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Towards an In Vitro Assay: Biomarker Validation for Facial Eczema Tolerance in Sheep

A thesis presented in partial fulfilment of the requirements for the degree of

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Claudia May Bramley

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Abstract

Introduction

Facial eczema (FE) is a photosensitisation disease of grazing ruminant livestock caused by the ingestion of the fungal metabolite sporidesmin which is found in the spores of the fungus *Pseudopithomyces chartarum.* It is of huge concern to the sheep industry as it effects animal production and welfare.

Several strategies to mitigate the effects of FE have been explored, however, breeding for increased tolerance has been recognised as the most effective management strategy. The current practice to test for FE tolerance is to dose rams with a controlled amount of sporidesmin and then observe the effects 21 days later which, from animal welfare and farming perspectives is unsustainable long-term and therefore an alternative test is needed. Recent advances have encouraged the development of an *in vitro* assay based on easily accessibly samples from individual animals which will remove the risks associated with animal welfare and accelerate FE tolerance in flocks.

Aim

The hypothesis for this project was that the differential expression and prognostic values of the nine biomarkers identified during earlier work can be reproduced in a different set of sheep.

Materials and methods

Peripheral blood mononuclear cells (PBMCs) were prepared from whole blood samples collected from sheep (n=290) on 13 farms enrolled in the Ramguard^{TM} programme, and an *in vitro* sporidesmin toxicity assay was performed. Total RNA was extracted from the PBMC pellets, subsequent to sporidesmin toxicity assay, using either the Qiagen Kit method or the Zymo Kit method. NanoString analysis of nine genes of interest and three reference genes was performed on 100 RNA samples and statistical analysis was carried out on each target gene between the FE tolerant and susceptible groups. The study animals were categorised into susceptible and tolerant groups based on the change in Gamma-Glutamyl Transferase levels (ΔGGT), before (day 0) and 21 (day 21) days subsequent to *in vivo* sporidesmin dosing.

Results

The change in GGT levels (ΔGGT) was found to be 916.90 ± 489.30 IU/L for susceptible animals and 8.57 ± 9.80 IU/L for tolerant animals. Variability in GGT levels in animals between farms revealed different proportions of tolerant and susceptible rams across farms. There was no difference in RNA

yield according to FE status (susceptible or tolerant), however there were differences in RNA yield and ΔGGT between the 13 farms.

Of the nine biomarkers[1] examined in this study, only four (ACP7, CCL27, LOC106990188 and TXN2) were significantly different between the susceptible and tolerant groups ($p<0.05$). Sensitivity and specificity were calculated for each of the four biomarkers individually and it was found that CCL27 had high specificity (0.80) and low sensitivity (0.34) while both TXN2 and LOC106990188 had high sensitivity (0.80) and low specificity (0.41). The four biomarkers were also combined in an attempt to improve individual prognostic values, and it was found that ACP7 combined with CCL27 resulted in a sensitivity of 0.37 and a specificity of 0.73. ACP7xCCL27 has a sensitivity of 0.31 and specificity of 0.75. Both ACP7+LOC106990188 and ACP7-TXN2 combinations have a sensitivity of 0.80 however, ACP7+LOC106990188 has a specificity of 0.46 while ACP7-TXN2 has a specificity of 0.23.

Conclusion

Of the nine biomarkers investigated, the four markers (ACP7, CCL27, LOC106990188 and TXN2) were differentially expressed between the susceptible and tolerant groups, thereby indicating their potential to be used in a prognostic test for FE tolerance in sheep.

[1] ACP7, CCL27, GLRX, GPR143, LOC106990188, PTGES2, TXN, TXN2 and UBB

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iii. List of Abbreviations

ACP7	Acid Phosphatase 7
ANOVA	Analysis of Variance
AUC	Area under the ROC curve
BCS	Body condition score
bME	beta-mercaptoethanol
BMP2	Gene
B12	Cobalamin
BV	Breeding value
C	Control
CCL27	Chemokine C-C motif ligand 27
cDNA	Complementary deoxyribonucleic acid
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
ELISA	Enzyme-linked immunosorbent assays
ETP	Epipolythiodioxopiperazine
FBS	Fetal Bovine Serum
FDR	False Discovery Rate
FE	Facial Eczema
FETT	Facial Eczema Tolerance Test
FFPE	Formalin-fixed paraffin-embedded
FNR	False negative rate
FOR	False omission rate
FPR	False positive rate
GGT	Gamma-glutamyl transferase
ΔGGT	Change in gamma-glutamyl transferase
GLRX	Glutaredoxin
GLDH	Glutamate dehydrogenase
GPR143	G Protein-Coupled Receptor 143

GPx	Glutathione peroxidase
LIC	Livestock Improvement Corporation
LOC106990188	Gene locus identified in the sheep genome
LPA	Lysophosphatidic acid
LR+	Positive likelihood ratio
LR-	Negative likelihood ratio
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
MT	Metallothioneins
N	Number
NPV	Negative predictive value
OAR1	*Ovis aries* chromosome 1
OAR13	*Ovis aries* chromosome 13
OH	Hydroxy radical
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PDGFD	Gene
PPV	Positive predictive value
PT	Prevalence threshold
PTGES2	Prostaglandin E Synthase 2
qPCR	Quantitative polymerase chain reaction
Q-Q plot	Quantile-Quantile plot
QTL	Quantitative trait loci
R	Resistant
RNA	Ribonucleic acid
ROC	Receiver Operating Characteristic
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
S	Susceptible

SLC	Solute carrier (enzymes)
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
StDev	Standard Deviation
TPR	True positive rate
TPR	True negative rate
TXN	Thioredoxin
TXN2	Thioredoxin 2
UBB	Ubiquitin B
UV	Ultraviolet (light)

Chapter 1: Introduction

1.1 Introduction

Facial eczema (FE) is a photosensitisation disease affecting grazing ruminant livestock (sheep, cattle, goats, deer, and alpacas) in New Zealand. FE is caused by the ingestion of the fungal metabolite sporidesmin found in the spores of the saprophytic fungus *Pseudopithomyces chartarum* (formerly *Pithomyces chartarum*) which is found, and grows, in the litter at the base of certain pasture swards in northern parts of New Zealand during the summer and autumn months (Di Menna et al., 2010). When ingested by livestock, sporidesmin is absorbed into the blood circulation and results in several symptoms leading to decreased animal production and welfare. New Zealand has among the most severe FE outbreaks in the world, and in 2019, it was estimated to cost $266 million dollars annually (Beef & Lamb, 2019).

FE was first recorded in 1897 (Brook, 1969) however, it was only in 1958 when the toxin produced by *P. chartarum,* sporidesmin, was identified (Percival & Thornton, 1959). Since then, several strategies to mitigate the effects of FE have been explored (Beef & Lamb, 2019); however, breeding for increased tolerance to FE has become recognised as one of the main tools breeders and farmers can deploy to effectively manage this disease (Di Menna et al., 2010). Testing for FE is important as it helps farmers make informed decisions on purchasing, breeding, and culling, reduces costs that arise from lost production, labour, treatment and deceased animals and improves animal welfare. The current practice to test for FE tolerance is to dose rams and bulls with a controlled amount of sporidesmin then observe the effects. Whilst this tolerance test is recognised under the Code of Welfare Sheep and Cattle (Ministry for Primary Industries, 2018), from animal welfare and farming perspectives, the test is unsustainable long-term, and an alternative test is needed.

Recent scientific and technological advances have encouraged the development of an *in vitro* assay based on easily accessible samples from individual animals. The availability of a cell culture-based FE tolerance test will remove the risk to farmers from being associated with the undesirable treatment of animals as part of the traditional tolerance test. It will also allow screening of larger numbers of animals including females, consequently accelerating breeding for FE tolerant flocks.

The research of which this thesis forms a part had objectives broken down into four phases, each representing the year the work was undertaken i.e., Phase 1 was Year 1 of the project and so on. Phases 1-3 and Part 1 of Phase 4 preceded the work described here, which took place in Part 2 of Phase 4 of the larger project.

Phase 1 focused on the development of an *in vitro* sporidesmin stimulation assay suitable for identifying potential biomarkers using transcriptomics (analysis of all RNA molecules expressed by an organism), proteomics (analysis of the structure, function, and interactions of proteins in various cells), and metabolomics (analysis of small metabolites in an organism, cell, or tissue). This involved collecting saliva, fibroblast, and blood samples from sheep and cattle at AgResearch Aorangi farm near Palmerston North, for analysis. Fibroblast samples came from archived samples. Gene expression, protein abundance, and metabolite profiles were assessed in response to sporidesmin treatment and differentially expressed genes. Proteins and metabolites were identified, many of which were associated with inflammatory response and immune defence however, no correlation was found between sporidesmin, gene expression, protein abundance and metabolite profiles. However, the purpose of the study was not to find a correlation between the four, rather, the purpose was to identify the feasibility of the approach (differentiating between tolerant and susceptible animals). The *in vitro* assay showed potential for differentiating between sporidesmin tolerant and susceptible animals, thus allowing progress to Phase 2.

Phase 2 aimed to identify potential biomarkers for FE tolerance using the *in vitro* sporidesmin stimulation assay developed in Phase 1. Cells from three groups of sheep: FE tolerant, FE susceptible, and FE naive, were used in the assay. MicroRNAs (miRNAs) and messenger RNA (mRNA) transcripts from each experimental group were analysed, resulting in the identification of candidate biomarkers for FE tolerance (17 miRNAs and 14 mRNA transcripts were differentially expressed with an additional six mRNA transcripts showing differential expression between untreated cells of tolerant and susceptible sheep).

Phase 3 confirmed the potential of the candidate biomarkers identified in Phase 2. RNA expression was quantified using NanoString technology, thus confirming the potential of some of the biomarkers identified in Phase 2; however, the quantification of lysophosphatidic acid (LPA) in supernatants using enzyme-linked immunosorbent assays (ELISA) and miRNA expression levels measured using PCR did not yield satisfactory results. There were no significant differences observed in the miRNA expression levels between tolerant and susceptible animals. A panel of 42 RNA biomarkers from 89 animals split into tolerant and susceptible groups was analysed. There were nine biomarkers that were differentially expressed and found to be statistically significant (p value was between 0.01 and 0.05) and were therefore considered to have potential as candidates for *in vitro* assay.

The aim of Phase 4 of this project was to select from these nine biomarkers, the biomarkers suitable for a facial eczema tolerance test (FETT). Phase 4 was split into two parts: Part 1 predated the work

done in this thesis. It involved the selection of biomarkers which were suitable for the development of a low-cost, high-throughput, non-invasive *in vitro* FETT. After being analysed using the FE status criteria (gamma-glutamyl transferase levels), nine biomarkers were chosen for assessment as described above: ACP7 (Acid Phosphatase 7), CCL27 (Chemokine C-C motif ligand 27), GPR143 (G Protein-Coupled Receptor 143), LOC106990188 (gene locus identified in the sheep genome), PTGES2 (Prostaglandin E Synthase 2), TXN (Thioredoxin), TXN2 (Thioredoxin 2), GLRX (Glutaredoxin), and UBB (Ubiquitin B). Blood samples from 282 rams were collated with previously collected and analysed samples from 89 animals resulting in a comprehensive dataset of 371 samples. Selection of predictive markers considered statistical significance of the biomarkers analysed in the panel mentioned in Phase 3 and clinical relevance. Clinical relevance was assessed based on effect size, sensitivity, and specificity, predictive value, and clinical utility. Statistical analysis (the Benjamini-Hochberg procedure was used to correct for multiple comparisons, ensuring the false discovery rate (FDR) was controlled) was used to determine whether a marker showed significant differences in expression between tolerant and susceptible groups. Gene expression analysis identified five out of the nine biomarkers: ACP7, CCL27, GPLR143, TXN2 and LOC106990188, that displayed differential expression between susceptible and tolerant groups. The gene expression of ACP7, CCL27, and GPLR143 in susceptible ram cells was significantly lower when compared to tolerant ram cells while the expression of TXN2 was higher. This indicates that rams with no or low expression of ACP7, CCL27, and GPLR143 are more susceptible to FE than rams with those markers, whilst rams with high expression of TXN2 are less susceptible to FE. It is important to note, however, that the identified biomarkers have no direct, well-established links to the molecular mechanisms of FE tolerance. While the association between biomarker expression and FE susceptibility is statistically significant, it does not necessarily imply a causal relationship or a specific defensive mechanism against FE. These markers were selected for further analysis. LOC106990188's differential expression was not as pronounced when compared to the other four biomarkers. It does not have a known protein or function allocated to it and may not even exist as an expressed protein however, it was also selected for further validation. Receiver Operating Characteristic (ROC) curves were calculated for ACP7, CCL27, GLRX and TXN2 to assess their predictive value. ACP7 achieved the highest specificity and sensitivity, with a cut-off determined at 102 RNA copies. Combinations of multiple markers were also evaluated, but none surpassed the predictive power of ACP7 alone (A. Heiser, personal communication, 2023).

Part 2 of Phase 4 is the focus of this thesis. In preparation for Part 2 of Phase 4, 290 blood samples from 290 sheep were collected in August 2022 from farms in the North Island involved in this project. These samples were processed and stored in an -80 °C freezer.

RNA Extractions of the 290 samples collected from throughout the North Island was performed. These samples were subsequently measured on the Nanodrop to assess the quality and quantity of RNA present. From these 290 samples, 100 samples were chosen (50 tolerant samples and 50 susceptible samples) based on Nanodrop results to undergo further analysis. NanoString Technology was used to analyse these 100 samples.

The hypothesis for this project was that the differential expression and prognostic values of the nine biomarkers identified in Phase 3 can be reproduced in a different set of sheep.

Chapter 2: Literature Review

2.1 Introduction

Mycotoxins are a large group of naturally occurring secondary metabolites produced by filamentous toxigenic fungi. Mycotoxins are a significant problem globally as they contaminate crops, pasture or stored feed and are often active at low concentrations (Council for Agricultural Science and Technology, 2003). Many are also heat stable meaning that they are resistant to food processing techniques like cooking, frying, baking, distillation, and fermentation.

Modern mycotoxin research began in the early 1960s after it was discovered that aflatoxins were responsible for the deaths of 100,000 young turkeys in the United Kingdom (Pitt & Miller, 2017); however, it was suspected that mycotoxins have had effects on animal health since they were first described in the 1800s (Brook, 1969).

There is a wide variety of mycotoxins produced by different fungi. Several species may produce the same toxin and conversely, one species may produce several toxins. Currently, over 100 species of fungi have been recorded which produce mycotoxins; however, there are potentially 20,000-300,000 unique mycotoxins undiscovered (Council for Agricultural Science and Technology, 2003). Most mycotoxins of concern to animal health are produced by five genera of fungi: *Aspergillus, Penicillium*, *Fusarium, Claviceps* and *Stachybotrys* (Council for Agricultural Science and Technology, 2003). Within toxins produced by these genera there are six major classes of mycotoxins: aflatoxins, trichothecenes, fumonisins, zearalenone, ochratoxins and the ergot alkaloids (ergotism) (Skládanka et al., 2010). Unlike certain bacterial toxins, mycotoxins are non-antigenic i.e., exposure doesn't induce an immune response, and typically have a low molecular weight (Bennett & Klich, 2003). *Claviceps* is most notable for its ability to produce toxic alkaloids. Otherwise known as a replacement parasite, it replaces plant structures with fungal tissue called ergots or sclerotia. These fungal tissues often contain toxic amounts of alkaloids leading to ergotism (poisoning) in animals consuming the tissue (Council for Agricultural Science and Technology, 2003). Toxic alkaloids e.g., Lolitrem B, are also produced by the genera Epichloe and Neotyphodium, both of which can be endophytic in certain plant species such as fescue and ryegrass (Council for Agricultural Science and Technology, 2003).

Mycotoxins are typically present in tropical environments as the fungi producing them rely on warm and humid conditions to grow. Animal diseases caused by mycotoxins are known as mycotoxicosis. These toxicoses are the results of exposure to toxic fungal metabolites through dietary, respiratory,

or dermal pathways. Mycotoxicoses are not to be confused with mycoses, diseases caused by the growth of fungi on animal hosts (Bennett & Klich, 2003). Many mycotoxins are present in different forages. If ingested, they cause livestock to show a variety of effects, from acute to chronic, thus, reducing production gains, economic profits, and animal welfare.

FE is one such mycotoxicosis caused by sporidesmin metabolites and affecting grazing ruminant livestock in countries with temperate climates. These countries include New Zealand, Australia, France, South Africa, Western China, Spain, and several South American countries (Collin & Towers, 1995; Liu et al., 2023; Hansen et al., 1994; Fernández et al., 2021). There are nine types of sporidesmin metabolites, which vary in their structure and toxicity. Varying toxicity is due to the sulphur-bridged epidithiodioxopiperazine ring (**Table 1**) which has wide biological reactivity (Jordan, 2020). Sporidesmin A is the metabolite that causes FE (Jordan, 2020). Sporidesmin A is found in the spores of the saprophytic fungus *Pseudopithomyces chartarum* (formerly *Pithomyces chartarum*) which obtains energy from decaying organic matter. *P. chartarum* grows on the dead/decaying organic matter that accumulates at the base of certain pasture swards, including perennial ryegrass (*Lolium perenne*). Perennial ryegrass forms the foundation of New Zealand's pastoral grazing system and has a high moisture content which triggers the mass production of *P*. *chartarum* spores. This has led perennial ryegrass to become the sward most often associated with FE (Mostrom, 2021).

Globally, FE outbreaks have been recorded as the most severe in New Zealand. However, a few countries with warm temperate climates, where ruminants are intensively grazed on pasture, have reported increased frequency and severity in FE cases (Di Menna et al., 2010).

In intensively grazed farming systems, ruminants ingest the toxin sporidesmin, which is produced by spores from *P. chartarum* once the pasture has been grazed right down to the base of the sward. Once ingested, sporidesmin is absorbed into the blood circulation, damaging the liver, and blocking the bile ducts, causing a build-up of phylloerythrin (a photodynamic breakdown product of chlorophyll). In affected animals, phylloerythrin no longer gets excreted, but rather circulates in the blood, causing lesions on unpigmented skin when the affected animal is exposed to sunlight. Lesions do not manifest until at least a week after the ingestion of sporidesmin, a delay that contributed to the slow identification of the causative agent. The causative agent was first thought to have been an abnormal metabolite of rapidly growing ryegrass (Di Menna et al., 2010). Other clinical and sub-clinical signs that an animal is affected by sporidesmin/FE are decreased production, inflammation, swelling, aversion to sunlight (constantly seeking shade), and death.

The existence of FE has been recognised for over 100 years and it was first recorded in 1897 (Brook, 1969), however, it was only in 1958 when the toxin produced by *P. chartarum,* sporidesmin, was

identified (Percival & Thornton, 1959). Various ruminants can be affected by FE including deer, alpacas, goats, horses, cattle, and sheep.

In 2019, FE was estimated to cost New Zealand $266 million dollars every year (Beef & Lamb, 2019). Over the years, a significant effort has been made to understand FE in order to combat the disease; however, there is currently no cure for FE, only preventative measures including zinc supplementation (giving a zinc bolus to animals which lasts six weeks, drenching animals with zinc oxide weekly/fortnightly, providing zinc in drinking water or spraying zinc on pasture), managing pasture and grazing by building up feed reserves, aiming for light rotational grazing, conducting annual spore counts (which help farmers identify safer parts of the farm i.e., the shady, windy parts of the farm) and spraying fungicide onto pasture before the onset of the FE season which reduces spore counts for five to six weeks (Beef & Lamb, 2019). These measures are not ideal due to the significant time and cost required to implement and maintain them; therefore, they remain as preventative measures. Fortunately, tolerance to sporidesmin is known to be moderately to highly heritable (McRae et al., 2021). Breeding animals for increased tolerance to FE (i.e., continually improving the genes associated with tolerance to FE in the flock) is considered to be the most effective method of long-term control (Di Menna et al., 2010; Beef & Lamb, 2019). However, it takes years to establish a fully resistant flock or herd, even if sires resistant to FE are continually chosen every year by farmers.

NanoString Technology is a powerful tool that allows detailed and accurate gene expression analysis. We used NanoString Technology to investigate blood samples collected from rams to confirm the prognostic value of the nine biomarkers identified in Phase 3 of this project. This approach (using all nine biomarkers) was used for practical reasons. NanoString's smallest assay measures 12 genes. Three housekeeping genes in an assay is an ideal number which leaves nine potential markers. NanoString allowed cheap, high volume, reliable processing, and identification of genetic merit with respect to FE.

There are several mycotoxins in forage grasses which affect livestock. These mycotoxins, the forages harbouring the fungi associated with them and the effects on livestock will be discussed in more detail in the following sections below. The types of sporidesmin, pathology associated with FE, diagnosis, treatment, and alleviation will also be discussed.

2.2 Mycotoxins in forage grasses

2.2.1 Fungal species

Mycotoxin production is stimulated in the field from fungal species during the growing season when the weather is humid and warm, (typically in late summer and early autumn months), but can also be stimulated during harvest, drying, transportation, processing, ensiling (turning pasture into silage by causing it to ferment in a closed pit or silo), and storage (Ogunade et al., 2018). During ensiling, most fungi can be eliminated due to the low availability of oxygen and high levels of organic acids and carbon dioxide typical of the ensiling process; however, there are a few species, including those in the *Aspergillus, Penicillium* and *Fusarium* genera, which can tolerate the high levels of organic acids and carbon dioxide as well as low availability of oxygen (Gallo et al., 2015). Likewise, the presence of oxygen in some parts of silage during storage, or oxygen penetration during feed-out and aerobic spoilage, could allow mould growth and mycotoxin production (Gallo et al., 2015).

There are several predisposing conditions for mycotoxin growth including geographical region, growth on crops or pasture, growth in storage, moisture, optimal temperature, and oxygen levels. The toxigenic fungi from which mycotoxins are formed can be split into two different groups (Nonzom & Sumbali, 2017). The first includes the genera *Fusarium* (trichothecenes, zearalenones and fumonisins) (Skládanka et al., 2010) and *Aspergillus* (aflatoxins), which produce preharvest (field level) toxins. These fungi invade the growing plants and produce mycotoxins before harvesting. The second group includes species in the genus *Penicillium* (ochratoxins, patulin and citrinin), which produce post-harvest (storage) toxins. These fungi produce toxins after harvesting and during crop storage, transportation, and marketing. Mycotoxins are usually present in tropical environments since their production relies heavily on the availability of water and temperature (humidity and warmth). The more water available, the more humid and warm the environment, the higher the mycotoxin production will be. Insects have also been found to increase fungal growth by providing access routes for fungi into the forage (Magnoli et al., 2019)

2.2.2 Fungi-harbouring forages

Forages harbour different fungi which affect livestock differently. Forages that contain mycotoxins include cereal grains (ochratoxin), wheat, barley, oats, and corn (trichothecenes, more specifically deoxynivalenol (DON or vomitoxin)), hay (zearalenone), perennial ryegrass and tall fescue (lolitrem B from the genera Epichloe) (Gallo et al., 2015), sporidesmin (FE), mouldy sweet potato toxicosis (*Fusarium* spp) and paspalum (*Claviceps paspali*).

The severity of clinical signs of mycotoxicosis (a consequence of ingestion of grains or forage containing toxic metabolites produced by certain fungi) varies between affected individuals and relates to the period of exposure, amount of toxin ingested (this is often vague), and the organ affected - this differs according to the toxin and what system it affects in the body e.g., the liver or the central nervous system (CNS). Susceptibility to the toxins also varies by age, sex, species, strain, and nutritional status of the animal (Council for Agricultural Science and Technology, 2003). Likewise, recovery depends on a range of factors including the duration of exposure (outbreaks are often seasonal and sporadic).

2.2.3 Sporidesmin types (cellular and molecular toxicity)

Pseudopithomyces chartarum (*P. chartarum*) is a fungus that thrives on dead plant material at the base of pasture swards. This fungus releases spores which produce sporidesmin, a toxin that is, once ingested, harmful to grazing ruminants. There are nine types of sporidesmin: A, B, C, D, E, F, G, H, and J (Jordan, 2020). Sporidesmins are pyrrolidines. Each differs based on its structure as shown in **Table 1**. **Table 1** shows that each sporidesmin metabolite possesses a 2,5-dioxopiperazine skeleton formed from tryptophan and alanine as the basic structure. Likewise, each has differences in the number of carbon, hydrogen, oxygen, and sulphur atoms which relate to the toxicity of the metabolite. The biologically active centre of these molecules is a sulphur-bridged dioxopiperazine ring that is also present in other families of fungal metabolites that are collectively called epidithiodioxopiperazines (ETPs) (Jordan, 2020).

Table 1. Types of sporidesmin and their chemical formula (Di Menna, 2010).

Sporidesmin Type	**Formula**
Sporidesmin A	$C_{18}H_{20}ClN_3O_6S_2$
Sporidesmin B	$C_{18}H_{20}ClN_3O_5S_2$
Sporidesmin C	$C_{22}H_{24}ClN_3O_8S_3$

Sporidesmin D	$C_{20}H_{26}ClN_3O_6S_2$
Sporidesmin E	$C_{18}H_{20}ClN_3O_6S_3$
Sporidesmin F	$C_{19}H_{22}ClN_3O_6S$
Sporidesmin G	$C_{18}H_{20}ClN_3O_6S_4$
Sporidesmin H	$C_{18}H_{20}ClN_3O_4S_2$
Sporidesmin J	$C_{17}H_{18}ClN_3O_5S_2$

Biological activities of the ETPs include antiviral, antibacterial, antifungal, antiparasitic, anticancer and immunosuppression, but their mammalian toxicity has prevented clinical development. Gliotoxin is the most commonly reported ETP. Sporidesmin is the second most reported ETP. *P.*

chartarum is the only organism known to produce sporidesmin metabolites with sporidesmin A being the metabolite that causes FE (Fernández et al., 2021).

The number of sulphur atoms in the bridge varies between the types of sporidesmin. Disulphides are the most abundant products of *P. chartarum,* therefore they are considered the most toxic. Sporidesmins A, B, D, F, H and J fall into this category. However, monosulphides and polysulphides can also be found. Sporidesmin C and E are trisulphides while sporidesmin G is a tetrasulphide. These other sporidesmin metabolites (those that are monosulphides, trisulphides, and tetrasulphides i.e., sporidesmin C, E and G) have lower toxicity due to the smaller amounts present in the environment (Jordan, 2020). In sporidesmin A and sporidesmin B the dioxopiperazine ring is bridged with a disulphide chain, with a trisulphide chain in sporidesmin E, and a tetrasulphide chain in sporidesmin G to form epidithio-, epitrithio-, and epitetrathio-dioxopiperazine. These sulphide bridges are eliminated or modified in sporidesmins C, D, and F.

The centrality of the sulphur-bridged ring for biological activity has been extensively reported, including the loss or absence of reactivity/toxicity when the sulphurs are eliminated or modified as in sporidesmin D. Sporidesmin B has only one hydroxy group, and sporidesmin E has an epitrithio group. Sporidesmin D has extra absorptions for the two S-methyl (in the C-H stretching region). Sporidesmin D and E have two hydroxy groups while sporidesmin B only has the tertiary 10b hydroxy group (i.e., one hydroxy group) (Jordan, 2020).

2.3 Effects of mycotoxins on livestock

Exposure to the mycotoxins mentioned above adversely affects the performance and health of various livestock in New Zealand. Ruminants are less susceptible to mycotoxins than monogastric animals, due to their rumen microbiota and the feed particle contained in the rumen compartment which may be effective in the degradation, deactivation, and binding of these toxic molecules (Gallo et al., 2015). The usual route of mycotoxin exposure is ingestion as food, with inhalation and dermal exposure being secondary routes. Most mycotoxins have a primary effect on a specific body system, so it is possible to classify mycotoxins based on which organ they affect. There are eight major classifications: immunotoxin, dermatoxins, hepatotoxins, cardiotoxins, nephrotoxins, neurotoxins, pulmotoxins and carcinogenic compounds. Aflatoxicosis, caused by aflatoxins of the *Aspergillus* genera, damages the liver of sheep, horses, and pigs (Bennett & Klich, 2003). Aflatoxicosis results in hepatoxicity, i.e., inappetence, stupor, jaundice, ruminal stasis, and death. In contrast, penitrem staggers occur because of mycotoxins in both the *Penicillium and Aspergillus spp.* Ruminants are the most affected and show symptoms of neurotoxicity: muscular tremors, incoordination, convulsions,

and collapse. Likewise, ochratoxin A is produced primarily by the *Aspergillus* and *Penicillium spp* and causes kidney damage and urinary tract cancers (nephrotoxicity) (Nonzom & Sumbali, 2017).

Symptoms of mycotoxicosis vary from acute (severe and sudden in onset with an obvious toxic response) to chronic (low-dose exposure over a long period resulting in cancers and other irreversible effects) (Bennett & Klich, 2003). Beef cattle fed feeds high in mycotoxins can suffer from digestive disorders, reduced dry matter intake, and increased susceptibility to disease, i.e., immunosuppression. Acute symptoms in bovines include, but are not limited to, a drop in milk production, lethargy, a dull or rough coat, increased evidence of mastitis and metritis, an increase in reproductive problems, decreased growth rate, stool variability (loose, diarrhoea), increased salivation, increased respiration rates and increased internal body temperatures. Chronic symptoms in bovines include immunosuppression, increased susceptibility to diseases, damage to organs, and abortions due to *Aspergillus*. Sheep have similar symptoms to cows, in that they also show reduced feed intake and nutrient absorption, an impaired metabolism, changes in hormone secretion and immunosuppression. Pigs often experience reduced reproductive performance and subsequently reduced fertility because of the mycotoxin zearalenone and its oestrogenic activity (Skládanka et al., 2010).

2.4 Facial eczema in cattle and sheep

2.4.1 Factors that aggravate facial eczema

FE is aggravated by several factors including weather (patterns), season, region, rainfall, and pasture type. Specific regions, particularly those that have a warm temperate environment, are associated with higher incidences of FE. These regions include, but are not limited to, the North Island in New Zealand, the East Coast of Australia (Victoria, Tasmania, and East Gippsland), areas of South Africa (Fernández et al, 2021) and areas in Western China (Liu et al., 2023). In these regions, the temperature is warmer (more humid) which promotes the growth of the fungus *P. chartarum* and the release of its spores. Season plays a significant role in aggravating FE. The months of January through to May in both New Zealand and Australia (these are the late summer/early autumn months), are the hottest months so the incidence of FE is increased during this time.

Weather (patterns) and rainfall also aggravate FE. Weather is linked to season via temperature. The higher the temperature, the more severe the FE outbreak and subsequently the symptoms of FE. FE is more severe on pastures where perennial ryegrass is dominant, where pastures have been grazed to the base of the sward and where the pastures have dried out during a hot, dry period and then been flushed rapidly after rain. Some pastures contain spores from *P. chartarum* for several months.

Continued exposure to spores and consequently, the toxin, will aggravate FE in ruminants. However, Morris et al. (2004), found that the length of time that a particular pasture continues to contain spores depends on air and soil temperatures, rainfall, or humidity, and on the quantity of dead material in the pasture. The quantity of dead matter is dependent on the pasture species present and on grazing management practices applied to each pasture over previous months (Morris et al., 2004). As the climate warms, it is likely the incidence of FE will increase.

2.4.2 Pathology

Once the spores are ingested, sporidesmin is absorbed via the upper gut and reaches the liver where it is excreted into the bile ducts (Di Menna et al., 2010). Typically, phytoporphyrin, a potent photodynamic compound, otherwise known as phylloerythrin, is produced through the metabolism of chlorophyll in the rumen and excreted in the bile. However, sporidesmin damages the epithelial cells lining the bile ducts (the biliary epithelium) causing the rapid reduction of biliary secretion (Di Menna et al., 2010). The intrahepatic bile ducts then become blocked with cell debris, inflammatory exudates, and necrotic material of thickened consistency. The lower biliary ducts become dilated and biliary excretion ceases (Di Menna et al., 2010). After the cessation of bile excretion, phytoporphyrin is retained in the blood where it builds up and gets transported to the skin and peripheral tissues of the animal. This condition, known as photosensitisation, is exacerbated by sunlight, particularly affecting non pigmented or less woolly areas of sheep and cattle such as the face, ears, and eyes. This is seen through oedemas and inflammation of the skin, face, ears, lips, udders of cattle, and white or pale areas of skin. Sloughing of the skin and swelling are also observed (Di Menna et al., 2010). In sheep, the face is the most exposed to ultraviolet (UV) light, leading to the condition known as FE (De las Heras et al., 2022; Mostrom, 2021).

Sporidesmin also diffuses into the liver parenchyma damaging the blood vessels and hepatocytes, leading to atrophy, necrosis, and fibrosis of the liver. Eventually, fibrosis can lead to cirrhosis. Liver regeneration may start as early as two weeks after toxin insult and usually leads to the clinical recovery of affected animals. Sporidesmin is also excreted into the urinary tract and bladder which may cause cystitis and frequent micturition (Di Menna et al., 2010).

Clinical signs for FE are seen 10-14 days after the ingestion of sporidesmin. Animals will become irritated in sunlight, seek shade, and may suffer stress. The photosensitisation process takes 7-24 days and animals may appear symptomless up to the stage of photosensitisation. In dairy cattle, an immediate drop in milk yield is observed, with diarrhoea and decreased appetite also evident. Other clinical manifestations in cattle include a severe decrease in milk production, crusting around the

muzzle and eyes, sunburnt teats and refusing to stand (will not leave the shade) (Beef & Lamb, 2019).

Clinical manifestation in sheep includes lesions on non-pigmented or wool-less skin, eyelids, muzzle, and ears. Inflammation, swelling, exudation, scab formation, photophobia (may see necrosis and sloughing of skin), concurrent jaundice, decreased production and decreased movement (constantly seeking shelter/shade) (Beef & Lamb, 2019). It is important to note that for every sheep or cow showing clinical signs, there are approximately ten sub-clinically affected animals (Beef & Lamb, 2019; DairyNZ, 2023). It is also important to note that cattle can contract FE, however it is not as common. FE toxins work on a per kilogram basis: the heavier the animal, the more toxin is needed to have an effect (Beef & Lamb, 2019).

2.4.3 Diagnosis

Animal behaviour is the first indicator that an animal is sick. If FE is suspected because the farm is in a region of known concern and the ruminant is showing clinical signs of FE such as refusing to stand, photosensitisation (refusing to leave shade), decreased production, lesions on non-pigmented skin, inflammation and swelling on non-pigmented skin during the late summer/early autumn months, this prompts testing. Several tests can be conducted to confirm a diagnosis of FE as described below.

The first test is a gamma-glutamyl transferase (GGT-serum) test. Gamma-glutamyl transferase (GGT) is an enzyme found throughout the body but mostly in the liver. If the liver is damaged, GGT may leak into the bloodstream (Di Menna et al., 2010). The GGT-serum test measures the amount of GGT in the blood. Elevated levels of GGT in the blood are potentially a sign of liver disease or damage to the bile ducts consistent with FE (De las Heras et al., 2020; Fernández et al., 2021). Elevated levels of GGT-serum are associated with intrahepatic bile duct obstruction and a range of biliary diseases such as sporidesmin toxicity in ruminants (Gribbles Veterinary, 2023). Typically, GGT is measured on its own however, a recent study conducted by Laven et al. (2022) showed that adding glutamate dehydrogenase (GLDH), which is another enzyme that indicates the level of liver damage, measurements to measurements of GGT significantly increases the accuracy of diagnosis of sub-clinical FE in cattle compared to using either test alone. This is significant as it minimises the likelihood of incorrect categorisation of FE status and requires fewer animals to be positive before a diagnosis of FE at the herd level could be made (Laven et al., 2022).

The second test is a necropsy. For every animal showing clinical signs, there are usually ten animals sub-clinically affected (DairyNZ, 2023). This means that occasionally animals who are not detected early, die unexpectedly. A necropsy allows various organs in the affected animal to be examined. A

necropsy conclusively diagnoses the presence of FE. The key organs that must be examined are the liver, bile ducts and gall bladder. If the animal has FE, the liver will be enlarged, its right lobe will show hypertrophy and its left lobe will show atrophy (Lawrence et al., 2022). The bile ducts will also show hypertrophy. If cut open, they will show an extra thickness to them. The gall bladder will be distended (Fernández et al., 2021).

Histopathology is intricately linked to a necropsy. Things to watch out for include periportal fibrosis, damage to, and loss of, the medium-sized bile ducts and biliary reduplication. Cattle will have a subcutaneous oedema of the head, particularly around the eyes and ears. Large areas of necrosis will be seen, and non-pigmented skins will show sloughing. Skin on teats will be red. Sporidesmin is so toxic for the bile ducts that the histopathological examination will show the presence of intimal mural changes in the hepatic arteries and portal veins on the sides adjacent to the bile ducts (Di Menna et al., 2010). High sporidesmin dose rates may also cause hepatocellular injury prior to the bile duct lesions but the main lesions are caused by the concentration of sporidesmin in the bile ducts (Di Menna et al., 2010). Hepatobiliary injury (injury to the liver, bile ducts, or the gall bladder), is a hallmark of the damage caused by the toxin although other organs including the kidney, urinary bladder, and adrenal glands are also affected.

GGTs, necropsies, and a histopathological examination are all tests that can be performed on the animal to diagnose FE. However, there is also a test that can be done on-farm to confirm the presence of the fungus *P. chartarum* in pasture or faeces known as a spore count. Spore counts from both pasture and faeces are used as they were thought to be equivalent to the amount of sporidesmin. More recent research has shown that spores may not contain any sporidesmin suggesting that spore counts may be unreliable indicators (Collin & Towers, 1995). In general, the higher the spore count, the higher the possibility that FE is present on-farm. Thus, it is more likely the animal has contracted FE. While spore counts are not a definitive diagnosis, they contribute to the information derived from GGT, necropsy and histopathological examination, providing a definitive diagnosis of FE.

2.4.4 Treatment

There is no cure for FE and consequently no treatment if animals fall ill. However, there are preventative measures that can be put in place to prevent the incidence of FE. These will be discussed in greater detail in section 2.5.1. Briefly, these preventative measures include zinc supplementation, fungicide application, pasture management, grazing management, and breeding (for tolerance). To be completely effective, these preventative measures must be in place before FE

spores are found. It is important to note that management (preventative measures) and treatment are different aspects of addressing the issue, both requiring distinct approaches and considerations.

2.4.5 Alleviation

As mentioned previously, there is no cure or treatment for FE once animals contract it, there are only methods of alleviation. If caught early, alleviation may result in the animal's recovery; however, it may take up to 12 months to recover (DairyNZ, 2023) during which time, there is a loss of production and associated economic loss.

As soon as clinical signs of FE are observed, affected cows must be dried off immediately to reduce pressure on the liver and zinc cream applied to the white areas of the coat and udder (DairyNZ, 2023). Affected livestock should be moved into dense shade (Mostrom, 2021). Ideally, an indoor space with a good water supply where supplementary feed can be provided, either a hay-barn, calf-rearing or implement shed. Cattle should be fed at night, so they are not exposed to sunlight. Hard grazing must be stopped so cattle do not come into close contact with the dead plant material at the base of the swards that contain the spores from *P. chartarum*.

Extremely sick cattle can be given a starter drench to boost metabolic function alongside a vitamin B12 supplement; however, veterinary advice should be sought regarding additional pain relief (DairyNZ, 2023). As mentioned, the drench will not treat FE, but it will provide an energy boost.

The last option if animals become severely affected to alleviate pain is culling. However, this decision should be made before the body condition score (BCS) and the severity of the condition causes unnecessary distress and suffering. It must be noted that abattoirs will not accept animals with a substantial number of lesions and inflamed skin.

2.5 Managing facial eczema

2.5.1 Preventative Measures

As mentioned previously in section 2.4.4 there is no cure for FE however, there are several preventative measures that can be put in place to reduce the incidence of FE. It must be re-iterated that management and treatment are different aspects of addressing the issue, both requiring distinct approaches and considerations. These preventative measures include zinc supplementation, fungicide application, pasture management, grazing management, and breeding (for tolerance). Zinc supplementation is widely used by farmers throughout New Zealand and is a highly effective preventative measure (Mostrom, 2021). Zinc does not stop the ruminants ingesting the toxin, but it does prevent the symptoms of FE from developing by binding to the sulphur ions of the sporidesmin

in the animal's rumen, blocking the redox reactions occurring at the sulphur bridge, therefore rendering the toxin inactive (Beef + Lamb, 2020; Hawkins & Moors, 2023). There are three methods commonly utilised to administer zinc. These are zinc boluses, zinc in water and zinc drenching (Hawkins & Moors, 2023). The first method, zinc boluses, are a slow-release capsule, administered orally which can protect against FE for up to six weeks. Both sheep and cattle can be given zinc boluses, but zinc can interact with copper so a veterinarian should be consulted before the bolus is given to cattle. This may become redundant as a study conducted by Berry et al. (2023) found that sheep given elemental zinc boluses and copper capsules did not show reductions in serum zinc values, suggesting that the simultaneous treatment does not result in antagonistic effects. This may influence changes to dosing strategies in the future. The second method, zinc in water (adding zinc sulfate to drinking water) is another effective method, particularly for cattle. However, it is less effective for sheep due to their lower water intake. The third method, zinc drenching (regular drenching i.e., weekly, or fortnightly with zinc oxide) can protect livestock, but this method is labour-intensive and can stress the animals. Sheep in particular may be at risk of developing pneumonia by frequently running them in and out of hot, dusty yards (Beef + Lamb, 2020).

Spraying pastures with fungicides before the peak spore season can reduce the growth of *P. chartarum* and lower spore counts, thereby decreasing the risk of FE. Pasture management, in particular sward management is another effective preventative method. Maintaining taller swards and avoiding overgrazing can reduce the exposure of livestock to *P. chartarum* spores located at the base of the pasture. If feed is being supplemented with silage or hay, properly managing the ensiling process to minimize fungal contamination, and ensuring that stored hay is kept dry can prevent the growth of mycotoxin-producing fungi. Implementing rotational grazing practices can also help in maintaining pasture quality and reducing the buildup of dead plant material where *P. chartarum* thrives. Incorporating pasture species that are less conducive to *P. chartarum* growth, such as chicory and plantain, can help reduce the risk of FE.

Breeding will be discussed in the subsequent sections.

2.6 Genetic improvement of resistance to facial eczema

2.6.1 Historical and current methods of genetic improvement

Prior to the 1960s, the focus of FE research was on understanding the pathology and conditions under which the disease proliferated. However, during that decade it became well-established that FE was caused by sporidesmin (Mortimer, 1969), and that breeding animal resistance was one of the options to control infection. Breeding resistant animals rested on the assumption that there were

highly variable individual responses for animals exposed to toxic pasture or to sporidesmin (Jordan et al., 1990) and that these responses were heritable. Sheep breeders and farmers could then exploit these differences to select for FE-resistant individuals and flocks. Many factors needed to be accounted for when making breeding decisions, such as the differences in resistance among breeds of cattle and sheep, as well as substantial heritable differences within breeds.

Tolerance to sporidesmin is known to be moderately to highly heritable; 0.44 $\pm$ 0.03 (McRae et al., 2021) and once this was discovered, it allowed genetic options to be explored. Historically, approaches for selecting for FE resistance relied on the assessment of the degree of liver damage following challenge with sporidesmin (Jordan et al., 1990). Liver damage was assessed post-mortem and scored. The scoring system used varied depending on the publication and the observer doing the scoring (Towers & Stratton, 1978; Di Menna et al., 2010). This process to select for FE resistance was less than ideal, considering that liver damage was assessed post-mortem. To address this deficiency, researchers began to search for genetic markers of disease resistance. This search was predicated on the assumption that a strong component of resistance is due to processes that affect the production and maintenance of liver damage. Progeny testing was also explored for use in selecting for FE resistance (Morris et al., 2013).

Several methods were used to search for potential genetic markers. Jordan et al. (1990) explored the use of DNA fingerprinting (which detects variability in the length of a repeated core DNA sequence at several sites scattered across the genome) and two-dimensional electrophoresis of blood or tissue proteins in which differences among up to 2-3000 proteins present in a sample can be detected. Towers and Stratton (1978) used performance testing with liver damage to assess the release of the liver enzyme GGT into blood after sporidesmin exposure. Activity of GGT in serum rises within about two weeks after a sporidesmin challenge. The offspring of resistant sires crossed with susceptible ewes or of susceptible sires crossed with resistant ewes showed intermediate levels of sporidesmin-induced liver damage. Through this research it was discovered that the indicator trait for FE (logGGT) is moderately heritable (0.45$\pm$0.05) (McRae et al., 2021) and responds readily to selection.

There were advantages and disadvantages in choosing FE as a trait for selection using molecular markers. One of the advantages resulting from the research done on sporidesmin toxicity was that the biochemical and physiological basis of disease resistance was more clearly established than the basis of many of the other economically important livestock traits. Significant disadvantages included the likely multigenic nature of resistance (it was later concluded that a single gene is not an obvious explanation of sheep highly resistant to FE (Morris et al., 1989)) and several uncertainties in the recognition of resistance phenotypes. Despite these disadvantages, Jordan et al. (1990)

concluded it seemed highly likely that the biochemical and molecular biological studies would lead to an improved understanding of resistance genetics and to the development of new methods for recognition of resistant individuals. It was also concluded that this approach would be of use to sheep and cattle breeders. A realistic evaluation of current progress up to and including 1990 conducted by Jordan et al. (1990) concluded that it would be several years before selection based on molecular technologies could become a viable option for the control of FE.

2.6.2 Commercial kits/methods to detect genetic resistance

The most common commercially available kit to detect genetic resistance to FE in ruminants for ram breeders is called Ramguard™ (McRae et al., 2021). Ramguard™ uses sporidesmin exposure and subsequent development of physiological symptoms to identify rams that demonstrate a particular level of genetic resistance to FE. The test is administered as a GGT test. The result of the GGT test is used as an indicator of damage to the liver. The higher the level of GGT in the blood, the less 'resistant' the animal, while the lower the level of GGT, the more 'resistant' the animal is.

A GGT test can also be conducted in cattle. Bull breeders commonly use the GGT to select sires showing FE resistance. The GGT test can be performed by a veterinarian or scientist in-field. The results from the GGT test are then used to generate breeding value (BV) estimates for tested and related animals. Farmers can then compare these breeding values with published values (e.g. on LIC (Livestock Improvement Corporation)) and make choices for the future of their herds.

2.6.3 Examples of genetic progress achieved

In New Zealand, AgResearch has been at the forefront of successful attempts to advance knowledge about FE. A long-term sheep breeding project was established at the Ruakura Research Centre in Hamilton in 1974 to examine breeding outcomes for FE resistance. Two lines of Romney sheep were divided into resistant (R) and susceptible (S) groups. 165 rams from five sources were progeny tested, producing lamb crops from 1974-1975 (Campbell et al., 1981). The rams with the best FE resistance, i.e., the lowest progeny mean liver injury score, were used as R-line sires, and those with the worst FE resistance, i.e., the highest progeny mean liver injury score, were used as sires in the S-line.

Beginning in 1975 and 1976, the selected rams were mated to randomly assigned Romney ewes, and then replacements were retained within their respective flocks. At least 100 breeding ewes per selection line were mated each year between 1975 and 2004. A control line (C) was also established in 1982 and continued to be maintained until 2004. This breeding programme led to the heritability estimate of 0.45± 0.03 (Morris et al., 2013), based on GGT levels in blood 21 or more days after a

sporidesmin challenge. This long-term breeding programme continued until at least 2002 (Longley, 1998; Morris et al., 2004). The R line was estimated to be roughly 11 times more resistant to sporidesmin challenge than the S line (this conclusion was drawn from the 2001 and 2002 lamb crop) (Morris et al., 2004).

To allow for the increasing difference between the R and S lines, indirect comparisons with the C line were made each year. C line animals were divided at random into low- and high-dose rate groups for direct comparison with the S line at the low dose rate and with the R line at the high dose rate. An approximately 20% faster selection response was achieved in the R than in the S line, partly because of differences in ram fertility (Morris et al., 2004). By 2002 there was no evidence that selection progress was slowing in either direction; however, it is believed that different loci may be under selection in the two lines because alleles affecting resistance or susceptibility may be segregating at different loci (Morris et al., 2004).

Further, though somewhat limited, evidence of genetic progress can be seen in dairy cattle. Research conducted at the Ruakura Research Centre with dairy cattle resulted in evidence that the heritability for FE in cattle was 0.31. Performance testing (i.e., dosing potential sires and then using those most resistant) and selection can be applied in cattle (Morris et al., 1991), opening possibilities for continuing the research in this area.

2.6.4 Recent research aimed at genetic improvement

Current approaches to FE focus on genetic improvement i.e., breeding animals for FE resistance, as it is believed to be the best long-term method of effective control. Recent research has focused on identifying the genes or loci associated with FE resistance (McRae et al., 2021 reported two loci for tolerance to FE on chromosomes 15 and 24). Several approaches have been used to search for causative genes or loci underlying FE tolerance, including a candidate gene approach, genome-wide scans for quantitative trait loci (QTL) (Phua et al., 2009) and scanning for selective sweep signatures (Phua et al., 2014). The development of single nucleotide polymorphism (SNP) arrays has also facilitated significant increases in the number of genotyped animals, driving the development of genomic selection. As a result, the number of animals with SNP genotypes and FE phenotypic measurements has increased (McRae et al., 2021).

Candidate genes are genes that, from their known biochemical function, have the potential to explain variation in an observed phenotype, in this case FE resistance. Candidate genes are used to map the relevant chromosomal region which contributes to or controls FE resistance. Since a proposed mechanism of sporidesmin toxicity is through the production of reactive oxygen species (ROS), genes coding for or controlling antioxidant enzymes and metallothioneins are functional

candidate genes in this instance. Genes involved in the detoxification processes, drug transporter genes and solute carrier (SLC) enzymes are also thought to be potential candidate genes. Searching for these candidate or causative genes has been promising, albeit limited to date, as the scope of the method relies heavily on existing knowledge. It must also be noted that although the genes are associated biologically with FE resistance, they may be minor genes of small effect (Morris et al., 2013).

Genomic screening for chromosomal regions linked to FE resistance has resulted in the identification of several regions, including two on chromosomes 15 and 24 of sheep (McRae et al., 2021). A total of 11 SNPs on chromosome 15 reached genome-wide significance along with two SNPs on chromosome 24 (McRae et al., 2021). These two regions explain around 5% and 1% of the phenotypic variance in response to FE, respectively. Of the significant SNPs in the region on chromosome 15, one has been identified as a missense variant within the haemoglobin subunit beta gene. Scanning for selective sweep signatures has provided limited results. Only two of the selective sweep regions in the study conducted by McRae et al., 2021, on chromosomes OAR1 and OAR13, were found within suggestive QTL regions (the F statistic value was over 10.2). The F statistic is a ratio used in ANOVA tests to determine if there are significant differences between group means. The higher the F statistic, the greater the disparity between groups relative to the variation within the groups which can be indicative of a significant effect or relationship being tested.

2.7 Detoxification pathways in mycotoxin resistance

Mycotoxin resistance is determined primarily by the ability of a ruminant to detoxify the secondary metabolites generated by the metabolism of mycotoxins, with respect to FE, the metabolism of sporidesmin. As a xenobiotic (a substance that is foreign to the body), mycotoxins undergo multi-phase metabolic detoxification through a coordinated series of enzymatic processes (Zhang et al., 2017). The initial phase of this metabolic breakdown, otherwise known as the modification phase, converts the xenobiotic to intermediary metabolites, which in turn are then conjugated by a broad range of transferases and thus readied for excretion via efflux membrane transporters (Zhang et al., 2017).

There are a number of candidate genes discussed with relation to FE resistance. These work by affecting enzyme production, helping to facilitate biotransformation of free radicals thus, detoxifying the metabolites (e.g., sporidesmin). These enzymes will be discussed in more detail below.

It is thought that Phase I and Phase II enzymes play important roles in the detoxification of mycotoxins. Phase I enzymes include the P450 family of enzymes (Zhang et al., 2017) which consists

of N- and O-dealkylases, aromatic, N-hydroxylases, and S-oxidases. Phase II enzymes are hepatic detoxification enzymes and include catalase, glutathione, glutathione peroxidase (GPx) and S-transferases. Phase I enzymes (if present in elevated levels) often catalyse oxidation, reduction, and hydrolysis reactions, thereby helping animals to metabolise barbiturates rapidly, while Phase II enzymes detoxify xenobiotics by increasing their aqueous solubility and facilitating their excretion in the urine (Morris et al., 2013).

Epipolythiodioxopiperazine (ETP) are a class of toxic secondary metabolites made only by fungi. Unlike several other structurally distinct groups of mycotoxin, the ETP class is produced by multiple fungi, all of which share a characteristic and reactive disulphide bond. This disulphide bond creates toxicity by targeting protein thiol groups and additionally generating reactive oxygen species (ROS) (Zhang et al., 2017) including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxy radical (OH). Sporidesmin is one such mycotoxin which has this disulphide bond, and its reduced (dithiol) form undergoes autooxidation readily *in vitro* in a reaction to generate the superoxide free radical (Munday, 1982). It is the increased generation of these free radicals and/or the suppression of antioxidant protective pathways, overwhelming the ability of the cellular enzymes to reduce and detoxify them, that is the cause of the sporidesmin toxicity (Longley, 1998).

Phase I enzymes that have been significantly expressed in animals exhibiting a higher tolerance to a challenge by sporidesmin include members of the cytochrome P450 (CYP) 2C family (Zhang et al., 2017). The 2C family is heavily implicated in being the primary agents of biotransformation after a sporidesmin challenge, in particular, 2C8 and 2C9 (Zhang et al., 2014). Biotransformation is key to oxidising the free radical. By undergoing biotransformation, the free radical can be excreted without harming the animal.

Loci coding for SOD (superoxide dismutase) enzymes are also candidate genes for FE resistance because they are responsible for reducing superoxide to hydrogen peroxide through autooxidation (Zhang et al., 2017). Phase II enzymes, specifically catalysing together with glutathione peroxidase, convert hydrogen peroxide to water and oxygen in the liver by using the catalase heme Fe^{3+} to first reduce one hydrogen peroxide molecule to water (Munday, 1982). This generates a porphyrin cation radical called compound I, which is then oxidised by a second hydrogen peroxide molecule to give oxygen and water. These can then be readily excreted from the body in the urine (Zhang et al., 2017).

Metallothioneins (MT) are also thought to be involved in the detoxification of ROS (alongside detoxifying heavy metals and metabolising essential ions like zinc and copper) (Carpenè et al., 2007).

MT genes are also candidates for FE resistance through ROS effects on the metabolism of sporidesmin. Four classes of MT, MT-1, -2, -3 and -4, are known (Carpenè et al., 2007).

Animals have adapted to include MT in their bodies to maintain metal homeostasis while mitigating the detrimental effects of excess metal ions. MT have a small molecular weight (Carpenè et al., 2007) and are intracellular proteins that bind to metals found in various organs in the body, typically, the liver and kidney. MT lack aromatic amino acids, are rich in reduced cysteine residues (Carpenè et al., 2007) and have a high affinity to bind to metal ions. Supplemental zinc protects cattle and sheep from FE, while the addition of copper increases the generation of superoxide radicals from sporidesmin cultures *in vitro.*

2.8 NanoString gene expression analysis and its applications

NanoString Technology was used to analyse 100 samples (50 tolerant and 50 susceptible) selected from the 290 samples used in Part 2 of Phase 4. This section will introduce NanoString Technology, its approach, and applications.

Since its conception in 2003, NanoString has rapidly gained traction and become more widespread in its use to analyse gene and protein expression in laboratory samples. Unlike other methods like qPCR or microarrays, NanoString's nCounter® technology employs a unique and sophisticated method involving molecular probes to analyse gene expression and RNA expression analysis from purified RNA without the need for amplification (Malkov et al., 2009). Amplification steps in qPCR microarrays greatly increase error and the risk of contamination.

NanoString's approach is based on the use of custom ProbeSets, which consist of two types of specifically designed probes for each gene of interest: a capture probe and a reporter probe. Each capture probe is biotinylated, allowing it to attach to a surface for easy separation and processing. The reporter probes are tagged with unique, color-coded, fluorescent molecular barcodes that correspond to specific RNA targets. The level of gene expression is measured by counting the number of codes for each tagged mRNA using digital imaging (Geiss et al., 2008). This process allows the analysis of multiple genes from the same sample, otherwise known as multiplexing.

This multiplexing capability significantly enhances throughput and data accuracy as it allows for the parallel processing of numerous interactions within a single reaction. Alongside this, the detection of multiple gene expressions in a single run greatly saves reagent use and costs. Pre-made gene expression panels which can examine up to 770 genes and custom ProbeSets which can analyse up to 800 genes are available for purchase from NanoString (NanoString Technologies, 2015). The minimum number of genes for this system depends on the type of assay and kit being used.

NanoString's popularity in the past decade has been greatly aided by the easy-to-use, extremely sensitive, precise, and reproducible manner in which it detects these multiple gene expressions.

When a sample is prepared and introduced to the NanoString system, these probes hybridize to their corresponding RNA molecules. The technology then utilizes a proprietary imaging system to count each unique barcode, allowing for the direct quantification of RNA molecules present in the sample. This direct digital counting eliminates the need for amplification, thus avoiding the common constraints, associated errors and contamination risks that are typical in polymerase chain reaction (PCR) based methods (Eastel et al., 2019).

Further enhancing the robustness of the nCounter system, NanoString also provides comprehensive data analysis tools, such as the nSolver application, which helps in processing and normalizing the raw data, conducting statistical analyses to identify differentially expressed genes, and visualising these changes via heatmaps.

The constraints of the other molecular techniques currently available include limited sample types for biomarker analyses, limited detection of a small number of genes expressed in a single run by quantitative polymerase chain reaction (q-PCR), the lack of reproducibility and cost-effectiveness in microarrays when formalin-fixed paraffin-embedded (FFPE) samples are considered (NanoString Technologies, 2015). NanoString overcomes all of these.

Applications for NanoString technology are widespread, ranging from identifying viruses and use in microbial diagnostics to gene expression analysis and disease prognosis estimation. However, there are some limitations to this technology. These limitations lie in the normalisation of expression level, probe design and its scale of detection being more narrow than other advanced technologies (Godoy et al., 2019).

2.9 Conclusion

The review of the literature provided above underscores the global challenge posed by mycotoxins, particularly FE, highlighting their pervasive presence in agriculture and their significant impact on animal health. The historical literature and ever evolving understanding of mycotoxins, particularly those affecting livestock through diseases like FE, reveal both the progress made and the persistent hurdles that complicate effective management strategies. Despite the advancements in genetic research and the development of innovative diagnostic tools like NanoString Technology, significant gaps remain in our ability to predict and mitigate the impacts of these toxins effectively.

The literature indicates a pressing need for improved methods that can pre-emptively identify and manage the risks associated with mycotoxin exposure in ruminants and that don't rely on exposure to the toxin. The complexity of interactions between mycotoxins and animal health is evident, further revealing the necessity for multidisciplinary approaches that integrate genetics, biochemistry, and environmental science. The insights gained from past research must inform the development of robust, practical solutions that safeguard livestock health and welfare and, by extension, the agricultural industry at large. This thesis aims to address these gaps through the application of recently developed genomic tools and assays (NanoString Technology, RNA sequencing and qPCR assays). All three of these genomic tools and assays were pivotal in identifying and validating the biomarkers. By advancing our understanding of genetic resistance mechanisms and enhancing diagnostic capabilities, this research will contribute to the global effort against mycotoxins, ultimately improving animal welfare and agricultural productivity.

Chapter 3: Materials and Methods

3.1 Animals

This study involved sheep (n=290) enrolled in the Ramguard™ programme, which is dedicated to assessing and improving genetic resistance to FE. Sheep were enrolled from 13 North Island farms participating in the Ramguard™ breeding programme. Blood samples were collected by local veterinarians. The AgResearch Animal Ethics Committee approved the animal manipulations (Animal Ethics number: 2022-0386-AE-1041) as per the New Zealand Animal Welfare Act 1999. The sheep were kept on their home farms under normal husbandry practices throughout the study.

3.2 Definition of Susceptible and Tolerant Animals Using GGT Values

The Gamma-Glutamyl Transferase (GGT) results utilized in this study were sourced from the Ramguard™ Programme, which obtained them from commercial veterinary diagnostic laboratories in New Zealand. These results were instrumental in categorising the sheep into susceptible and tolerant groups based on their response to sporidesmin, a toxin associated with FE.

The animals were retrospectively grouped based on their change in GGT levels (ΔGGT), which was calculated as the GGT level at day 21 after *in vivo* sporidesmin dosing minus the GGT level at day 0 (before sporidesmin dosing). This change in GGT levels served as a biomarker for determining the FE status of the sheep. The GGT concentration in blood was determined using the GGT-serum test (Towers & Stratton, 1978). All animals are dosed with sporidesmin at an agreed dose rate (between 0.25-0.64 mg/kg depending on the level of tolerance already obtained) with the Ramguard™ co-ordinator to identify the most susceptible animals. For new breeders, this will be a relatively low dose while breeders who have been in the Ramguard™ programme for longer will have increased dose rates. No correlation between dose rate and marker expression was detected.

Two different FE statuses were defined:

- **FE Tolerant**: Sheep with $\Delta GGT \leq 70$ IU GGT/L after sporidesmin dosing were classified as FE tolerant.
- **FE Susceptible**: Sheep with $\Delta GGT > 70$ IU GGT/L after sporidesmin dosing were classified as FE susceptible.

This classification enabled a clear differentiation between animals that showed resilience to FE (tolerant) and those that were adversely affected (susceptible), providing a basis for further genetic and molecular analysis within the study.

3.3 Blood sampling and processing

Blood samples (total of ≤9 mL/animal) were collected into evacuated blood tubes containing a lithium heparin anticoagulant (Vacutainer; BD Bioscience, Plymouth, UK). Blood tubes were inverted 8 to 10 times to ensure mixing.

The blood samples were shipped directly (overnight) at ambient temperature to the Hopkirk Research Institute for the *in vitro* sporidesmin toxicity assay. All samples were processed on the day of receipt in the laboratory, i.e., within ≤ 36 hours of collection.

For the *in vitro* sporidesmin toxicity assay, peripheral blood mononuclear cells (PBMC) were prepared from whole blood using hypotonic lysis of red blood cells. Therefore, 5 mL of whole blood was transferred to a 50 mL tube and 45 mL of ultrapure water (Milli-Q® Direct Water Purification System, Merck, Burlington, MA, USA) was added. Tubes were incubated at room temperature for 1 minute and lysis was stopped by adding 5 mL of 10×PBS (100 mM PBS, pH 7.4, made by media kitchen at Hopkirk Research Institute) and gentle mixing. Tubes were centrifuged at 600 × g for 10 minutes at room temperature. The resulting supernatant was decanted, and the remaining cells counted using Trypan Blue exclusion and a TC20 cell counter (Bio-Rad, Hercules, CA, USA), according to the manufacturer's protocol. They were resuspended at 1,000 viable leukocytes/µL in RPMI 1640 medium, pH 7.2 (RPMI, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA), and immediately used for the *in vitro* assay.

3.4 *In vitro* sporidesmin toxicity assay

For each animal, 200,000 PBMC/200 µL/well were incubated in 96-well U-bottom plates for 2 hours at 37°C, either with 1 µg/mL sporidesmin (Batch 2) or left untreated. Plates were centrifuged at 600 × g for 2 minutes at room temperature, and supernatants transferred into new 96-well plates. Cell pellets were then resuspended in RLT buffer (Qiagen, Hilden, Germany) to lyse the cells and protect the RNA. All plates were sealed with seal film (Thermo Fisher Scientific, Waltham, MA) and stored at -80°C.

3.5 RNA Extraction

Total RNA was extracted from the cultured PBMCs using commercially available kits and two variations of the kits as described below. The kits used were the Qiagen RNeasy Mini Kit and the Zymo MiniPrep Plus kit. Both kits operate on the same fundamental principle of solid-phase extraction using silica-based membrane technology, however, while both kits use the same basic principle, there are some differences in their specific protocols and reagents which may affect the

efficiency of cell lysis, RNA binding, the yield, and the purity of the extracted RNA. Therefore, the decision to use two kits stemmed from the possibility of a higher yield and purity of the extracted RNA.

3.5.1 RNA extraction using Qiagen Kit Method

RNA extractions from the sporidesmin-stimulated and untreated PBMC lysates were undertaken using spin columns (RNeasy Mini, Qiagen, Hilden, Germany) following the manufacturer's recommendations.

The procedure began with the preparation of necessary reagents and equipment. A stock bottle of 70% analytical-grade ethanol was prepared, and a 1% stock lysis buffer consisting of beta-mercaptoethanol (bME) (Thermo Fisher Scientific, Waltham, MA) and RLT buffer (from the Qiagen RNeasy Mini Kit) was mixed in a fume hood. The appropriate amount of DNase (RNeasy Mini, Qiagen, Hilden, Germany) was also prepared from stock solutions. A heating block was set to 55°C to warm the RNase-free water for elution.

The kit was then opened, and RPE buffer was prepared. RW1 buffer and RNase-free water were selected, and columns, 2 mL collection tubes, 1.5 mL microcentrifuge tubes, and safe-lock tubes were labelled and arranged. A vial of RNase-free water was placed on the heating block.

Cell lysate samples were defrosted from -80°C storage by placing on ice before being moved to the bench. In a fume hood, 200 µL of the RLT and bME lysis buffer was carefully mixed into each well containing the cell lysate. The contents were transferred to 1.5 mL microcentrifuge tubes, and an aliquot (150 µL) was pipetted back into each well to ensure thorough collection of the cells, which were typically at the bottom of the wells. The aliquots were then combined with the initial transfer in the microcentrifuge tubes to make up a total volume of 350 µL.

The lysate was vortexed at medium speed for 20 seconds. Subsequently, 350 µL of 70% ethanol was added to the homogenised lysate, and the mixture pipetted to mix thoroughly. The mixture, including any precipitate formed, was carefully transferred to a marked QIAamp Mini spin column and tube. The samples were centrifuged for 30 seconds at 8000 x g, and the flow-through discarded. This step was repeated as necessary for each sample.

Next, 350 µL of buffer RW1 was added to each column, and the columns were centrifuged for 30 seconds at 8000 x g. The flow-through was discarded. DNase treatment was performed by adding 80 µL of a DNase mix (10 µL DNase stock and 70 µL Buffer RDD) to each column, followed by a 15-minute incubation at ambient temperature. Without discarding the previous solution, 350 µL of

buffer RW1 was added to each column, which was then centrifuged for 30 seconds at ≥8000 x g to wash the columns. The flow-through was discarded.

Subsequently, 500 µL of buffer RPE was pipetted into each QIAamp spin column, and the columns centrifuged for 30 seconds at 8000 x g. This step was repeated, and the flow-through and collection tubes discarded. The columns were then placed onto new 2 mL collection tubes and centrifuged at full speed (≥14,000 x g) for 3 minutes to ensure the columns were dry. Any wet collection tubes were centrifuged again until dry.

Finally, the collection tubes were discarded, and the QIAamp spin columns transferred into labelled 1.5 mL safe-lock tubes. To elute the RNA, 30 µL of warmed RNase-free water was pipetted directly onto the QIAamp membrane. The columns were centrifuged for 30 seconds at ≥8000 x g, and this elution step was repeated with another 30 µL of RNase-free water. The volumes of the eluates were checked, and any discrepancies addressed by additional centrifugation. The RNA concentration was measured using a NanoDrop spectrophotometer, and the RNA samples were stored at -80°C for further analysis.

3.5.2 RNA extraction using Zymo Kit Method

The process began with buffer preparation, where 96 mL of 100% ethanol was added to the 24 mL DNA/RNA wash buffer concentrate. Subsequently, an appropriate amount of DNase I was prepared by mixing 5 µL DNase I with 75 µL DNA Digestion Buffer. Additionally, 275 µL of DNase/RNase free water was added to the DNase I white powder, mixed by gentle inversion, and the solution was stored in a 4°C walk-in fridge.

A heating block was turned on and set to 55°C, and a vial of diethylpyrocarbonate (DEPC) water was placed in the block to warm. The extraction kit was opened, and one set of yellow spin away filters, green spin columns, and 1.5 mL sure-lock microcentrifuge tubes were labelled and arranged.

Cell lysate samples were defrosted from -80°C storage by placing on ice before being moved to the bench. If samples were stored in a 4°C walk-in fridge, (i.e., the plate had been stored overnight in a 4°C walk-in fridge while being processed) they were left until the end of the preparation to ensure the samples remained cold. A 200 µL aliquot of RNA Lysis Buffer was mixed into each well containing the cell lysate, and the contents transferred to labelled 1.5 mL microcentrifuge tubes. An additional 150 µL of RNA lysis buffer was used to ensure thorough cleaning of the wells, as cells tended to settle at the bottom. The lysed samples were then transferred to yellow spin away filters placed on collection tubes and centrifuged at 10,000 x g for 30 seconds to remove most genomic DNA. The flow-through was saved.

To the saved flow-through, 350 µL of 95-100% ethanol was added and mixed well by pipetting. This mixture was transferred into green Zymo spin columns in collection tubes and centrifuged at 10,000 x g for 30 seconds. The flow-through was discarded. The columns were washed with 400 µL of RNA wash buffer and centrifuged again at 10,000 x g for 30 seconds, followed by discarding the flow-through.

An 80 µL aliquot of DNase was directly added to each column matrix and incubated at room temperature for 15 minutes. Without discarding the DNase solution, 400 µL of RNA prep buffer was added to each column, which was then centrifuged at 10,000 x g for 30 seconds, and the flow-through discarded. This was followed by adding 700 µL of RNA wash buffer to the columns, which were then centrifuged at 10,000 x g for 30 seconds, and the flow-through discarded. Another 400 µL of RNA wash buffer was added, and the columns were centrifuged for 1 minute at 12,000 x g to ensure complete removal of the wash buffer. The columns were carefully transferred to nuclease-free microcentrifuge tubes.

To elute the RNA, 30 µL of warmed DEPC water was added directly to the column matrix and centrifuged at 10,000 x g for 30 seconds. This elution step was repeated to obtain a total of 60 µL of eluted RNA. The volumes were checked to ensure they were as expected, and any discrepancies were addressed by additional centrifugation.

The quality and quantity of the extracted RNA were assessed using a NanoDrop spectrophotometer. Only RNA samples with an A260/A280 ratio between 1.8 and 2.0 and intact 18S and 28S rRNA bands were used for further analysis. The RNA samples were then stored at -80°C.

Key differences in this method compared to the previous one included the initial preparation of DNase I and the use of yellow Spin Away filters to remove genomic DNA before ethanol precipitation. Additionally, the specific steps for washing and eluting the RNA highlighted procedural nuances that ensured higher purity and integrity of the final RNA samples.

3.5.3 RNA quality assessment

RNA quality was assessed using a NanoDrop spectrophotometer (De Novix Inc, Wilmington, DE, USA). Prior to measurement, the upper and lower measurement surfaces of the NanoDrop were cleaned with deionized water to ensure accuracy. Calibration was performed using a blank sample, typically RNAse free water or DEPC water, to set the baseline to zero.

For each of the 290 RNA samples, a 1-2 µL aliquot was pipetted directly onto the lower measurement pedestal. The sampling arm was then lowered, creating a liquid column between the optical fibres. The NanoDrop software was used to initiate a spectral measurement, during which

light from a xenon flashlamp passed through the sample. The internal spectrometer measured the absorbance across the UV-visible spectrum, focusing on the absorbance at 260 nm to calculate RNA concentration using the Beer-Lambert equation. The purity of the RNA was assessed by calculating the A260/A280 ratio, with values around 2.0 indicating high purity. Additionally, the A260/A230 ratio was evaluated to check for the presence of other contaminants.

3.6 Modifications to RNA Extraction methods

The following modifications were trialled to improve the ng/µL read out in each sample as well as the A260/280 and A260/230 ratios:

1. When discarding the flow-through**,** the tube was blotted gently on a clean paper towel to remove excess liquid.
2. When preparing the 70% ethanol, to increase the level of sterility and decrease the level of contamination, the 70% ethanol was prepared using DEPC (diethylpyrocarbonate) treated water.
3. Eluting in DEPC treated water rather than RNase free water to further decrease the possibility of any contamination.
4. Samples were vortexed for 20 seconds. If necessary, based on the colour of the sample and observation of the “snot” precipitate in the tube, the tube was vortexed for another 10 seconds.
5. A third RPE step was added to the pre-existing two to further wash/remove any traces of salts which may still be present in the columns due to the buffers used earlier in the Qiagen Kit method **(3.5.1)**. This was only tried twice at the very beginning of the RNA extraction process. Once it was determined it did not change the end result, this third RPE step was removed.

3.7 mRNA transcript analysis using NanoString

The mRNA transcript analysis was performed using the nCounter Analysis System (NanoString Technologies Inc., Seattle, WA). This method uses molecular barcodes on gene-sequence-specific probes and single molecule imaging to count RNA copies (Geiss et al., 2008). Multiplexed probes were designed with two sequence-specific probes for each of the nine genes of interest. The capture probe was coupled to biotin as affinity tag. The reporter probe was coupled to a color-coded tag. Each target molecule of interest was identified by the unique colour code generated by the ordered fluorescent tags on the reporter probe. The level of expression was measured by counting the number of codes for each mRNA using digital imaging. This allows the analysis of multiple genes

from the same sample (multiplexing) using a customized set of probes with distinct bar codes, called a ProbeSet.

3.7.1 Probe Design

The RNA samples prepared from blood were analysed using a ProbeSet consisting of probes specific for nine genes from *Ovis aries*. Additionally, three reference genes were included based on published recommendations. A complete list of genes and probe sequences is provided in **Table 2**.

Table 2: List of genes included in mRNA transcript analysis

Name	Accession	Position	Target Sequence
ACP7	XM_042232007.1	1120-1219	TACGACGCTGTTCTTCATGTGGGAGACTTCGCCTATAACATGGATCAGGACAATGCACGTGTCGGGGACAGATTCATGAAACTCATCGAACCCGTGGCTG
CCL27	XM_027964274.2	1601-1700	AGGTTGCAGACGGCATCCTTGAAACAACCTGATAAGGGCTGGATTATTGAGTTATGACTCAGGTTACATTTCACATAGAGAGCAGGACAGGAGGGTTGAA
GLRX	NM_001127288.1	91-190	GAGCTTCTCAGCCAACTGCCCTTCAAACAAGGGCTTTTGGAATTTGTCGATATTACAGCTGCCAGTAACACCAGTGAGATTCAAGATTACTTGGAGCAGC
GPR143	XM_015104790.3	672-771	CCGAAAGACAGTAACTGCAGTGGCCTCCTTACTGAAGGGAAGACAAGGCATTTACACGGAGAACGAGAGGCGCATGGGAGCCATGATCAAGACCCGATTC
LOC106990188	XM_027974854.1	585-684	ATGTCAGATGAAAAGGCTGGGATGGTACTGGCAAAACAATTGGCTTTTGAAAACGCTAACTCTGCTTGTCAAGCTGCTTTAAGACCTTATCGAAAAAAGG
PTGES2	XM_027966397.2	489-588	GAACGACTCCTCTGTGATCATCAGCGCCCTCAAGACCTATCTGGTGTCAGGGCAACCCCTGGCGGACATCATCACCTACTATCCACCCATGAAGGCGGTG
TXN	NM_001009421.1	204-303	CTGAAAAGTATTCCAACGTGGTGTTCCTCGAAGTAGATGTGGATGACTGTCAGGATGTTGCTGCAGAGTGTGAGGTCAAATGCATGCCAACCTTCCAGTT
TXN2	XM_042247237.1	211-310	GCCCGGTCTATATACACCACCAGAGTCTGTTCAACAACCTTTAACATCCAAGATGGACCTGACTTTCAAGACCGAGTTGTCAATAGTGAGACACCAGTGG
UBB	NM_001009202.1	476-575	AGATGGCCGCACTCTTTCTGATTACAACATCCAGAAGGAGTCGACCCTGCACCTGGTTCTCCGTCTGAGGGGTGGTATGCAGATCTTCGTGAAGACCCTG
Reference Genes			
RPL19	XM_015089125.1	335-434	TGGATGAGGAGGATGAGAATTCTGCACCGGCTGCTCAGACGATACCGTGAATCTAAGATTGACCGCCACATGTATCACAGCCTGTACCTGAAGGTGAAGG
SDHA	XM_012097183.1	1561-1660	GCTGGGGAAGAATCTGTCATGAATCTTGACAAATTGAGATTTGCCGATGGAAGCATAAGAACATCGGAATTGCGACTCAGCATGCAGAAGTCGATGCAGA
YWHAZ	NM_001267887.1	304-403	GAAAAGTTCTTGATCCCAAACGCTTCACAAGCAGAGAGCAAAGTCTTCTATTTGAAAATGAAAGGAGACTACTATCGCTACTTGGCTGAGGTTGCAGCTG

3.7.2 Probe Hybridisation

The gene expression analysis was performed using the NanoString nCounter Analysis System, which utilises Reporter CodeSet and Capture ProbeSet for specific target detection. RNA was hybridized

with the ProbeSets according to the manufacturer's instructions (Gene-Expression-Panels-and-Custom-CodeSet-User-Manual; MAN-10056-06). The following steps outline the hybridization protocol used in this study.

During the setup, the Reporter CodeSet and Capture ProbeSet were handled with care to avoid vigorous mixing. Instead of vortexing, the tubes were gently flicked or inverted to mix, and spun down using a picofuge or mini-centrifuge at less than 3000 x g for no longer than 10 seconds to prevent the probes from being spun out of solution.

The protocol began by pre-heating a thermal cycler to 65°C with the heated lid set to 70°C. This step is crucial as a thermal cycler with a heated lid is required to prevent condensation and ensure uniform temperatures.

Once the thermal cycler was ready, the Reporter CodeSet and Capture ProbeSet tubes were removed from the -80°C freezer and allowed to thaw at room temperature, shielded from light. After thawing, the tubes were mixed well by inverting or flicking, and briefly spun down. It was important to inspect the Reporter CodeSet for any coloured precipitate; if present, the entire tube was heated to 75°C for 10 minutes and cooled to room temperature before use.

A hybridization master mix was created by adding the hybridization buffer directly to the tube containing the Reporter CodeSet. For one nCounter assay (12 reactions plus two reactions of dead volume), the master mix included 42 µL of Reporter CodeSet and 70 µL of hybridization buffer, making a total volume of 112 µL. For samples using crude whole cell lysates (which were prepared using the *in vitro* toxicity assay mentioned in section 3.4), Proteinase K was added to the master mix at a final concentration of 200 µg/mL. The mixture was then flicked or inverted repeatedly to mix, and then briefly spun down.

Strip tubes provided with the nCounter Master Kits were used, ensuring proper positioning of the notch between tubes 1-2 and 8-9. Strip tubes were labelled.

Hybridization reactions were prepared using a new pipette tip for each step. First, 8 µL of the hybridization master mix was added to each tube of the prepared strip tube. Then, 7 µL of RNA sample containing approximately 600 ng (as recommended by NanoString) was added to each tube containing the master mix. The strip tubes were then capped tightly and mixed by inverting and flicking to ensure complete mixing. After a brief spin, the tubes were immediately placed in the pre-heated 65°C thermal cycler.

The hybridization reactions were incubated for at least 16 hours, with a maximum hybridization time of 48 hours to maintain consistency across experiments. Longer incubation times increased

sensitivity by raising total counts without significantly increasing background. Optionally, after the desired hybridization time, the reactions could be incubated at 4°C on the thermal cycler but not for more than 24 hours to avoid increased background.

3.7.3 Sample Alignment and Immobilisation

Once the hybridization reactions were completed, they were immediately processed on the nCounter Image Analyser, following the respective instrument-specific user manuals (nCounter Analysis System User Manual for MAX/FLEX Systems (MAN-C0035). This step ensured the proper alignment and immobilization of the probe-target complexes in the nCounter cartridge for subsequent data analysis.

3.7.4 RNA Counting

Sample cartridges were placed in the nCounter Digital Analyzer which counted and tabulated colour codes on the surface of the cartridge for each target molecule. Data were retrieved from the Analyzer as raw data (Reporter Code Count, RCC) files.

3.7.5 Nanostring Quality Control and Normalisation

For analysis, RCC files were imported into nSolver Analysis Software v4.0 (https://www.nanostring.com/products/analysis-software/nsolver) and underwent the software's sample quality control routine set to the following criteria: (1) imaging: fields of view registration < 75%; (2) binding density outside the 0.05 to 2.25 range; (3) positive control linearity: positive control R2 value < 0.95; and (4) positive control limit of detection: 0.5 fM positive control ≤ 2 standard deviations above the mean of the negative controls. All samples (n=100) used for statistical analysis passed the quality control routine.

Background subtraction was performed by subtracting the geometric mean of eight internal negative controls from each sample. Positive control normalization was performed using the geometric mean of six internal positive controls to compute the normalization factor. The normalization factor of all samples was inside the 0.15 to 15 range.

Reference gene normalization was performed using the geometric mean of counts for the 10 reference genes included in the ProbeSet. The average of these geometric means across all lanes was used as the reference against which each lane is normalized. A normalization factor was then calculated for each of the lanes based on the geometric mean of counts for the reference genes in each lane relative to the average geometric mean of counts for the reference genes across all lanes.

This normalization factor was then used to adjust the counts for each gene target and controls in the associated lane. The normalization factor of all samples was inside the 0.1 to 30 range.

3.8 Statistical Analyses

3.8.1 Data Analysis

Data were analysed for differential transcription of each target gene between the FE tolerant and susceptible groups. Initially, the distribution of the data was assessed to determine its suitability for parametric statistical tests. Histograms and Q-Q plots were visually examined to check the normality assumption, revealing that the data did not follow a normal distribution. To address this, a naturalised logarithmic transformation (base e) was applied.

Following the logarithmic transformation, the transformed data were used for further statistical analysis. The means between the two groups were compared using independent samples t-tests. The t-test was conducted at a significance level of $\alpha = 0.05$, and two-tailed p-values are reported here. The null hypothesis of equal means between the two groups was tested against the alternative hypothesis of unequal means.

To evaluate the value of mRNA transcription levels as predictive biomarkers, 15 parameters were defined and calculated including measures of test performance such as true positives, false positives, true negatives, and false negatives. The definitions of the parameters used were as follows:

3.8.2 True Positives

Here, "true positives" refers to the number of animals that have been identified by the Ramguard™test as tolerant, i.e. ≤70 IU GGT/mL, and the RNA copy number is larger than the cut-off.

3.8.3 False Positives

Here, "false positives" refers to the number of animals that have been identified by the Ramguard ™ test as susceptible, i.e. >70 IU GGT/mL, but whose RNA copy number is larger than the cut-off.

3.8.4 True Negatives

Here, "true negatives" refers to the number of animals that have been identified by the Ramguard ™ test as susceptible, i.e. >70 IU GGT/mL, and the RNA copy number is smaller than the cut-off.

3.8.5 False Negatives

Here, "false negatives" refers to the number of animals that have been identified by the Ramguard™ test as tolerant, i.e. ≤70 IU GGT/mL, but the RNA copy number is smaller than the cut-off.

3.8.5.1 Sensitivity, Recall, Hit Rate, or True Positive Rate (TPR)

Sensitivity is calculated as:

Sensitivity = true positives / (true positives + false negatives)

3.8.6 Specificity, Selectivity or True Negative Rate (TNR)

Specificity is calculated as:

Specificity = true negatives / (true negatives + false positives)

3.8.7 Other Parameters

- Precision or positive predictive value (PPV) = true positives / (true positives + false positives)
- Negative predictive value (NPV) = true negatives / false negatives
- Miss rate or false negative rate (FNR) = false negatives / (false negatives + true positives)
- Fall-out or false positive rate (FPR) = false positives / (false positives + true negatives)
- False discovery rate (FDR) = false positives / (false positives + true positives)
- False omission rate (FOR) = false negatives / (false negatives + true negatives)
- Positive likelihood ratio (LR+) = TPR/FPR
- Negative likelihood ratio (LR-) = FNR/TNR
- Prevalence threshold (PT) = $\sqrt{FPR} / (\sqrt{TNR} + \sqrt{FPR})$

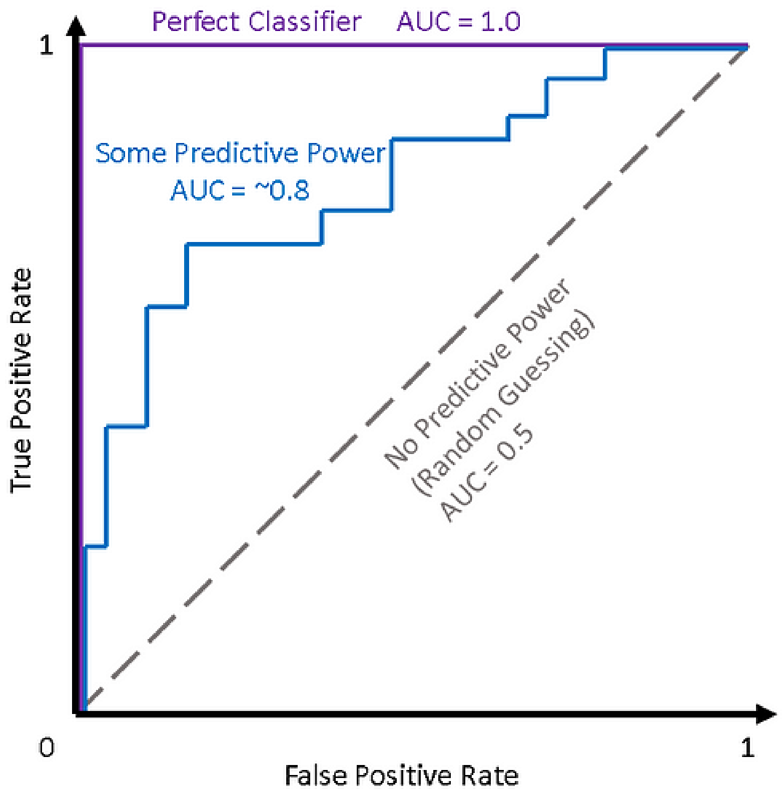

Figure 1. Theoretical Receiver Operating Characteristic curve (ROC) with Area Under Curve (AUC) scores.

The figure demonstrates how some theoretical classifiers would plot on an ROC curve. The gray dotted line represents a classifier that is no better than random guessing — this will plot as a diagonal line. The purple line represents a perfect classifier — one with a true positive rate of 100% and a false positive rate of 0%. Nearly all real-world examples will fall somewhere between these two lines — not perfect but providing more predictive power than random guessing.

3.8.8 Cut-off Value & Receiver Operating Characteristic (ROC) Curve Analysis

The cut-off value for various RNA counts and combinations thereof (models) were determined by finding the optimal balance between sensitivity and specificity for the particular marker(s) using receiver operating characteristic (ROC) curve analysis. The ROC curve is a graphical representation of the relationship between sensitivity and specificity at different cut-off values for a test as shown in (**Figure 1**). To construct ROC curves, the true positive rate (sensitivity) was plotted on the y-axis against the false positive rate (1-specificity) on the x-axis for different cut-off values. The resulting curves show how the sensitivity and specificity of the test change as the cut-off value is varied.

3.8.9 Combinatorial models for biomarker analysis

To enhance the predictive power or individual biomarkers, several combinatorial models were tested. These models involved mathematical combinations of the RNA copy numbers for the identified biomarkers (ACP7, CCL27, LOC106990188, and TXN2). The rationale behind this approach was to explore whether combining the expression levels of multiple biomarkers could improve the accuracy of differentiating between FE tolerant and susceptible animals.

The combinatorial models were constructed by applying simple arithmetic operations to the RNA copy numbers of the biomarkers. Specifically, the models were created by either adding, subtracting, or multiplying the values of two or more biomarkers.

1. **Addition model (+):** in this model, the RNA copy number of two or more biomarkers were added together. For example, the sum of ACP7 and TXN2 RNA copies was calculated as (ACP7 + TXN2).
2. **Subtraction model (-):** this model involved subtracting the RNA copy number of one biomarker from another. For instance, the difference between CCL27 and LOC106990188 was computed as (CCL27 – LOC106990188).

3. **Multiplication model (x):** in this model, the RNA copy numbers of two or more biomarkers were multiplied. For example, the product of ACP7 and CCL27 RNA copies was determined as (ACP7 x CCL27).

Each combinatorial model was then evaluated for its ability to distinguish between the FE tolerant and FE susceptible groups using receiver operating characteristic (ROC) curve analysis. The optimal cut-off values for each model were identified by balancing sensitivity and specificity, providing a comprehensive assessment of the combined biomarkers' diagnostic potential.

These combinatorial strategies were tested to determine if combining biomarkers could provide a more robust and reliable method for identifying FE tolerance, ultimately aiming to improve the accuracy of predictive methods used in this study.

Chapter 4: Results

4.1 Gamma-glutamyl transferase (GGT) values

GGT is an enzyme found throughout the body, but mostly in the liver. If the liver is damaged, GGT may leak into the bloodstream. Elevated levels of GGT in the blood are potentially a sign of liver disease or damage to the bile ducts consistent with FE (Morris et al., 1994; Morris et al., 1995).

The mean GGT level for susceptible animals increased from 58.22 ± 12.94 IU/L on day 0 to 975.10 ± 491.3 IU/L on day 21 after sporidesmin challenge. In contrast, tolerant animals showed mean GGT levels of 57.20 ± 11.61 IU/L on day 0 and 65.77 ± 13.61 IU/L on day 21. The change in GGT levels (ΔGGT) was 916.90 ± 489.30 IU/L for susceptible animals and 8.57 ± 9.80 IU/L for tolerant animals (**Table 3**). The difference in ΔGGT between the two groups was significant ($P < 0.001$).
Table 3 shows GGT values and summary statistics.

Table 3: Gamma-glutamyl transferase (GGT) values in susceptible and tolerant rams

Variable	**GGT d0***		**GGT d21***		**ΔGGT**	
FE Tolerance Status	Susceptible	Tolerant	Susceptible	Tolerant	Susceptible	Tolerant
N*	41	35	41	35	41	35
Mean [IU/L]	58.22	57.20	975.10	65.77	916.90	8.57
StDev [IU/L]	12.94	11.61	491.30	13.61	489.30	9.80
Minimum [IU/L]	22.00	37.00	153.00	38.00	91.00	-6.00
Maximum [IU/L]	95.00	89.00	1892.00	96.00	1825.00	32.00

*N indicates the number of sheep in each group (susceptible or tolerant) while d0 is day zero (prior to sporidesmin exposure) and d21 is 21 days later.

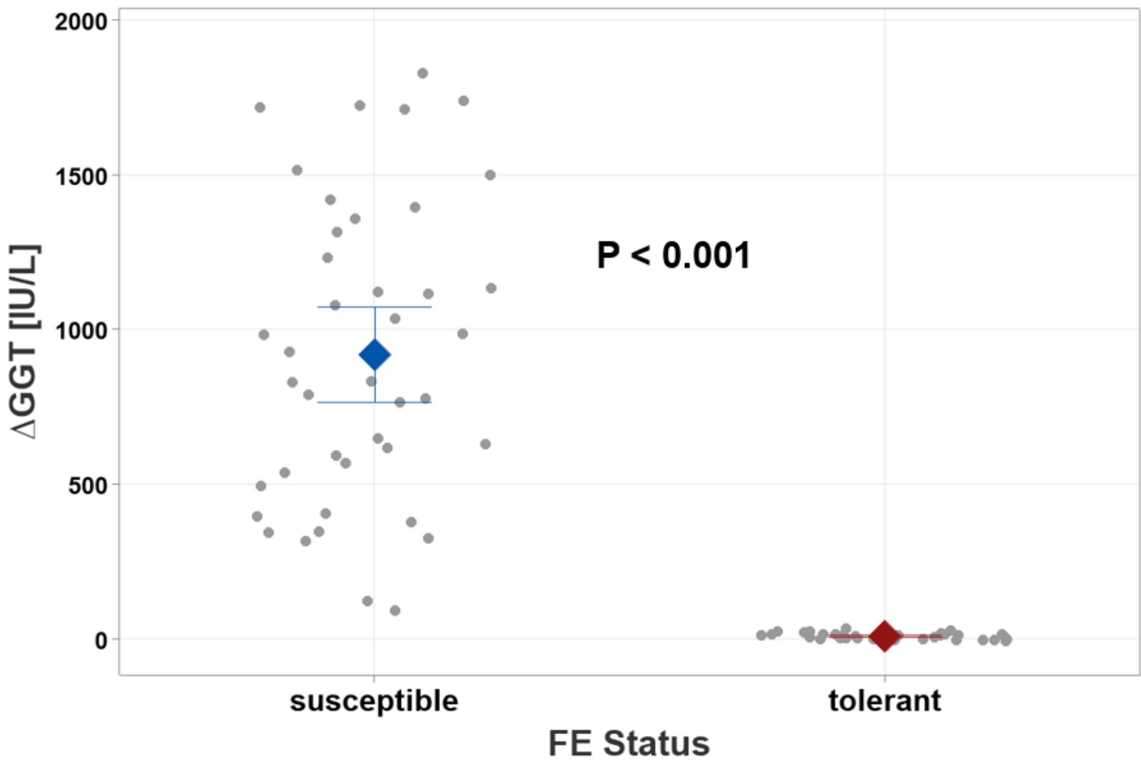

Figure 2. Interval plot for the change in gamma-glutamyl transferase (**ΔGGT**) measured from blood samples prior to exposure to sporidesmin (**d0**) and 21 days later (**d21**).

Grey dots indicate individual results. Diamonds show mean GGT levels for susceptible (**blue**) and tolerant (**red**) animals, as defined by having GGT levels above 300 IU/L and below 70 IU/L, respectively (**section 3.2**).

4.1.1 Farm variability

The variability in gamma-glutamyl transferase (ΔGGT) levels in animals between farms was analysed to assess the distribution of tolerant and susceptible animals. The results indicated that farms exhibited different proportions of tolerant and susceptible rams. Specifically, Farms 6 and 7 were comprised entirely of susceptible rams and Farms 2 and 11 had a high number of susceptible rams, as shown in **Figure 3**. Farms 3 and 4 had a mixed population of both tolerant and susceptible rams and Farms 1, 12, and 13 had a predominance of tolerant rams.

For the farms with the high number of susceptible rams, the ΔGGT for Farm 7 (all susceptible rams) was 653 $\pm$ 137.3 IU/L, 650.1 $\pm$ 479.9 IU/L for Farm 2, and 1493.4 $\pm$ 259.5 IU/L for Farm 11 as shown in **Table 4**. The ΔGGT for Farm 6 was 1393.00 IU/L however the sample size was 1. For farms with a mixed population, the ΔGGT levels at Farm 3 was 66.3 $\pm$ 137.3 IU/L and at Farm 4 120.00 $\pm$ 177.00 IU/L. For farms with a high proportion of tolerant rams, the ΔGGT was 155.00 $\pm$ 405.00 IU/L for Farm 1, 30.4 $\pm$ 52.7 IU/L for Farm 12, and 6.80 $\pm$ 8.90 IU/L for Farm 13. The values and summary statistics

for the ΔGGT levels across the 13 farms involved in this study are shown in **Table 4**. Farm 5 was not included in the table and graph above because although RNA was extracted from the rams, we eventually did not choose any of them for the NanoString analysis. This is because we aimed to conduct a specific number of NanoString analysis and initially extracted RNA from more animals than could be analysed. Ultimately, Farm 5 was excluded because it only had tolerant animals and no susceptible rams, leading us to prioritize samples from other farms.

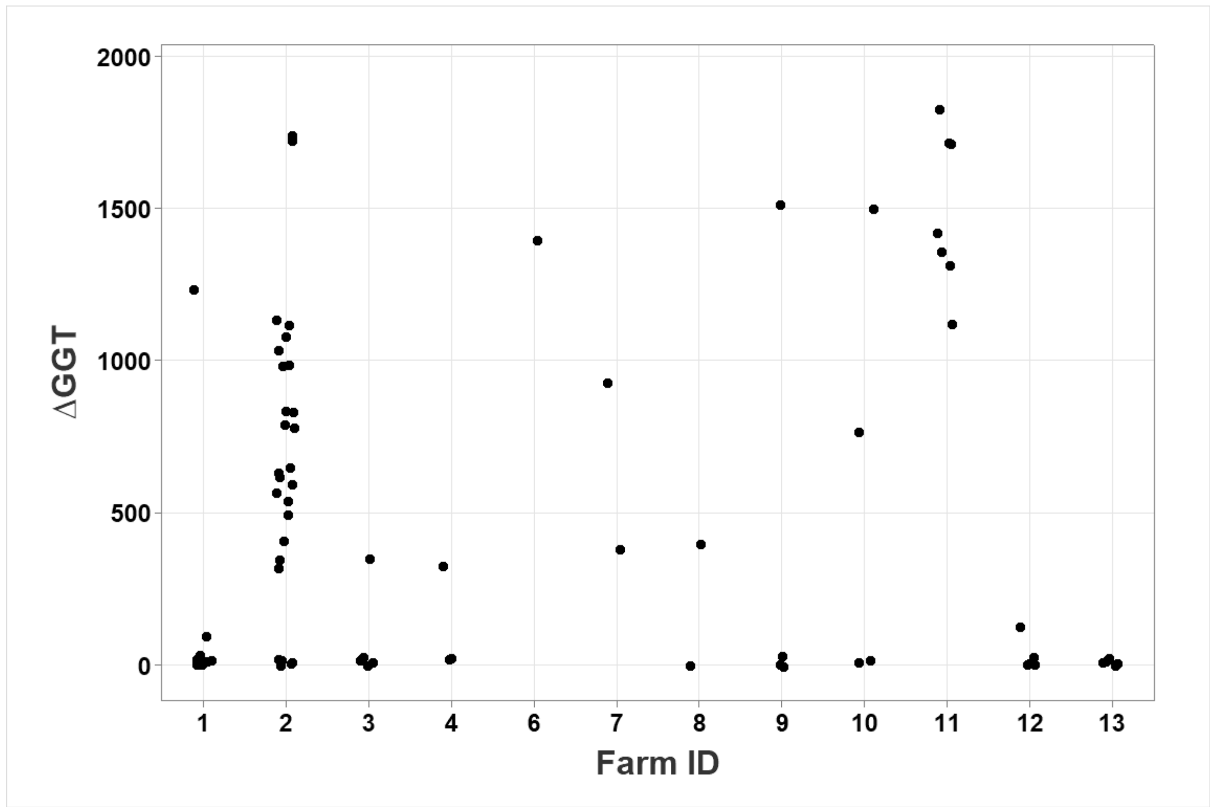

Figure 3. Interval plot of the change in gamma-glutamyl transferase (ΔGGT) relative to Farm ID. Farms had different levels of tolerance and susceptibility. Dots indicate change in GGT (ΔGGT) measured from blood samples prior to exposure to sporidesmin (d0) and 21 days later (d21), with dots closer to the top of the figure (higher change in GGT) indicating susceptibility and lower change in GGT indicating tolerance. Farm 5 was not included because although RNA was extracted from the rams on that farm, NanoString analysis was not done on those samples.

Table 4: ΔGGT levels for rams 21 days after sporidesmin challenge.

Farm ID*	n	Mean change in GGT	StDev	Minimum change in GGT	Maximum change in GGT
1	9	155.00	405.00	-1	1232
2	28	650.10	479.90	-4	1737
3	6	66.30	137.30	-4	346.00
4	3	120.00	177.00	16	324.00
6	1	1393.00	*	1393	1393
7	2	653.00	388.00	378.00	927.00
8	2	196.00	282.00	-4	395.00
9	4	383.00	753.00	-6	1513
10	4	570.00	713.00	5	1497
11	7	1493.40	259.50	1119.00	1825.00
12	5	30.40	52.70	1.00	123.00
13	5	6.80	8.90	-5.00	19.00
Total	76	476.42	332.39	240.67	944.25

* Farm 5 was not included because although RNA was extracted from the rams on that farm, NanoString analysis was not done on those samples.

4.2 RNA Preparation

4.2.1 Total RNA yield

The total average RNA yield of the samples used in this study (n=290) was 11.07 $\pm$ 17.92 ng/µL. The purity of the RNA was assessed by calculating the A260/A280 ratio, which is used to assess the purity of RNA by indicating protein contamination, with a ratio around 2.0 generally considered pure. Additionally, the A260/A230 ratio was evaluated to check for the presence of other contaminants such as carbohydrates or phenol, with a ratio above 2.0 indicating high purity (Koetsier & Cantor, 2019). The average A260/A280 ratio was 2.04 $\pm$ 1.23 while the average A260/A230 ratio was 0.79 $\pm$ 1.07.

4.2.2 FE status

RNA yield was measured in susceptible and tolerant groups from the samples collected (n=290). The ANOVA conducted showed no difference in RNA yield according to FE status (p=0.670) as depicted in Figure 4.

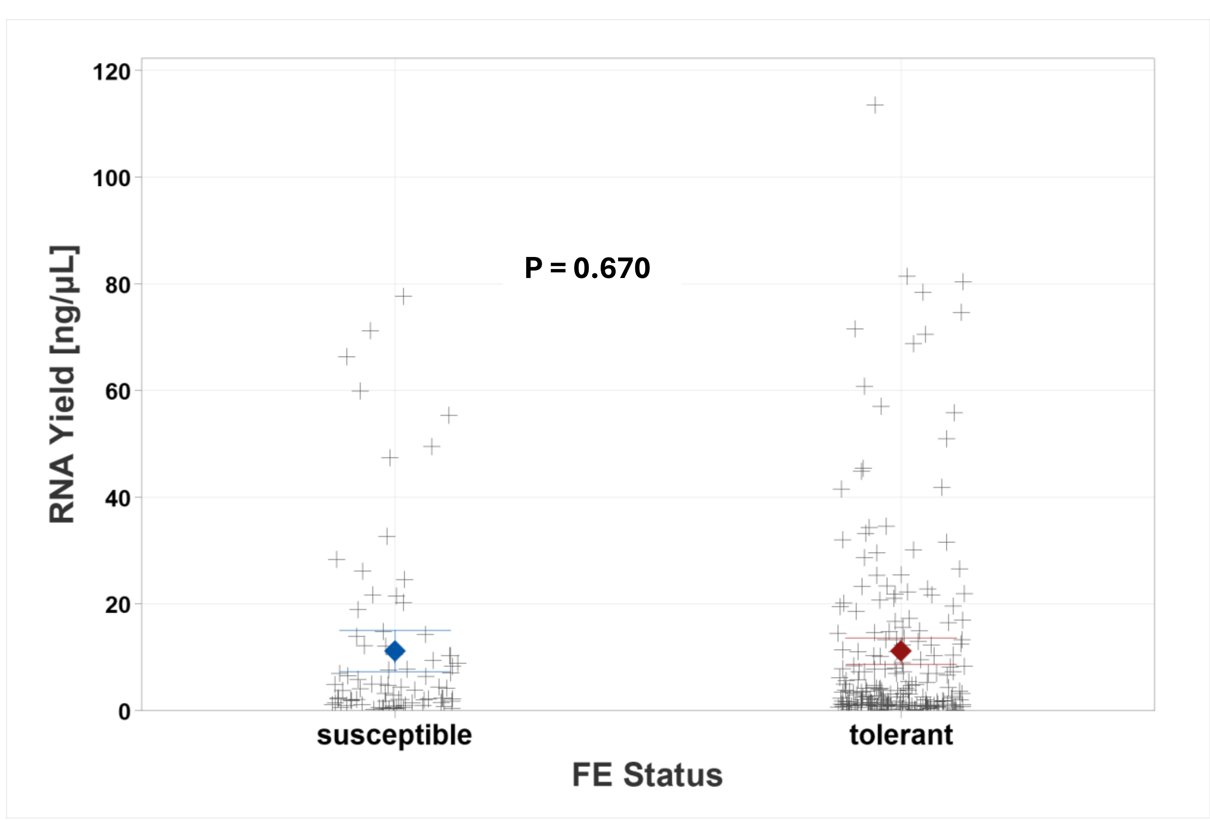

Figure 4: Interval plot of RNA yield (ng/μL) in susceptible and tolerant groups of rams.

Grey crosses indicate individual results. Diamonds show mean RNA yield for susceptible (blue) and tolerant (red) animals. The ANOVA test revealed the difference between the two groups was not significant (P=0.670).

4.2.3 Differences between Farms

When RNA Yield (ng/µL) for each of the 13 farms was compared, there were some differences: Farm 4 had the highest RNA yield, followed by Farms 5 and 6 as shown in **Figure 5**.

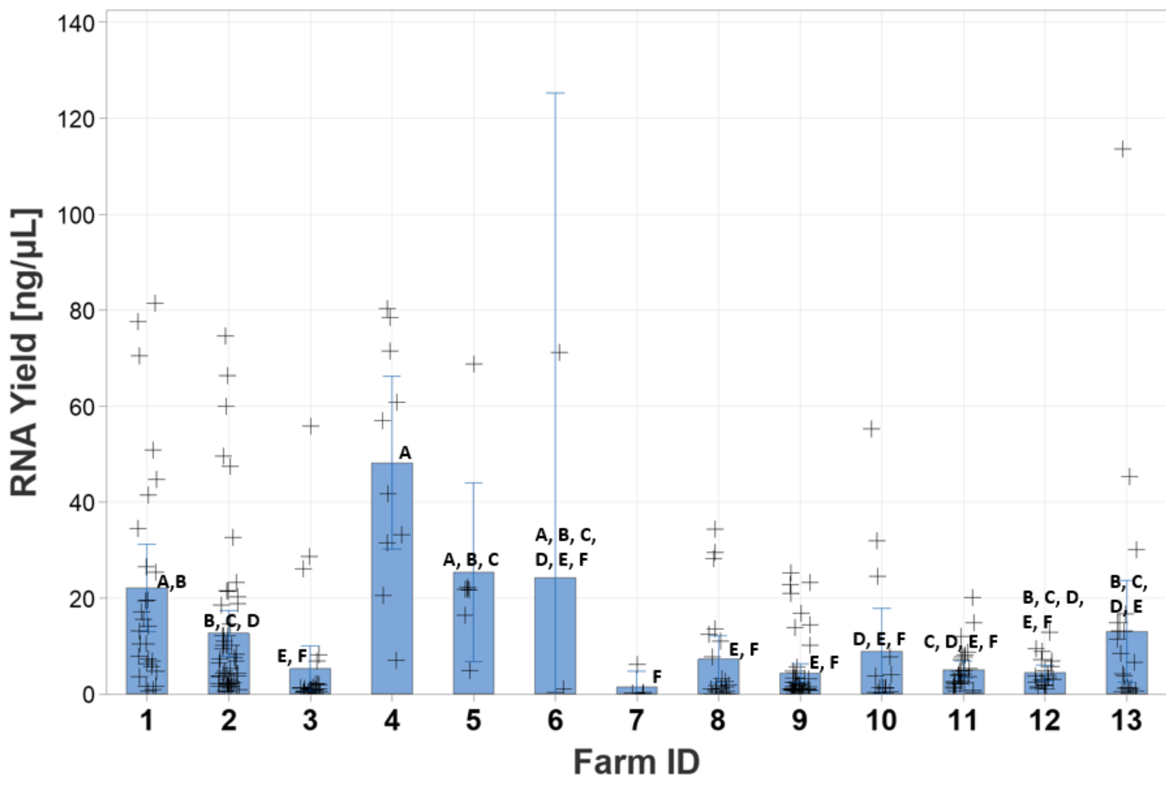

Figure 5: RNA yield for rams based on the farm of origin.

Grey crosses indicate individual results. Bars (blue) indicate the mean RNA yield for each farm. Letters (A, B, C, D, E, and F) are the result of the Tukey pairwise comparison. Means that do not share at least one letter were significantly (P<0.05) different.

4.3 Gene expression

4.3.1 Gene expression profile

Of the nine biomarkers[2] used in this study, only four (ACP7, CCL27, LOC106990188, and TXN2) were significantly different in expression between the susceptible and tolerant groups (p= <0.05) as shown in **Figure 6**.

The mean expression level of ACP7 was 85.4 ± 91.6 RNA copies in susceptible animals, compared to 162.2 ± 176.9 RNA copies in tolerant animals. Biomarker CCL27 exhibited the smallest difference between susceptible and tolerant groups, with mean levels of 17.81 ± 23.09 and 34.41 ± 38.31 RNA copies, respectively. Similarly, TXN2 showed a modest difference with 162.51 ± 56.72 RNA copies in susceptible animals and 203.3 ± 75.2 RNA copies in tolerant animals. In contrast, LOC106990188 displayed the largest difference, with mean levels of 149.3 ± 199.8 RNA copies in susceptible animals and 325.3 ± 398.7 RNA copies in tolerant animals as shown in **Table 5**.

The p values for group-wise comparison of each of these 4 biomarkers; ACP7 (p= 0.013), CCL27 (p=0.005), LOC106990188 (p=0.010) and TXN2 (p= 0.009) were significantly different (test, $p < 0.05$) as shown in **Figure 6**.

[2] ACP7, CCL27, GLRX, GPR143, LOC106990188, PTGES2, TXN, TXN2 and UBB

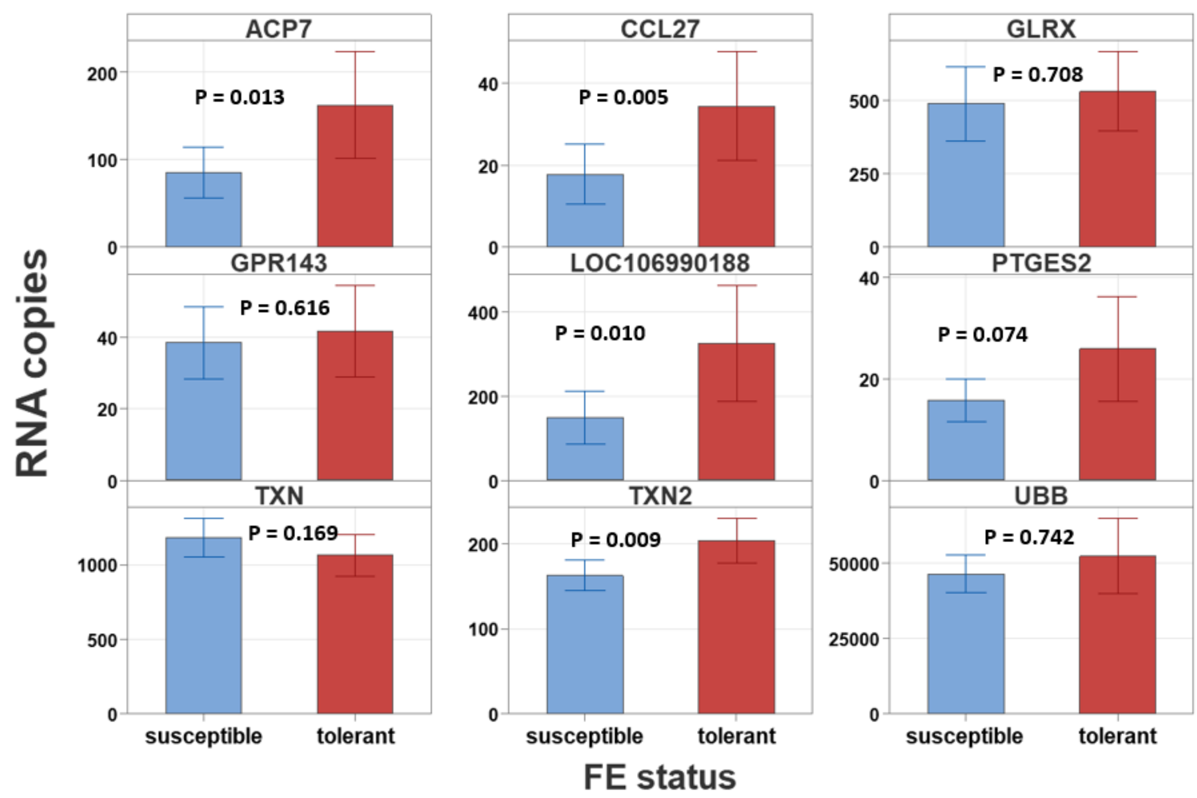

Figure 6. The Gene expression profile of all nine biomarkers, shown as RNA copies against FE status. In each individual plot, RNA copies are shown on the Y-axis and FE status on the X-axis, while the name of the biomarker is indicated on top. Bars show the mean RNA copies for susceptible (blue) and tolerant (red) animals. Standard errors are also depicted on top of individual bars.

Table 5. Mean RNA counts for four biomarkers of interest in FE susceptible and tolerant rams.

Biomarker	**FE Status**	**N**	**Mean**	**StDev**
ACP7	Susceptible	41	85.4	91.6
	Tolerant	35	162.2	176.9
CCL27	Susceptible	41	17.81	23.09
	Tolerant	35	34.41	38.31
LOC106990188	Susceptible	41	149.3	199.8
	Tolerant	35	325.3	398.7
TXN2	Susceptible	41	162.51	56.72
	Tolerant	35	203.3	75.2

For each of the four biomarkers (ACP7, CCL27, LOC106990188 and TXN2) of interest, **mean**, and standard deviation (**StDev**) of RNA counts in susceptible or tolerant rams are shown, along with the number of samples analysed (**N**).

4.3.2 Receiver Operating Characteristic (ROC) curves

ROC curves were performed on the four biomarkers of interest (ACP7, CCL27, LOC106990188 and TXN2) and the results are shown in **Figure 7**. The area under the ROC curve (AUC) indicates how well the test is able to classify the subjects correctly. AUC values ranged from 0.5 to 1.0.

The AUC for ACP7 (0.67), CCL27 (0.68) and LOC106990188 (0.66) are similar while that for TXN2 (0.64) is slightly lower as shown in **Figure 7**. These values suggest that the biomarkers provide a fair level of classification performance and represent a promising foundation for distinguishing FE-susceptible sheep from FE-tolerant ones, with potential for further refinement and optimization.

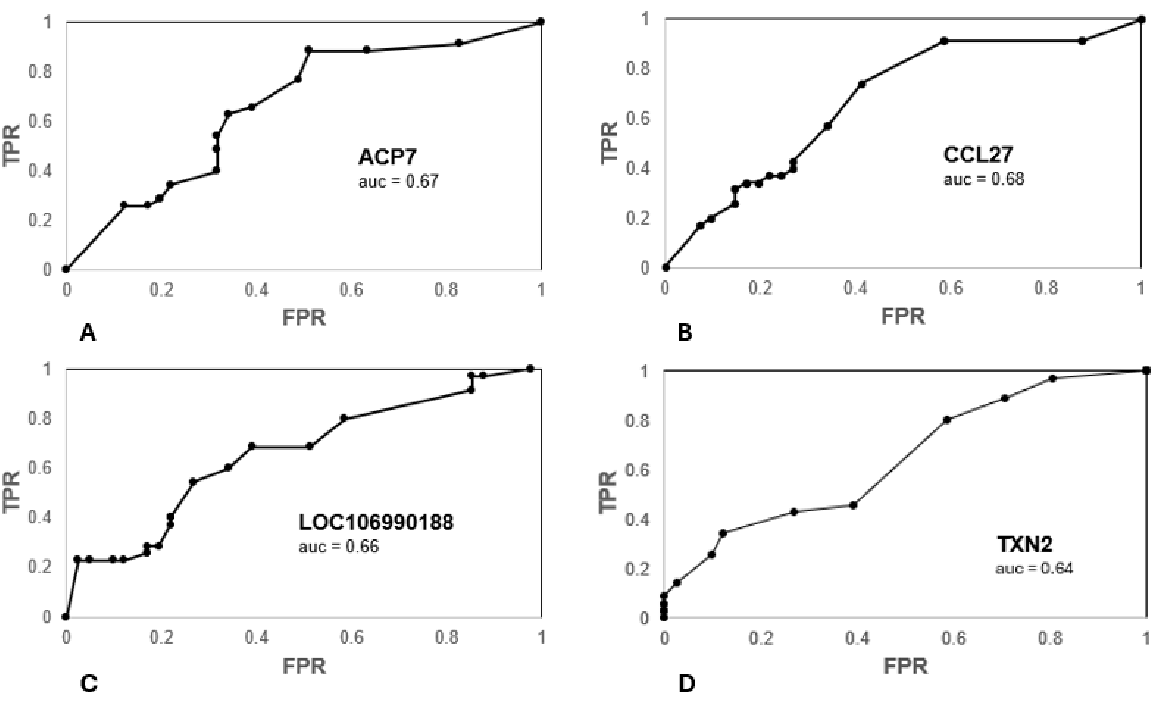

Figure 7. Receiver Operating Characteristic (ROC) curves for the four biomarkers of interest (ACP7, CCL27, LOC106990188, and TXN2).

The area under the ROC curve (AUC) is the measure of whether the biomarker has the ability to diagnose the animals with FE. A) the ROC and ACU for ACP7, B) the ROC and AUC for CCL27, C) the ROC and AUC for LOC106990188 and D) the ROC and AUC for TXN2. The y axis shows the TPR (True Positive Rate), and the x axis shows the FPR (False Positive Rate).

4.3.3 Prognostic values for individual markers

Prognostic values (sensitivity and specificity) were calculated for each of the 4 biomarkers of interest (ACP7, CCL27, LOC106990188 and TXN2). Sensitivity and specificity are used together to indicate how good the biomarkers are at determining whether the individual has a disease (FE) or not. A value of 0.8 is considered to have some predictive power.

CCL27 has high specificity (0.80) and low sensitivity (0.34) while both TXN2 and LOC106990188 had high sensitivity (0.80) and low specificity (0.41) as set out in **Table 6**.

Table 6. Prognostic values (**Sensitivity** and **Specificity**) for the four biomarkers of interest.

Marker	ACP7	CCL27	LOC106990188	TXN2
CutOff [RNA copies]	72	30	50	150
Sensitivity or True Positive Rate (TPR)	0.63	0.34	0.80	0.80
Specificity or True Negative Rate (TNR)	0.68	0.80	0.41	0.41

Biomarkers ACP7, CCL27, LOC106990188, and TXN2 were identified as significantly differentially expressed ($p<0.05$) in susceptible and tolerant animals. CutOff (RNA copies) values for each biomarker were determined by finding the optimal balance of specificity and sensitivity for each marker.

CCL27, LOC106990188 and TXN2 have only either high sensitivity or high specificity not both. This means as a diagnostic test, these markers, on their own are not strong enough predictors of whether the individual has a disease (FE) or not. ACP7 has similar levels of sensitivity (0.63) and specificity (0.68) so while not excellent, as a biomarker, it has the potential to be an indicator of whether the individual has the disease (FE) or not.

4.3.4 Combinatorial Strategies

Prognostic values (sensitivity and specificity) for selected pair-wise combinations of the four biomarkers of interest were also estimated, in an attempt to increase the sensitivity and specificity (**Table 7**). ACP7 combined with CCL27 resulted in a sensitivity of 0.37 and a specificity of 0.73. ACP7xCCL27 has a sensitivity of 0.31 and specificity of 0.75. Both ACP7+LOC106990188 and ACP7-TXN2 combinations have a sensitivity of 0.80 however, ACP7+LOC106990188 has a specificity of 0.46 while ACP7-TXN2 has a specificity of 0.23.

Table 7. Prognostic values (**Sensitivity** and **Specificity**) of selected pair-wise combinations of the four biomarkers of interest**.**

	ACP7+CCL27	**ACP7xCCL27**	**ACP7+LOC106990188**	**ACP7-TXN2**
CutOff [RNA copies]	125	3199	120	-22
Sensitivity or True Positive Rate (TPR)	0.37	0.31	0.80	0.80
Specificity or True Negative Rate (TNR)	0.73	0.75	0.46	0.23

Chapter 5: Discussion

Facial eczema (FE) is a significant issue for ruminant livestock and farmers in New Zealand, Australia, France, South Africa, Western China, and several South American countries (Collin & Towers, 1995; Liu et al., 2023). Currently, to determine the level of FE tolerance in rams, there is a programme called RamGuard™. This involves challenging rams with a controlled dose of sporidesmin and measuring GGT levels before (d0) and after (d21) the challenge. This is a costly, intensive process which affects the animal's welfare. Therefore, a research team at AgResearch, funded by B+LNZ and supported by farmers, is exploring the development of an *in vitro* test that will be non-invasive, cheaper and will not adversely affect animal welfare.

At the commencement of this project, nine biomarkers (ACP7, CCL27, GPR143, LOC106990188, PTGES2, TXN, TXN2, GLRX, and UBB) had been identified as candidates for determining tolerance to FE. This study has confirmed that using an *in vitro* assay, four (ACP7, CCL27, LOC106990188 and TXN2) of the nine biomarkers were differentially expressed between susceptible and tolerant rams ($p<0.05$) and may have the potential to be used in a non-invasive prognostic test to identify FE tolerance in sheep.

Phua et al. (2014) used the Illumina OvineSNP50 BeadChip to develop a prognostic test with an accuracy of 0.38 to predict FE tolerance in the Romney breed of sheep. This is far below the Ramguard™ test's prediction accuracy (0.64); however, the test is non-invasive and can also be provided as part of genomic testing for other traits at minimal cost. While this set a solid foundation, the results from the combinatorial strategies explored in this project indicate the potential to produce a test with a greater accuracy even though the sensitivity and specificity values vary between the four biomarkers.

5.1 Gamma-glutamyl transferase

GGT plays a pivotal role in identifying the level of resistance an animal has to FE. To provide the basis for further genetic and molecular analysis within the study, GGT levels were measured before (d0) and after (d21) rams were challenged with sporidesmin. As a result of the ram's response to the sporidesmin challenge, two different FE statuses were defined:

- **FE Tolerant**: Sheep with ΔGGT ≤ 70 IU GGT/L after sporidesmin dosing were classified as 'FE tolerant'.
- **FE Susceptible**: Sheep with ΔGGT > 70 IU GGT/L after sporidesmin dosing were classified as 'FE susceptible'.

As expected, FE susceptible animals showed a significant increase in GGT levels at day 21 (from 58.22 ± 12.94 IU/L at d0 to 975.10 ± 491.30) while tolerant animals showed only a minor increase over the same period (57.20 ± 11.61 IU/L at d0 to 65.77 ± 13.61 IU/L at d21) as shown in **Table 3**. This difference between groups was significant (p<0.001) and aligns with the findings from previous studies (Munday et al., 2021; Johnson & Amyes, 2020)**.**

The change in gamma-glutamyl transferase (ΔGGT) levels among farms was analysed to assess the distribution of tolerant and susceptible animals. The results, unsurprisingly, indicated that farms were home to different proportions of tolerant and susceptible rams. Farms 2 and 11 had high numbers of susceptible rams while Farms 1, 12 and 13 exhibited a higher proportion of tolerant rams as shown in **Figure 3**. In contrast, Farms 6 and 7 ram samples were comprised entirely of susceptible individuals, while Farms 3 and 4 had mixed populations of both tolerant and susceptible rams. The ΔGGT for all farms reflected which population (tolerant or susceptible) was dominant (**Figure 3**). The proportion of tolerant rams on each farm is also likely a reflection of each farm's progress in achieving a fully tolerant flock via selective breeding or other means. Given the farms in this study are involved in the Ramguard^{TM} programme it can be inferred that those farms with higher numbers of tolerant rams would likely show significant progress in achieving FE tolerance, whereas those farms with higher numbers of susceptible rams are likely to show little progress. It should be noted that the sample sizes from each farm were too low (range 1 – 28 rams) to apply robust statistical methods to these results. Therefore, these findings should be interpreted with caution and can be considered as indicative rather than conclusive.

5.2 Modifications to RNA extraction methods

Various modifications were made to both RNA extraction methods used (the two kits used were the Qiagen RNeasy Mini Kit and the Zymo MiniPrep Plus kit) in an attempt to increase the RNA (ng/µL) yield in each sample alongside the absorbance (A260/A280 and A260/A230) ratios. Two variations were used as these kits were repurposed from previous experiments. The A260/A280 absorbance ratio was used to assess the purity of the RNA while the A260/A230 absorbance ratio was used to check for the presence of other contaminants. Both methods resulted in similar yields of RNA. Key differences between the two methods included the initial preparation of DNase I and the use of Yellow Spin Away filters (Zymo MiniPrep Plus method) to remove the genomic DNA before ethanol precipitation. Other differences were the specific steps for washing and eluting the RNA which highlight procedural nuances that ensure higher purity and integrity of the final RNA samples.

Of the modifications, the third RPE step, which was added to the pre-existing two to further wash/remove any traces of salts that may still be present in the columns due to the buffers used

earlier in section 3.5.1 (RNA extraction using the Qiagen Kit Method), was the only modification discontinued. This method was only used twice at the very beginning of the RNA extraction process and once it was determined it did not change the result, it was discontinued.

Based on prior experience in the team, it was recommended that eluting with DEPC-treated water, rather than RNase-free water, would enhance both the yield and purity of total RNA. Consequently, this method was adopted and consistently used from the outset. DEPC[3] treatment is particularly important in molecular biology experiments where RNA integrity is critical as it helps prevent RNA degradation by RNases that might be present in water or on lab surfaces. DEPC works by modifying the histidine residues in RNases, thereby inactivating these enzymes. It is typically used to treat water which is then used to prepare RNA solutions or reagents that will come into contact with RNA (Pierson & Butler, 2007).

5.3 Quality of RNA

The blood samples used in this study were collected from rams enrolled in the Ramguard™ programme from 13 different farms around the North Island of New Zealand and shipped at ambient temperature to the Hopkirk Research Institute in Palmerston North for analysis. Once processed, using the extraction methods outlined in **sections 3.5.1** and **3.5.2**, it was evident there were differences in the total RNA yield for each farm. The total average RNA yield of the samples used in this study (n=290) was 11.07 $\pm$ 17.92 ng/µL. This yield is slightly lower than that reported in other studies (e.g., Pokharel et al., 2018). This poor yield may have been influenced by how the samples were processed (described in sections 3.3 and 3.4, Blood sampling and processing and *In vitro* sporidesmin toxicity assay, respectively). After being stimulated with sporidesmin, 200,000 cells/200 µL/well were incubated in 96-well U-bottom plates. It is possible that for occasional wells some cells were accidentally removed when removing the supernatant. Fewer cells will result in less RNA. It is also possible that there were variable amounts of supernatant remaining with the cell pellet. This would dilute the RLT buffer used to lyse the cells and result in incomplete lysis and consequently less RNA for analysis. To address these issues, others in the team switched (after this study) to “deep well” plates which take a larger volume allowing more cells to be added and more RLT buffer.

The average A260/A280 ratio for total RNA yield was 2.04 $\pm$ 1.23 which is within the ratio at which a sample is generally considered “pure” or “clean” (Koetsier & Cantor, 2019). The average A260/A230 ratio was 0.79 $\pm$ 1.07, i.e., well below the ratio of 2.0 at which a sample is considered free of

[3] Diethylpyrocarbonate (DEPC) is a chemical reagent commonly used to inactivate RNases which are enzymes that degrade RNA.

contamination. These values indicate significant variability between samples. This can be attributed to the assay used to process the samples (mentioned in **section 3.5** above).

Figure 4 shows that there was no difference in RNA yield between susceptible and tolerant groups ($p = 0.670$), i.e., there was significant overlap between the two groups. However, there were differences in the total RNA yield for some of the farms involved. This will be discussed in the following section.

5.4 Technical differences

As described above, there are technical differences introduced by the different people collecting samples in the field and processing the samples in the laboratory. These differences, while minor, may still have an impact. Shipping conditions also introduce slight differences. While the samples for this study were shipped at ambient to the Hopkirk Institute in Palmerston North, it is difficult to ascertain if they remained at ambient temperature or if there was either an increase or decrease in the temperature while they were being shipped. The samples themselves are from different farms which again may introduce non-random differences.

5.5 Choice of NanoString technology for biomarker quantification

Gene expression profiling is widely used to study metabolic and infectious diseases, particularly in humans (Fiorentina et al., 2021).

NanoString and RT-qPCR (Reverse Transcription Quantitative Polymerase Chain Reaction) are the two methods used to measure gene expression, each with its own strengths, depending on the research aims. In this study, NanoString was chosen because it is especially good at analysing many genes at once (multiplexing) and providing accurate counts of genes (quantification) (NanoString Technologies, 2015).

NanoString works by directly attaching barcodes to target molecules, which lets it count these molecules without needing to amplify them. This makes the process simpler and allows up to 800 targets to be analysed at the same time—something that's hard to do with other methods (Bergbower et al., 2020; NanoString Technologies, 2024). NanoString is also easy to use with small samples, making it a suitable approach when sample sizes are limited.

In contrast, RT-qPCR involves the synthesis of cDNA from RNA via reverse transcription and then amplifying it to measure gene expression. While RT-qPCR is very sensitive, especially for detecting small amounts of RNA, it doesn't handle multiple targets as well as NanoString (Pescarmona et al., 2018; Bergbower et al., 2020). NanoString's strength in analysing many gene expressions at once

and providing direct, accurate counts was crucial for this study, which needed to look at multiple biomarkers across many samples.

5.6 Gene expression

Four biomarkers (ACP7, CCL27, LOC106990188 and TXN2) were identified as being differentially expressed between the groups of FE tolerant and susceptible rams ($p<0.05$).

There was high variability in the RNA copies, both for each biomarker of interest and within the susceptible and tolerant groups. ACP7 expressed 85.4 ± 91.6 RNA copies in susceptible animals compared to 162.2 ± 176.9 RNA copies in tolerant animals ($P = 0.013$) as shown in **Table 5**. CCL27 exhibited the smallest difference between susceptible and tolerant groups, with mean levels of 17.81 ± 23.09 and 34.41 ± 38.31 RNA copies, respectively ($P = 0.005$). Similarly, TXN2 showed a modest difference with 162.51 ± 56.72 RNA copies in susceptible animals and 203.3 ± 75.2 RNA copies in tolerant animals ($P = 0.009$). In contrast, LOC106990188 displayed the largest difference, with mean levels of 149.3 ± 199.8 RNA copies in susceptible animals and 325.3 ± 398.7 RNA copies in tolerant animals ($P = 0.010$).

These findings are somewhat consistent with a previous study on a different set of samples, which also identified ACP7, CCL27, and TXN2 as significant biomarkers, but additionally found GPLR143 and GLRX to be significantly differently expressed (A. Heiser, personal communication). The discrepancy between the current study and the previous one is likely due to a combination of technical and biological factors. Technical variability, such as differences in sample handling conditions, RNA extraction protocols or media used during sample processing could have influenced the results. Biological differences, including variations in farm environments and animal cohorts, may also have contributed. The lack of differential expression of GPLR143 and GLRX in this study suggests that these genes may not be robust candidates for biomarkers in the context of FE tolerance. To gain further insights, it is recommended to plan to conduct a comprehensive analysis of gene expression data across both datasets.

5.7 Function of biomarkers

The following section discusses each of the four biomarkers of interest in more detail with respect to their functions and how they can be linked to FE. Understanding the functions of each of the biomarkers helps form connections between how the biomarkers could potentially be involved in the chemical processes that cause FE (i.e., sporidesmin toxicity) and thus dictate their inclusion (or exclusion) in the prognostic test.

5.7.1 ACP7

Acid Phosphatase 7 (ACP7) is an enzyme that plays a critical role in various biological processes, primarily through its acid phosphatase activity. ACP7 is part of the hydrolase class, the largest and most diverse class of enzymes with more than 200 enzymes that catalyse the hydrolysis of phosphoric monoesters, resulting in the release of inorganic phosphate. This activity is essential for multiple cellular processes, including dephosphorylation (important for signal transduction and energy metabolism) (Kalds et al., 2022) and the degradation of other molecules into smaller fragments.

Specific studies on ACP7 are limited in sheep, however general knowledge about the enzyme can be extrapolated from broader research (Kalds et al., 2022; GeneCards., 2024; Fabre et al., 2006). This allows connections to be made between what is known about this biomarker and how it could potentially be involved in the processes that contribute to the toxicity of sporidesmin (i.e., FE).

ACP7 is involved in the dephosphorylation of various substances, affecting cellular processes like growth, differentiation, and metabolic regulation. Given the importance of phosphate metabolism in cellular functions, it is likely that ACP7 plays a significant role in maintaining cellular homeostasis and function in sheep, like its role in other organisms (GeneCards., 2024).

Research in sheep genomics has revealed that various genes related to metabolic and phenotypic traits are subject to selection pressure that shapes their roles in biological functions. For example, the expression of genes involved in lipid metabolism, such as PDGFD and BMP2, affects traits like tail fat deposition and overall metabolism in sheep (Kalds et al., 2022). Although ACP7 has not been specifically highlighted in these studies, its role in phosphate metabolism suggests it may interact with these pathways indirectly by regulating the availability of phosphate groups for metabolic processes (Kalds et al., 2022; GeneCards., 2024).

ACP7 also plays a significant role in cellular detoxification and repair processes. It is implicated in detoxification pathways by dephosphorylating and inactivating potentially harmful phosphorylated compounds. This detoxification process is vital in preventing the accumulation of toxic substances in the cell, thereby maintaining cellular health and function. Enzymes like ACP7 help break down xenobiotics (foreign chemical substances) and metabolic by-products that could otherwise be harmful to cells (GeneCards., 2024).

Furthermore, ACP7 contributes to cell repair mechanisms by regulating the phosphorylation status of proteins involved in repair processes. Phosphorylation and dephosphorylation of proteins are crucial for signalling pathways that govern cell repair and regeneration. For instance, during DNA

repair, various proteins need to be activated or deactivated through phosphorylation. By removing the phosphate groups, ACP7 can help modulate these proteins' activities, ensuring proper repair mechanisms are in place. Additionally, ACP7's role in dephosphorylation is essential in managing oxidative stress responses. By participating in the deactivation of stress-related phosphorylated compounds, ACP7 aids in protecting cells from oxidative damage and facilitates recovery from such stress (GeneCards., 2024; Fabre et al., 2006).

Regardless of the limited specific studies on ACP7, its general role in detoxification and cell repair processes highlights its importance in maintaining cellular integrity and function. Research in model organisms and other mammals suggests that enhancing ACP7 activity could potentially improve cellular resistance to toxins like sporidesmin and enhance repair mechanisms, which is particularly relevant in agricultural settings where sheep may be exposed to various environmental stressors (GeneCards., 2024; Fabre et al., 2006). The results of this study corroborate this observation, as ACP7 was significantly differentially expressed between FE-tolerant and susceptible animals, indicating its potential role in mitigating the cellular damage caused by sporidesmin exposure. This finding supports the hypothesis that ACP7 contributes to cellular mechanisms associated with FE tolerance in sheep.

5.7.2 TXN2

Thioredoxin 2 (TXN2), like TXN, is an enzyme involved in redox regulation. Also known as TRX2, particularly when referenced in human and mouse studies, TXN2 is part of the thioredoxin superfamily and is primarily localized in the mitochondria where it is important for the control of mitochondrial reactive oxygen species (ROS) homeostasis, apoptosis regulation and cell viability (GeneCards., 2024). TXN2 is highly expressed in various tissues, including the heart, liver, skeletal muscle, and kidney (Chasapis et al., 2019; Spyrou et al., 1997) and contributes to cellular processes related to energy metabolism and oxidative stress response.

Like ACP7, specific studies of TXN2 in sheep are limited, however general knowledge about the enzyme can be extrapolated from broader research (GeneCards., 2024; Chasapis et al., 2019; Godoy et al., 2011). TXN2's primary function is in the regulation of the oxidative stress response. Oxidative stress is often characterized by the excessive production of ROS (Godoy et al., 2011) including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxy radical (OH). An important source of ROS is mitochondria (Spyrou et al., 1997). TXN2 possesses a dithiol-reducing activity, more specifically, a protein-disulphide reductase activity. This activity either reduces sulphide bonds or oxidises disulphide bonds. Disulphide bonds create toxicity by targeting protein thiol groups and generating ROS. These reactive disulphide bonds, like the bond found in the ETP class of metabolites,

is oxidized to generate superoxide free radicals. TXN2 is implicated in being able to reduce hydrogen peroxide and scavenge free radicals (Spyrou et al., 1997).

Mycotoxin resistance is determined primarily by the ability of a ruminant to detoxify the secondary metabolites generated by the metabolism of mycotoxins. While not explicitly named a detoxification enzyme involved in mycotoxin resistance, TXN2 is thought to work closely with glutathione and glutathione peroxidase (GPx) which are Phase II enzymes that detoxify xenobiotics (a substance foreign to the body) by increasing their aqueous solubility and facilitating their excretion in the urine (Morris et al., 2013).

Regardless of the limited specific studies on TXN2 in sheep, its general role in detoxification and oxidative stress response highlights its importance in regulating cell viability and ROS homeostasis. Research in model organisms and other mammals suggests that enhancing TXN2 activity could potentially improve cellular resistance to toxins, including sporidesmin, and improve the response to oxidative stress which is particularly relevant in agricultural settings where sheep may be exposed to various environmental stressors (GeneCards., 2024).

5.7.3 CCL27

Chemokine C-C motif ligand 27 (CCL27) is a chemokine that belongs to the C-C motif chemokine family. Chemokines can be divided into four major subfamilies based on the arrangement of the first two of the four conserved cysteine residues: CXC, CC, C and CX_3C (Sino Biological, 2024; Laing & Secombes, 2004). In the CXC subfamily, the two cysteines are separated by a single amino acid while in the CC subfamily, the two cysteines are adjacent. Most mammalian chemokines belong to the CXC and CC subfamilies (Laing & Secombes, 2004).

Chemokines have roles in immunoregulatory and inflammatory processes (GeneCards., 2024) and can be divided into two main categories: inflammatory or homeostatic. Homeostatic chemokines are produced and secreted constantly, and are generally involved in lymphocyte trafficking, immune surveillance, and localisation of lymphocytes with an antigen in the lymphatic system (Laing & Secombes, 2004). Inflammatory chemokines are only produced by cells during an infection or following a pro-inflammatory stimulus which prompts the migration of leukocytes to an injured or infected site (Laing & Secombes, 2004). These inflammatory chemokines can also activate cells to raise an immune response and begin the wound-healing process (Laing & Secombes, 2004).

Much like the other biomarkers, specific studies of CCL27 in sheep are limited however, general knowledge about the chemokine can be extrapolated from broader research (Laing & Secombes, 2004; GeneCards., 2024). CCL27's role focuses on the recruitment and activation of immune cells like

T cells and dendritic cells in the skin (this is also known as T cell-mediated skin inflammation). This means CCL27 is expressed in the skin and lymph nodes. It also plays fundamental roles in the development, homeostasis and function of the immune system and has effects on the cells of the central nervous system (CNS) as well as on endothelial cells involved in angiogenesis (tissue regeneration) or angiostasis (regulation of the creation of new blood vessels).

CCL27 specifically binds to the chemokine receptor (CCR10) and upregulates it promoting the recruitment of leukocytes. These leukocytes hone in on the area where CCR10 is being upregulated and mount an inflammatory response. This process of inflammation can be linked to the inflammation caused by sporidesmin in FE.

Regardless of the limited specific studies on CCL27, its general role in inflammatory processes and homeostasis highlights its importance in regulating processes in the body. Research in model organisms and other mammals suggests that enhancing CCL27 activity could potentially improve cellular response to inflammation caused by toxins like sporidesmin.

5.7.4 LOC106990188

LOC106990188 has no specific functional annotations available for this gene locus that could be identified.

5.8 Receiver Operating Characteristic curves and AUC

The Receiver Operating Characteristic (ROC) curves are used to assess how well a test can discriminate between two conditions e.g., healthy, and sick animals. On the ROC curve, the true positive rate (sensitivity) is plotted against the false positive rate for different threshold values. This helps us understand how good a test is at distinguishing between the two conditions (Shapiro, 1999; Florkowski, 2008).

The ROC curves are used in conjunction with the area under the curve (AUC) to assess the discriminatory ability of a test. The AUC provides a summary measure of the prognostic method's overall discriminatory power with an AUC of 0.5 meaning the test has no discriminative power, while an AUC of 1.0 means the test is perfect. An AUC between 0.7-0.8 is considered fair or acceptable i.e., the test is reasonably accurate (Mandrekar, 2010). An AUC between 0.8 and 0.9 is considered very good or excellent i.e., this indicates high accuracy. An AUC above 0.9 is considered outstanding i.e., the test is extremely accurate (Mandrekar, 2010). By looking at the AUC, researchers can figure out how well a biomarker can diagnose a condition like FE in sheep. The ROC curves and AUC help in choosing the best biomarkers for diagnostic tests by showing how well they balance sensitivity and specificity at different thresholds.

The ROC curves and the area under the curve (AUC) were calculated for each of the four biomarkers of interest. The AUC for ACP7 (0.67), CCL27 (0.68) and LOC106990188 (0.66) are similar while the AUC for TXN2 (0.64) is slightly lower (Figure 6). All the four biomarkers of interest are slightly below the threshold for a test considered fair or acceptable which may mean that if used in a prognostic test, these biomarkers will have less than ideal discriminatory power.

Prognostic values (sensitivity and specificity) were also calculated for each of the biomarkers. The CutOff (RNA copies) was determined by locating the optimal balance of sensitivity and specificity for each biomarker. Sensitivity measures the ability of the prognostic method to correctly identify individuals with the target condition i.e., FE tolerance, while specificity measures its ability to correctly identify individuals without the target condition, i.e., susceptibility. Both are calculated by comparing the results of the prognostic test with a gold standard reference test or clinical diagnosis. In this study, the Ramguard test, which involves sporidesmin dosing and subsequent measurement of GGT levels, was used as the reference method. Like the AUC curves, a value of 0.8 and upwards is considered excellent. Of the four biomarkers identified, CCL27 demonstrated high specificity (0.80) and low sensitivity (0.34) while both TXN2 and LOC106990188 had high sensitivity (0.80) and low specificity (0.41) (Table 4). ACP7 had the highest combined sensitivity (0.63) and specificity (0.68). Ideally, both sensitivity and specificity would be between 0.7-0.8 for a test to be considered for use and as these results indicate, none of the four biomarkers are strong enough to be used on their own in the prognostic test i.e., the likelihood the test definitively identifies a sheep with increased tolerance to FE is reduced. Therefore, various combinatorial strategies were explored.

5.9 Combinatorial strategies

ACP7 proved to be the biomarker with the most potential for standalone use, as evidenced by the sensitivity (0.63) and specificity (0.68) values. While below the threshold to be considered excellent, or even fair, of the four biomarkers, it had the highest sensitivity and specificity i.e., the hallmarks of a test with the potential to be an indicator of whether the individual has the disease (e.g., FE) or not.

For these reasons, ACP7 was combined with the other three biomarkers (**Table 7**). Like the individual results, the combinations also indicated either fair sensitivity or specificity, but not both. ACP7+CCL27 showed a sensitivity of 0.37 and a specificity of 0.73 while ACP7xCCL27 showed a sensitivity of 0.31 and a specificity of 0.73. ACP7+LOC106990188 showed a sensitivity of 0.80 and a specificity of 0.46 while ACP7-TXN2 showed a similar sensitivity at 0.80 and a lower specificity of 0.23. While both combinations had similar sensitivity values there was a significant difference between the specificities.

5.10 Specificity of Biomarkers to Facial Eczema

Although the expression of these biomarkers is significantly different between susceptible and tolerant animals, it is important to consider whether these biomarkers are specific to FE or if they could also respond to other conditions affecting the animal. Based on their known roles and functions, there is a possibility that these biomarkers may reflect a broader capacity to resist liver toxicity, which can be triggered by various factors beyond sporidesmin exposure, such as other toxins or inflammatory conditions. Further studies are necessary to investigate the specificity of these biomarkers to FE by examining their expression in animals exposed to different hepatic stressors or diseases. However, this potential overlap does not diminish their utility in the context of FE, as the ability to identify animals with enhanced liver resilience is highly relevant for breeding programs aimed at improving tolerance to FE.

5.11 Drawbacks/limitations of study

RNA extraction yielded lower quantities than anticipated but remained sufficient for reliable analysis using the NanoString approach. Despite tweaking several aspects of the extraction process, the RNA yield remained variable, likely due to several reasons. Firstly, the cells used for RNA extraction had been exposed to the toxin sporidesmin, and by the time the extraction happened, these cells were at various stages of dying. When cells are dying or dead, they tend to release less RNA and what they do release is often poor quality (Thomas et al., 2019) which may explain the variability in yield observed here.

Also, only a small number of cells were used in the assay, so any inefficiency in the extraction process had a bigger impact on the amount of RNA measured than it would have if larger numbers of cells were used. Another issue was that it was difficult to completely remove all the supernatant before adding the lysis reagent. Any leftover liquid would dilute the lysis buffer, making it less effective at breaking open the cells and further reducing the RNA yield.

The quality of the RNA measured by the A260/A280 and A260/A230 ratios was also inconsistent. The average A260/A280 ratio was 2.04 + 1.23 and the A260/A230 ratio averaged 0.79 + 1.07. This variation likely reflects the issues with cell lysis and leftover supernatant both of which can affect RNA quality as described above.

Even though the RNA yields were not as high as hoped, they were still enough for NanoString analysis which only requires small amounts of RNA (only 6-7 µL or about 600ng). This outcome shows how important it is to fine-tune the cell lysis and sample preparation processes, prior to RNA collection, especially when dealing with stressed or limited cell samples.

5.12 Further study

RT-qPCR is a laboratory technique used to detect and quantify the amount of RNA molecules present in a sample. It combines two processes: reverse transcription and quantitative PCR. The first step, reverse transcription, involves the conversion of RNA into complementary DNA (cDNA) using an enzyme called reverse transcriptase. This step is necessary because PCRs require DNA as the starting material, and many biological samples contain RNA instead. Once the RNA has been converted into cDNA, the quantitative PCR (qPCR) step follows.

Developing a multiplex qPCR to simultaneously detect, amplify and quantify multiple RNA targets in a single reaction will save time, reduce sample consumption, and provide a cost-effective approach for analysing multiple RNA and DNA targets. Presently, a lack of consensus exists on how to best perform and interpret qPCR experiments (Bustin et al, 2009). Typically, this results in a lack of sufficient experimental data in many publications, thus impeding the reader's ability to critically evaluate the quality of the results presented or repeat the experiments. The Minimum Information for Publication of Quantitative Real-Time PCR experiments (MIQE) guidelines layout the minimum information necessary for evaluating qPCR experiments thus encouraging improved experimental practice and allowing more reliable and unequivocal interpretation of qPCR results (Bustin et al, 2009).

Developing a quantitative RT-qPCR for the four biomarkers of interest using archived RNA samples from Phase 3 and Phase 4 would validate their predictive capabilities and subsequently their inclusion in a prognostic test.

To improve RNA yield and quality, it is recommended to modify the *in vitro* sporidesmin assay by using cell culture plates that allow for larger volumes of blood. This change would allow collection of more cells which should lead to higher RNA yields and reduce issues with leftover supernatant relative to the number of cells. Additionally, using a larger volume of lysis buffer should help address the problem of incomplete lysis, further improving RNA extraction.

Given the narrowed focus to fewer biomarkers (four rather than nine), using multiplex RT-qPCR might be a better approach for future studies. This method is not only more cost-effective than NanoString but is also more commonly available in veterinary diagnostic labs which often don't have the capability to run NanoString.

Developing a multiplex RT-qPCR that can detect and measure multiple RNA targets in one go would save time, reduce the amount of sample needed, and be a more affordable way to analyse several biomarkers at once.

Other members of the team in AgResearch are planning to develop a quantitative RT-qPCR for the four biomarkers identified in this study, using RNA samples archived from Phases 3 and 4. This would help validate the biomarker's effectiveness and decide whether they should be included in a prognostic test for FE tolerance.

5.13 Conclusion

In conclusion, this project has demonstrated that of the nine biomarkers identified, four biomarkers have the potential to be used in a prognostic test to identify tolerance to facial eczema. Future research will continue to investigate whether the sensitivity and specificity of the nine biomarkers can be improved which will thus improve the accuracy of the prognostic test. It will also focus on developing the RT-qPCR to validate the biomarkers predictive capabilities and subsequently their inclusion in the prognostic test.

This project also demonstrated the variability of the RNA yield measured from the samples obtained from each of the 13 farms involved. Unfortunately, there was no further information on the breed, age, or weight of the rams used in this project so the difference in RNA yield cannot be extrapolated from the individual rams used however, the RamGuard^{TM} test typically involves testing ram hoggets (young male sheep), usually before they are used for mating. These ram hoggets are often tested in the autumn season to determine their tolerance to FE before they are selected for breeding. It is known that the farms involved in this project are based in the North Island of New Zealand as this is where the incidence of FE is the highest.

Farm 4 had the highest RNA yield, followed by Farms 5 and 6. RNA yields in Farms 4 and 5 were significantly higher than those in Farms 3, 7 and 9. RNA yield in Farm 1 was also significantly higher than that in Farms 3, 7, 8, 9, 10 and 11. It is unlikely that biological differences between the animals or specific farming practices were the primary cause of these observed differences in RNA yield. Instead, the most plausible explanation for the variation in RNA yields is related to differences in transport and handling conditions that could have led to cell damage prior to RNA extraction.

Variability in factors such as the duration of transport, temperature fluctuations and handling during transit could have affected the integrity of the samples, thereby affecting the RNA yield. Understanding these logistical factors and implementing standardised protocols for sample collection and transport would be crucial to minimising such variations. Future studies should aim to control these variables more tightly to ensure that RNA yield differences reflect true biological variations rather than artifacts introduced during sample handling.

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