws, 2012) ensuring persistence and escape from antibody control (Andrésdóttir, 2003). This study establishes for the first time a consistent link between post-vaccine secondary effects and SRLV infections. Post-vaccination granulomas are composed of abundant activated macrophages that sustain viral replication as evidenced by molecular, virological and pathological methods. A similar relationship was established with infectious granulomas formed by *Mycobacterium tuberculosis* and HIV in humans (Diedrich *et al.*, 2020). Occurrence of granulomas and SRLV infection in the same animal is highly likely and may account for altered biodistribution and/or pathogenesis, due to the generalized use of vaccines and the worldwide SRLV spread. Viral genetic populations in granulomas resembled those in circulating PBMCs, suggesting a role for granulomas in favoring SRLV biodistribution, using the well-characterized *Trojan horse* mechanism (Minguijón *et al.*, 2015). As a consequence, antibody titers increased in both, healthy lambs born in SRLV-infected commercial flocks and in sheep chronically affected by SRLV-induced arthritis. On top of that, arthritic sheep inoculated with Al-containing vaccines showed a significant accelerated progression of the arthritis. Putting all these results together it is clear that

Al-induced granulomas can afford SRLV replication, likely favoring biodistribution and modifying pathogenesis, potentially becoming a favoring factor for disease acceleration. This description is very important, as it opens the door for a brand-new, unexpected field of research. Future work will assess this interaction by analyzing iatrogenic granulomas derived from oil-based adjuvant inoculation as well as infectious granulomas due to *Mycobacterium avium subspp. paratuberculosis* infection infection.

A recent growing body of studies focused on SRLV identification and prevalence in different geographical regions prompted the systematic review and meta-analysis, providing an interesting overview of the SRLV worldwide distribution and summarizing important environmental and host-dependent factors. In the absence of vaccines or treatments, control is based on early diagnosis, typically by serological determinations. However, and despite high antigenic SRLV heterogeneity, most prevalence studies and eradication programs are based on a single diagnostic test, a strategy that underestimates the number of infected animals leading to inappropriate segregation of infected and non-infected individuals, which finally jeopardize control strategies as well as evaluation of productive and clinicopathological parameters (Echeverría *et al.*, 2020).

The results presented in this PhD thesis may set up the basis for other research topics. Safety concerns related to the persistence of Al-induced granulomas, translocation of ABAs to the CNS and SRLV replication within Al-loaded macrophages demand the design of new adjuvants that can help producing safer vaccines in sheep. Current approaches of our research group consists on the development of biodegradable and biocompatible adjuvants that might substitute ABAs in the future. Among these, CaP and microcrystalline tyrosine (MCT) will be tested as possible future adjuvants for sheep vaccines. CaP is a biocompatible compound usually found within bones and teeth that shows high tolerability and safety when used as adjuvant and it is approved by the WHO for human use (Masson *et al.*, 2017). Our research group is currently devoted to the development of CaP nanoparticles as they have demonstrated the same tolerability as amorphous CaP with a better balance between T_h1/T_h2 response and high rates of protection against infectious agents (He *et al.*, 2000; Sun *et al.*, 2021). Moreover, CaP nanoparticles are simple and cheap to synthesize (Behera and Swain, 2011) and of practical use as they are resistant to variable pH and temperatures (Huang

et al., 2017). MCT are crystallized microparticles of a non-essential amino acid that is used in humans to replace the cumulative effect derived from the use of Al in allergen-specific subcutaneous immunotherapy (Klimek *et al.*, 2017). The injection-site reaction last only 7-10 days and its half-life in the body is only 48 hours with no risk of bioaccumulation (Baldrick, Richardson and Wheeler, 2002). The derived immune response is characterized by similar IgG levels to Al, with a potent T_h1 response and a lower T_h2 response showing low IgE levels (Cabral-Miranda *et al.*, 2017). As a final approach to develop safer vaccines, our group will also explore a different avenue, consisting in the development of new vaccines based on viral vectors. First *in vitro* results indicate that Sendai virus vectors (a murine paramyxovirus) show promising results in blocking SRLV infection. Next steps will require to evaluate the *in vivo* efficacy after administration of a modified Sendai virus encoding SRLV immunodominant epitopes.

The interaction between SRLV and ABAs in sheep needs to be further addressed in a more controlled experimental model since in this study, SRLV-naturally infected animals showed a high variability degree due to natural infection, host susceptibility factors or circulating strains. To overcome these difficulties, *in vivo* experimental infection with SRLV before and after granuloma induction is prospected. Infection with a controlled dose of virus and a well characterized viral strain will ease to monitor viral load and the evolution of viral quasi-species along time. On top of this, this experiment will provide samples from granulomas induced before and after SRLV-infection, allowing immunohistochemical characterization of these macrophages.

To sum up, the results of the present PhD Thesis provides new light into the mechanism of action and toxicokinetics of Al-oxyhydroxide alone or in vaccines and points towards the search of alternative vaccine adjuvants in sheep to develop safer vaccines. Moreover, the present work opens the door to an unknown field of research based on the interaction between macrophage-tropic pathogens and chronic inflammatory reactions.

CONCLUSIONS

1. Repetitive inoculation of Al-oxyhydroxide in lambs, either alone or in vaccines, induces a decrease in the growth rate. This fact is more evident when Al-oxyhydroxide is administered as part of a vaccine.

2. Repetitive inoculation of Al-oxyhydroxide in lambs, either alone or in vaccines, is necessary but is not sufficient to fully reproduce the ovine ASIA syndrome.

3. Repetitive inoculation of Al-oxyhydroxide in lambs, either alone or in vaccines, leads to increased accumulation of Al-deposits in the lumbar spinal cord. Al-deposits are more evident in the gray matter and they are usually associated to glial-like cells.

4. Repetitive inoculation of Al-oxyhydroxide in lambs, either alone or in vaccines, leads to the upregulation of genes related with pro-inflammatory signaling pathways at the injection-site granuloma and the regional lymph node and the upregulation of genes associated with innate and adaptive immunity in the spleen. In regional lymph node and spleen, these changes are more evident when Al-oxyhydroxide is administrated as part of a vaccine.

5. Al-induced subcutaneous granulomas sustain SRLV replication thereby increasing serum antibody titers against SRLV.

6. In SRLV naturally-infected sheep, the repetitive inoculation of Al-oxyhydroxide, either alone or in vaccines, leads to an accelerated progression of the arthritis. These changes are more evident when Al-oxyhydroxide is administrated as part of a vaccine.

7. Small ruminant lentiviruses are worldwide distributed. SRLV distribution is markedly heterogeneous with extreme differences in animal and flock prevalence between different continents, countries and regions.

CONCLUSIONES

1. La inoculación repetida de oxihidróxido de Al en corderos, ya sea solo o como parte de una vacuna, induce una disminución de la tasa de crecimiento. Este efecto es más evidente cuando el oxihidróxido de Al se administra como parte de una vacuna.

2. La inoculación repetida de oxihidróxido de Al en corderos, ya sea solo o como parte de una vacuna, es un factor necesario, pero no suficiente para reproducir completamente el síndrome ASIA ovino.

3. La inoculación repetida de oxihidróxido de Al en corderos, ya sea solo o como parte de una vacuna, conduce a una mayor acumulación de depósitos de Al en la médula espinal lumbar. Los depósitos de Al son más evidentes en la sustancia gris y suelen estar asociados a células de tipo glial.

4. La inoculación repetida de oxihidróxido de Al en corderos, ya sea solo o como parte de una vacuna, induce una mayor expresión de genes relacionados vías de señalización proinflamatorias en el granuloma del punto de injección y el linfonodo regional e induce una mayor expresión de genes relacionados con la inmunidad innata y adaptativa en el bazo. Estos efectos son más evidentes cuando el oxihidróxido de Al se administra como parte de una vacuna.

5. Los granulomas subcutáneos inducidos por Al permiten la replicación de SRLV, aumentando así los títulos de anticuerpos séricos contra el SRLV.

6. En ovejas infectadas de forma natural por SRLV, la inoculación repetida de oxihidróxido de Al, ya sea solo o como parte de una vacuna, conduce a una progresión acelerada de la artritis. Este efecto es más evidente cuando el oxihidróxido de Al se administra como parte de una vacuna.

7. Los lentivirus de los pequeños rumiantes están ampliamente distribuidos por el mundo. La distribución de SRLV es marcadamente heterogénea, con diferencias extremas en la prevalencia individual y colectiva entre diferentes continentes, países y regiones.

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