# An association between circulating copper concentrations and gammaglutamyl transferase activity in sheep after exposure to the toxin sporidesmin

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## Abstract

Copper is a potent catalyst of the reaction in which sporidesmin generates damaging free radicals causing facial eczema in grazing livestock in New Zealand. This study sought to investigate whether circulating copper at the time an animal is dosed with the toxin, influenced the way the animal responds to sporidesmin and whether the action of sporidesmin on the liver in susceptible animals results in changes to circulating copper. Serum copper concentrations (CU) together with gamma glutamyl transferase (GGT) activity, an indicator of bile duct damage, were opportunistically collected on over 400 animals from nine flocks in which the animal's tolerance to sporidesmin was commercially assessed through measuring its response to a known dose of the toxin. The association between CU concentration before the animals were dosed and natural log transformed GGT activity measured 21 days after the animals had been dosed was not significant, but there was a suggestive (P=0.06) interaction between flock and CU. There was a significant positive association between natural log transformed GGT activity and CU concentrations measured 21 days after the animals had been dosed with the toxin (P<0.0001). For some individuals, the CU concentrations approached or met copper-toxicity thresholds (above 40 µmol/L). The specific biological mechanisms causing this association remain unknown, but given the role of copper in catalysing the sporidesmin reaction and the potential of toxic copper concentrations, further research is warranted.

**Keywords**: sheep; facial eczema; copper; gamma glutamyl transferase

## Introduction

Facial eczema is caused by ruminants ingesting the toxin sporidesmin, which is produced by the fungus *Pithomyces chartarum* that proliferates in the presence of dead plant matter in warm humid conditions in parts of New Zealand. The primary site of action of the toxin in sheep is in the liver and bile ducts of animals that ingest the toxin, with the production of superoxide radicals ($O_2^-$) and a by-product of the reaction, hydrogen peroxide (Munday 1982).

*In vitro* copper has been shown to be a potent catalyst of the autoxidation of reduced sporidesmin in producing $O_2^-$ (Munday 1985). It has been hypothesised that a mechanism through which zinc prevents facial eczema may be through decreasing intestinal copper absorption (Munday 1985). The liver is the major storage site of copper. There is evidence that under stressful conditions copper can be released from storage in the liver (Farquharson 1984). Alternatively, copper can be released from the liver as a result of damage to hepatocytes (Nederbragt et al. 1984). In high enough concentrations, copper can be toxic and can cause damage to the liver and bile duct through the generation of hydroxyl radicals (Nederbragt et al. 1984). Gamma glutamyl transferase which is used as indicator of bile duct damage in facial eczema (Amyes & Hawkes 2014), is similarly used in the diagnosis of copper toxicity in farmed animals (Johnston et al. 2014).

Despite the known *in-vitro* relationship, no *in-vivo* testing of the role of copper in the sporidesmin reaction has been conducted previously. The most appropriate study to test for a role would be to measure circulating and liver stored copper concentrations; however, as a first step, serum samples from sheep that were being dosed with sporidesmin as part of the Ramguard FE tolerance test were available to test for serum copper concentrations in addition to the routine gamma glutamyl transferase testing. The aim was to investigate whether circulating copper concentrations at the time of sporidesmin dosing influenced the way in which an animal responded to sporidesmin and/or whether the action of sporidesmin in susceptible or tolerant animals resulted in changes to circulating copper levels.

## Materials and methods

This study utilised blood samples collected through the Ramguard programme. The Ramguard programme is described in detail by Amyes and Hawkes (2014). Briefly, an animal's tolerance to sporidesmin is determined through an animal being dosed by intraruminal intubation with a pre-determined amount of sporidesmin. The chosen dose rate reflects previous response levels in the flock the animal came from, and the animal's live weight and expressed as mg sporidesmin per kg live weight. Blood samples are collected either on the day the animal is dosed with the toxin (D0) or seven days prior (D-7; to check for pre-existing liver damage in animals grazed in areas where natural sporidesmin exposure had been possible) and again 21 days (D21) after exposure. For simplicity of reporting the pre-sporidesmin dosing time point will be referred to as D0. Ramguard blood samples are routinely analysed for serum gamma glutamyl transferase activity (IU/L 37°C; GGT) through a commercial laboratory (IDEXX, Hamilton, New Zealand).

Initially, a single Ramguard client was contacted (Flock 1), and permission obtained to also allow measurement of

serum copper concentrations (µmol/L; CU) on the blood samples being collected for RamGuard testing. A total of 55 animals had been selected by the breeder for testing. The results from this flock supported the collection of more CU data in addition to the routinely collected GGT data.

An additional eight Ramguard clients (Flocks 2-9) were identified from the total Ramguard client list, with the aim of representing the mix of New Zealand's maternal breeds, including Romney, Perendale, and composites including Finnish Landrace and Texel genetics; a range of sporidesmin dose rates ranging from 0.27 to 0.55 mg per kg live weight; and geographical locations ranging from Northland to Otago. In all cases the number of, and specific animals dosed were chosen by the breeder as part of their breeding programme, and thus, in some instances, only low numbers of animals were tested. In total, between September 2018 and September 2019, CU data were collected for 414 lambs from nine flocks, representing approximately one third of all Ramguard-tested animals for that year. The breeders have been breeding for tolerance to FE from five to more than 30 years. The dose rates varied between flocks dependent on how many years testing they had undertaken, and the estimated tolerance of their flock (based on results from Ramguard testing in previous years). Day 21 CU data were available for all animals in the study, however, D0 data were only available for animals from seven breeders, representing 382 animals.

Data used in the analysis were D21 serum GGT (GGTD21) activity and D0 and D21 serum copper (CUD0 and CUD21) concentration. The ratio of CUD21:CUD0 was also calculated (CURATIO) where both CUD0 and CUD21 data were available. Due to D21 serum GGT activity data exhibiting a non-normal distribution (Amyes & Hawkes, 2014), the data were natural log-transformed prior to analysis (lnGGTD21). Each flock represented a unique combination of breed and dose rate, and as such breed and sporidesmin dose-rate data were confounded with flock, and therefore, only flock was fitted in models described below, with no attempt to interpret the results with respect to breed or sporidesmin dose rate. The GLM Procedure in SAS (SAS 2011) was used to undertake analysis of variance analysis to investigate the association between copper and GGT. Three models were fitted. In the first model lnGGTD21 was fitted as the dependent variable, with CUD0, flock and their interaction fitted as explanatory variables. CUD0 was fitted as a covariate, whilst flock was fitted as a fixed effect. In the second and third models CUD21 and CURATIO were fitted as dependent variables, with lnGGTD21, flock and their interaction fitted as explanatory variables. lnGGTD21 was fitted as a covariate. The results are presented whereby the $R^2$ of reduced models are reported in addition to the $R^2$ of the full model to determine which explanatory variables were explaining the largest amount of the variation reported. The first reduced model did not include flock or the interaction between flock and the covariate fitted, with the second reduced model also including flock but not the interaction, with the full model including the interaction. The $R^2$ of the reduced models were calculated by dividing the reported type-I sums of squares for the explanatory variables included in the reduced model (summed for the second reduced model) by the total model sums of squares. Two data sets were used in the analysis, the first just exploring relationships in Flock 1, as was carried out to inform the decision to do further data collection, with the second data set including data from all nine flocks. For the first data set, flock was not fitted as a fixed effect.

To graphically represent the data, individual animals were assigned to tolerance/susceptibility groups (GGTGRP) based on their GGTD21 results according to the standard groupings used within the Ramguard programme to estimate liver/bile duct damage, with GGT<71 IU/L low; GGT 71-300 IU/L slight; GGT 301-700 IU/L moderate and GGT> 700 IU/L high, with the CUD21 and CURATIO trait data plotted in a boxplot using GGTGRP as the category on the X axis.

## Results

The summary statistics for GGTD21, CUD0, CUD21 and CURATIO for the nine Ramguard flocks are presented in Table 1. Flock 1 was the first flock where data were collected, and a preliminary analysis of these data led to the decision to collect additional data from additional flocks. For Flock 8 most animals exhibited high tolerance to their dose of sporidesmin with an average GGTD21 of only 73±53 IU/L, with a maximum GGTD21 of only 343 IU/L. For the other flocks there was a large range in GGTD21 concentrations, with all flocks recording GGTD21 levels greater than 1000 IU/L. The average CUD21 was elevated in most flocks compared to CUD0, except for Flock 4 which was a flock in which the tolerance to sporidesmin was also high, with an average GGTD21 of only 128 ± 170 IU/L. Serum copper concentrations >11 µmol/L are considered to indicate adequate copper, while those above 25 µmol/L are considered to be high (Laven & Smith 2008). Two animals in Flock 1 exceeded the serum copper toxicity concentration of 40 µmol/L, with a further 13 above 30 µmol/L across all flocks. In all flocks with CURATIO (except Flock 4), there was at least one animal that had double the concentration of copper between the two measurements.

In the analysis only including data from Flock ,1 which was the flock which copper data was first collected, there was a significant positive association between CUD0 and lnGGTD21 ($P<0.05$), lnGGTD21 and CUD21 ($P<0.0001$) and lnGGTD21 and CURATIO ($P<0.0001$). The results from the analyses including data from all nine flocks are presented in Tables 2 and 3. For the model considering the association between CUD0 and lnGGTD21 (Table 2), CUD0 was not significant, however, the interaction between flock and CUD0 was approaching significance ($P=0.06$). For the model considering the relationship between lnGGTD21 and CUD21 (Table 3), all explanatory variables were significant (lnGGTD21, flock and the interaction; $P<0.05$). For the model considering the relationship between lnGGTD21

**Table 1** Summary statistics for gamma glutamyl transferase activity 21 days (GGTD21) and its natural log-transformed value (lnGGTD21) after animals were being dosed with the toxin sporidesmin and serum copper concentrations before (Day 0 or Day -7: CUD0) and after (Day 21: CUD21) dosing, together with their ratio (CURATIO) in nine Ramguard flocks.

| | | GGTD21 | | lnGGTD21 | | CUD0 | | CUD21 | | CURATIO | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Flock | n | Mean ± SD | Range | Mean ± SD | Range | Mean ± SD | Range | Mean ± SD | Range | Mean ± SD | Range |
| 1 | 55 | 224 ± 299 | 41 - 1341 | 4.8 ± 1.1 | 3.7 - 7.2 | 19.9 ± 3.9 | 12 - 31 | 21.7 ± 7.0 | 12 - 44 | 1.1 ± 0.4 | 0.6 - 2.3 |
| 2 | 17 | 624 ± 525 | 43 - 1195 | 6.0 ± 1.2 | 3.8 - 7.1 | 14.6 ± 2.1 | 11 - 19 | 24.5 ± 6.9 | 13 - 35 | 1.7 ± 0.5 | 1 - 2.5 |
| 3 | 10 | 582 ± 563 | 49 - 1316 | 5.6 ± 1.5 | 3.9 - 7.2 | na | na | 22.3 ± 8.6 | 13 - 33 | na | na |
| 4 | 146 | 128 ± 170 | 45 - 1079 | 4.5 ± 0.6 | 3.8 - 7.0 | 14.7 ± 3.2 | 10 - 30 | 14.6 ± 3.4 | 11 - 27 | 1.0 ± 0.2 | 0.6 - 1.9 |
| 5 | 75 | 375 ± 349 | 44 - 1405 | 5.5 ± 1.0 | 3.7 - 7.2 | 14.5 ± 2.9 | 9.8 - 23 | 20.4 ± 4.8 | 11 - 32 | 1.4 ± 0.4 | 0.8 - 2.4 |
| 6 | 28 | 235 ± 297 | 41 - 1187 | 4.8 ± 1.0 | 3.7 - 7.1 | na | na | 15.8 ± 5.2 | 8.7 - 27 | na | na |
| 7 | 33 | 673 ± 608 | 48 - 1955 | 5.9 ± 1.3 | 3.9 - 7.6 | 17.0 ± 3.3 | 13.1 - 28.6 | 20.2 ± 6.1 | 9.2 - 31.4 | 1.2 ± 0.4 | 0.6 - 2.1 |
| 8 | 44 | 73 ± 53 | 41- 343 | 4.2 ± 0.4 | 3.7 - 5.8 | 13.3 ± 2.5 | 10 - 21 | 14.4 ± 4.1 | 7.4 - 28 | 1.1 ± 0.3 | 0.6 - 2.1 |
| 9 | 6 | 535 ± 289 | 59- 812 | 6.0 ± 1.0 | 4.1- 6.7 | 13.0 ± 2.1 | 9.7 - 15 | 22.3 ± 4.8 | 16 - 28 | 1.7 ± 0.4 | 1.1 - 2.3 |

na: Pre-dose CU was not measured in this flock

**Table 2** Results for model fitted to determine relationship between explanatory variables, serum copper concentrations (CUDO) prior to dosing animals with the toxin sporidesmin, and flock, and the dependent variable gamma glutamyl transferase activity (natural log transformed) 21 days (GGTD21) after dosing in nine Ramguard flocks.

| | Significance | | | Solution Est. | Model $R^2$ | | |
|---|---|---|---|---|---|---|---|
| | CUDO | Flock | CUDO*Flock | CUDO | Red. 1[1] | Red. 2[1] | Full[1] |
| lnGGTD21 | 0.41 | <0.0001 | 0.06 | -0.08 | 0.00 | 0.32 | 0.34 |

[1]Reduced model 1 only included CUD0; Reduced model 2 also included flock; Full model also included the interaction.

**Table 3** Results for models fitted to determine relationship between explanatory variables, gamma glutamyl transferase activity (natural log transformed) 21 days (GGTD21) after dosing animals with the toxin sporidesmin and flock, and dependent variables of serum copper concentrations after dosing (CUD21) and the ratio of copper levels prior to and at day 21 after dosing (CURATIO) in nine Ramguard flocks.

| | Significance | | | Solution Est. | Model $R^2$ | | |
|---|---|---|---|---|---|---|---|
| | lnGGTD21 | Flock | lnGGTD21*Flock | lnGGTD21 | Red. 1[1] | Red. 2[1] | Full[1] |
| CUD21 | <0.0001 | <0.0001 | 0.03 | 4.87 | 0.54 | 0.67 | 0.69 |
| CURATIO | <0.0001 | <0.0001 | 0.09 | 0.32 | 0.55 | 0.64 | 0.65 |

[1]Reduced model 1 only included GGTD21; Reduced model 2 also included flock; Full model also included the interaction.

and CUD21 (Table 3), all explanatory variables were significant (lnGGT21 and flock; $P<0.0001$) or approaching significance (the interaction between lnGGTD21 and flock; $P<0.10$). Considering the $R^2$ values for reduced models relative to the full models, lnGGTD21 explained most of the variation in both CUD21 and CURATIO, with flock explaining less of the variation, and the interaction between flock and GGTD21 contributing very little of the variation.

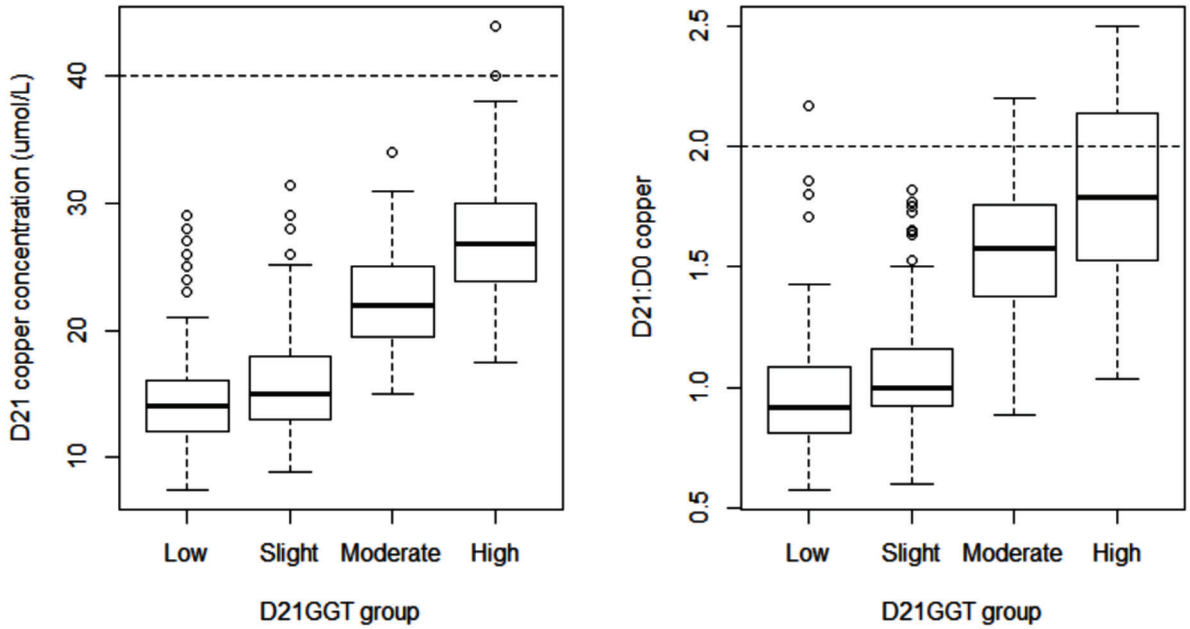
Graphical representation of the data is provided in Figure 1, with CURATIO trait data plotted in a boxplot using GGTGRP as the category on the X axis. This illustrates the relationships described in Table 2, in that animals with highly elevated GGTD21 had an elevated CURATIO; however, it also visualises that those with non-elevated GGTD21 had, on average, reduced CUD21 compared to CUD0 (CURATIO below one). Additionally, this plot illustrates that several animals that had moderate to highly elevated GGTD21 exceeded the ratio of 2.0 (n=15), above which animals can be at risk of copper toxicity.

## Discussion

The range in GGTD21 (Table 1) was as expected for animals exposed to the toxin through the Ramguard programme. For CUD0 and CUD21 individual animals had values both below and above the adequate range for CU, although the flock average concentrations were within the adequate range. There was greater variability in CUD21, and one flock had an average CUD21 that was sitting on the "high" threshold for the adequate range.

The initial results from Flock 1 suggested that a significant association existed between lnGGTD21 and CUD0, with a very significant association between lnGGTD21 and CUD21 and CURATIO, supporting the collection of further copper data which was carried out,

**Figure 1.** Left: boxplot of the distribution of the copper concentration (μmol/L) measured 21 days (D21) after animals have been dosed with the toxin sporidesmin grouped by the level of liver response estimated by activity of Gamma Glutamyl Transferase (GGT) where Low: GGT<71IU/L; Slight: GGT 71-300 IU/L; Moderate: GGT 301-700 IU/L and High GGT> 700IU/L. Right: boxplot of the distribution of the ratio of copper concentration measured before animals were dosed with the toxin sporidesmin (D0) and 21 days later (D21) grouped by level of liver response estimated by GGT activity. There is a risk of copper toxicity where copper concentrations are above 40μmol/L or D21 concentrations are double or above D0 concentrations (indicated by the horizontal dashed line).




forming the full data set used in this study. In the full data set, the association between CUD0 and lnGGTD21 was not replicated (Table 2), although there was a significant (P=0.06) interaction between flock and CUD0, indicating that an association may exist in some flocks. There was a highly significant positive association observed between lnGGTD21 and CUD21 and CURATIO suggesting that the association is consistent across a range of dose rates, breeds and geographical regions. In addition to visualising the elevation in CUD21 in animals with high lnGGTD21, Figure 1 illustrates that animals with limited liver damage (low GGTD21) had similar or even lower CUD21 compared with CUD0 (values below one).

The level significance of the association between CUD21 and GGTD21 were a surprise. The absolute concentrations of CUD21 and the CURATIO, for some individuals put them at concentrations/ratios associated with copper toxicity in sheep (Figure 1). Serum copper concentrations of greater than 40 μmol/L (Gribbles 2020) can indicate toxicity, and two-fold changes in CU concentrations have been suggestive of an early onset of a copper toxicity event (McCosker 1968).

Copper toxicity has been observed in sheep in association with the ingestion of other plant secondary metabolites, such as a toxin associated with lupins (Gardiner 1966) and pyrrolizidine alkaloids produced by plants including *Echium plantaglneum* and *Heliotropium europaeum* (Seaman 1987). Whilst the most likely explanation for the observed result is liver hepatocyte damage caused by the sporidesmin toxin resulting in the release of stored copper (Nederbragt et al. 1984) alternative hypotheses do exist. An alternative hypothesis is that the increased CU is the result of lower copper excretion through the gall bladder, once it is damaged by the toxin, leading to a build-up of CU (even though the rate of excretion of the copper through bile is considered to be low (Søli & Rambaek 1978)). Another alternative is that the copper is actively released from the liver in response to the physiological stress of exposure to the toxin as described by Farquharson (1984). Whilst elucidating the cause of the rise is important, equally important is determining whether the rise in circulating copper goes on to play a role in catalysing further sporidesmin reactions, or if the increased circulating copper directly causes the generation of superoxide radicals and further liver and bile duct damage as can be observed in cases of copper toxicity (Johnston et al. 2014), in addition to that already caused by sporidesmin.

There is no relevant literature to consider potential mechanisms through which reduced circulating copper occurred in some tolerant animals.

The finding of a significant association between CU concentration and GGT activity after exposure to sporidesmin is novel. However, further studies are required to understand the cause and implications of this association. Stored liver copper could not be measured in this opportunistic data set. Although the relationship between lnGGTD21 and CUD0 was not consistent, it does not preclude stored liver copper at the time of dosing

from contributing to the results observed at Day 21 given circulating and stored copper levels are not well correlated in normal animals (Johnston et al. 2014), and future studies should include measurement of liver copper concentration.

Although the underlying reason for the association between CUD21 and GGTD21 is not known, the results of this study do have practical relevance, as copper supplementation is common place, particularly in copper-deficient regions in New Zealand, and supplementation could further exacerbate the potential for copper toxicity in animals if given whilst copper concentrations are already elevated. As such, assessment of copper concentrations after facial eczema challenge should be made prior to any supplementation. Alternatively, if zinc supplementation has occurred, there is also the possibility that liver and serum copper concentrations may be low due to zinc supplementation driving decreased storage of copper (Smith et al. 2010). This further highlights the importance of testing both serum and liver copper concentrations to understand the status of a flock (or herd).

This study has shown, using an opportunistic data set, that a significant association exists between CUD21 and GGTD21. The cause of the association cannot be inferred from this study, however, irrespective of the cause, increased circulating concentration of copper is of potential importance given the proposed role of copper in catalysing the sporidesmin reaction, and the fact that copper can be toxic to the animal in high concentrations which were reached or approached in some animals in this data set. Further investigation of the association with blood samples collected at multiple time points between D0 and D21, including collection of liver copper data, is warranted to confirm whether the circulating copper elevations occur before or after rise in GGT activity. In addition, irrespective of the order, further investigation should determine whether the additional circulating copper further exacerbates liver and bile duct damage, either directly, or through further stimulation of the sporidesmin reaction. Ultimately, we seek to gain further understanding of biological mechanisms associated with sporidesmin toxicity to contribute towards the discovery of alternative preventative strategies.

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