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Assessing changes in serum IgG subclass antibodies in horses following a Phase I clinical trial of Antigen Specific Oral Immunotherapy of *Culicoides spp.* salivary gland proteins.

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Abstract

Insect Bite Hypersensitivity has been extensively demonstrated as a IgE mediated Type I hypersensitivity caused by biting insects of *Culicoides spp.* in horses. The horse has the most IgG subclasses of any studied mammalian species, but their individual functions are not fully known. The blocking activity of human IgG4 in allergy provided the aim of this study to determine any changes in serum IgG subclass antibody levels after a phase I oral immunotherapy trial of common midge salivary gland proteins. This phase I trial took place over a period of 2 years. In the first year, 10 horses (cohort 1) were blood sampled, then 5 received a 1nMol immunotherapy dose containing the most allergenic midge salivary gland proteins, while the remaining 5 received a placebo dose (0nMol). Doses were given bi-weekly for 3 months before the cohort were re-blood-sampled. In the second year, 20 horses (cohort 2) were blood sampled. 5 received the placebo dose, with the remaining 15 receiving a 2nMol immunotherapy dose containing the most allergenic midge salivary gland proteins. After a month, 10 receiving the 2nMol dose moved up to a 4nMol dose, then after a further month, 5 receiving the 4nMol dose moved up to an 8nMol dose leaving 5 horses in each dosage group for one month. Dosing was once again performed bi-weekly, and blood sampling was performed at the conclusion of the immunotherapy. The sera collected was analysed for IgG1, IgG4/7, IgG5, IgG6, and IgE binding to 30 recombinant *Culicoides spp.* salivary gland proteins via indirect ELISA. Individual ODs were totalled, and the ratios calculated by dividing post-treatment sera by pre-treatment sera to obtain a single figure. These figures were analysed using a Kruskal Wallace test or a Mann-Whitney U test. Year 1 horses for IgE antibody levels demonstrated a significant difference (P=0.05) and year 1 horses for IgG1 demonstrated a borderline significance (P=0.0548). The other subclasses apart from IgG6 may have showed a dose effect. This study demonstrated that the administration of oral immunotherapy is safe in horses, as none of the measured adverse reactions (lip smacking, chewing and tongue movements, swelling of the tongue, urticaria (hives), and anaphylactic shock) were observed. It also demonstrates that equine serum IgE antibody levels are influenced by oral immunotherapy and may influence serum IgG subclass antibody levels.

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED:  DATE: 23/10/2022

Dedication and Acknowledgments

I would like to thank my supervisor Dr Douglas Wilson for accepting me as a masters student onto this project and proceeding to teach me about Insect Bite Hypersensitivity and its immunological mechanisms. As well as this I would like to thank him and Dr Fran Whittington for their invaluable lessons in the laboratory, teaching me new techniques that will be translatable in future projects.

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1.0 Introduction

1.1 Background of Equine Insect Bite Hypersensitivity

Equine Insect Bite Hypersensitivity (IBH) is the most common and best characterised allergic disease of the horse (Schaffartzik, Hamza, Janda, Crameri, Marti, & Rhyner, 2012). It is a Type I hypersensitivity allergic response to bites of blood feeding insects from the genus *Culicoides* (Pilsworth, & Knottenbelt, 2010). Only female *Culicoides spp.* feed on blood (Wilson, Harwood, Björnsdottir, Marti, & Day, 2001). When feeding, they use their mouth parts to lacerate the surface of the skin and secrete saliva containing the salivary gland proteins that causes the allergy (Wilson, Harwood, Björnsdottir, Marti, & Day, 2001). Due to the lifecycle of *Culicoides* populations, clinical symptoms emerge in the spring and summer and go into remission during the winter (Pilsworth, & Knottenbelt, 2010). IBH is characterised by inflamed skin being hot to touch with intense pruritis. This leads to broken hair and loss, bleeding sores on the skin which heal to grey scabs. Chronic lesions are characterised by hyperkeratosis, thickened skin ridges due to fibrosis (Wilson, Harwood, Björnsdottir, Marti, & Day, 2001). In general, most clinical signs are not present before the ages of 2-4 (Wilson, Harwood, Björnsdottir, Marti, & Day, 2001).

Prevalence of IBH varies worldwide due to the distribution of *Culicoides* populations (Schaffartzik, Hamza, Janda, Crameri, Marti, & Rhyner, 2012), from 3% in the UK (McCraig, 1973), to 37% in German Shire Horses (Littlewood, 1998), and 60% in Queensland, Australia (Riek, 1953). Icelandic horses imported to Continental Europe as adults develop IBH at a rate of >50% within 2 years post *Culicoides* exposure (Broström, Larsson & Troedsson, 1987). Due to the absence of *Culicoides* species in Iceland these horses have no prior exposure to *Culicoides* antigens (Broström, Larsson & Troedsson, 1987) (Birras et. al., 2021). However, horses that have been imported from Iceland under the age of 7 months old have the same risk of developing IBH as local horses (about 5%), which suggests early exposure to *Culicoides* is key to remaining immunologically tolerant (Sommer-Locher, Endriss & Fromm, 2012).

Horses exposed to *Culicoides* bites develop antibodies to multiple salivary gland proteins (Birras et. al., 2021). IBH affected individuals produce allergen specific IgE antibodies which bind to FceR receptors expressed on innate granulocytic effector cells: basophils and mast cells. Interaction between the allergens and cell bound IgE release granular contents including histamine, prostaglandin, and leukotriene mediators to act on target tissues, causing an immediate physiologic response (Waserman, Bégin, & Watson, 2018).

When Shetland ponies were injected with *Culicoides spp.* whole body extracts, an allergen-specific Th1 immune response was associated with protection against the development of IBH, while an allergen-specific Th2 immune response led to the development of IBH (Meulenbroeks et al., 2015). (*Appendix A* shows the differences between Th1 and Th2 responses). When skin biopsies were examined, both IBH and healthy horses had an increased infiltration of lymphocytes with a significantly stronger infiltration in IBH horses (Meulenbroeks et al., 2015). Granular contents from eosinophils were present in higher numbers in the middle dermis of IBH-affected horses compared to healthy horses, with mast cells also being present in higher numbers in IBH horses (Meulenbroeks et al., 2015). However, the frequency at which mast cells entered the injection site remained consistent in both groups which suggests that injected allergen did not provide a significant movement of mast cells into lymph nodes (Meulenbroeks et al., 2015). In contrast, T cells were shown to be moving into the skin after injection of allergen (Meulenbroeks et al., 2015).

1.2 IgE in Allergic Responses

IgE's involvement in allergy has been long documented in the sensitisation process and re-exposure of allergens (Hamza et. al., 2008). During the early sensitisation process, injury to epithelial cells caused by the feeding activity of *Culicoides* stimulates the release of Thymic Stromal Lymphopoietin (TSLP) – an interleukin-like cytokine that has a key role in the differentiation of T Helper 2 (Th2) cells (Cvitas, Galichet, Ling, Müller, & Marti, 2019). This in turn leads to production of pro inflammatory mediators IL-5 and IL-13 which bias dendritic cell antigen presentation towards Th2 cells. When compared to healthy epithelial cells, IBH-affected epithelial cells have a substantial upregulation of IL-13 and involvement of the hypoxic pathway (Cvitas et. al., 2020). Genes involved in the metabolism of epidermal lipids, pruritus development, and IL-25 in the epidermis, are significantly differentially expressed between healthy horses and horses with IBH (Cvitas et. al., 2020). Th2 cells promote IL-4, IL-5, and IL-13, triggering B-cell class switching to IgE (Heimann et. al., 2011). Once established, mast cell degranulation releases more mediators including IL-4 to create a positive feedback loop. Following re-exposure, the binding of allergen to IgE causes an aggressive and rapid memory immune response mediated by Th2 cells (Hamza et., al., 2008) (Wagner et. al., 2006) (Heimann et. al., 2011), which leads to the production of allergen specific IgE and promotion of infiltrating eosinophils (Heimann et. al., 2011). Fully activated eosinophils degranulate and secrete mediators stored in their granular contents (Adamko, Lacy, & Moqbel, 2004), and later exposure to allergens cross-links bound IgE which causes degranulation and secretion of

mediators like histamine (Navinés-Ferrer, Serrano-Candelas, Molina-Molina, & Martín, 2016). *Figure 1.1* describes this process. The effects of histamine release include pruritus, prostaglandin generation, and increased vascular permeability (White, 1990). With pruritus being a key clinical sign of IBH (Wilson, Harwood, Björnsdottir, Marti, & Day, 2001), and prostaglandin production and increased vascular permeability being key signs of inflammation (White, 1999), it is clear the role in which IgE has in the allergic process of Insect Bite Hypersensitivity.

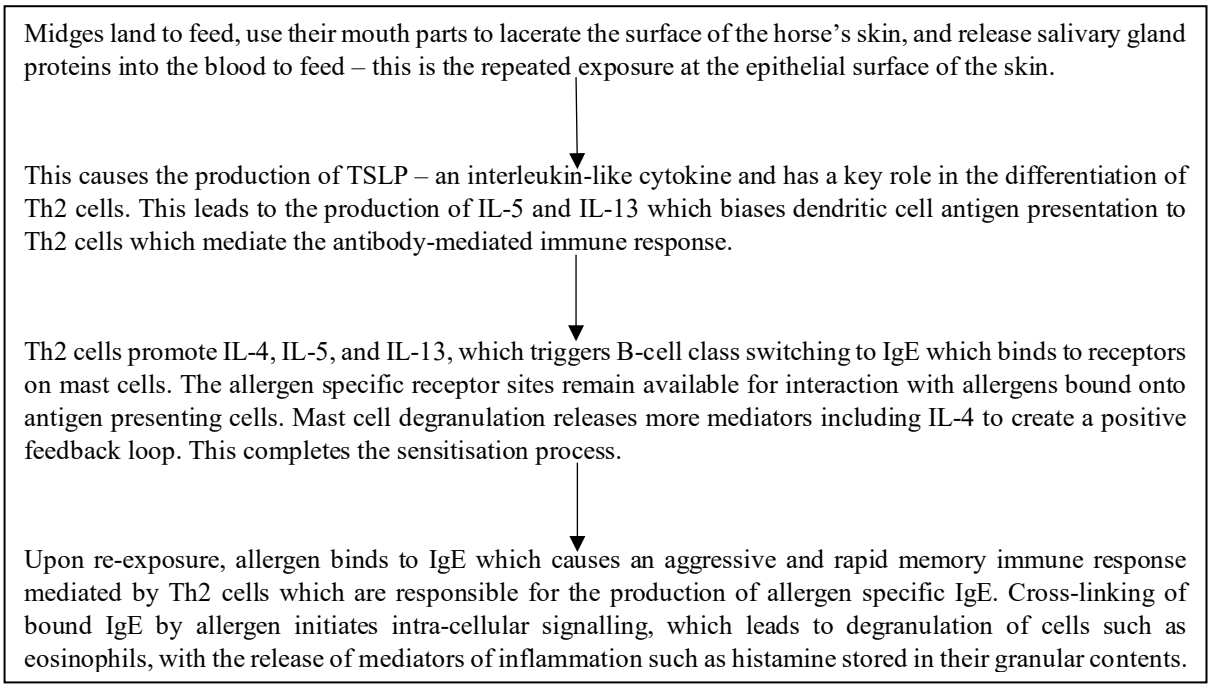

Figure 1.1 The sensitisation process, demonstrating the production of allergen specific IgE to cause allergy (Cvitas, Galichet, Ling, Müller, & Marti, 2019), (Heimann et. al., 2011), (Hamza et., al., 2008), (Wagner et. al., 2006), (Adamko, Lacy, & Moqbel, 2004), (Navinés-Ferrer, Serrano-Candelas, Molina-Molina, & Martín, 2016).

Culicoides salivary gland protein specific IgE was shown to be higher in IBH horses compared to healthy controls, but it is still detectable, with 26 of 27 tested allergens having significantly different levels in Novotny et. al., (2021).

1.3 Identification of allergens involved in Insect Bite Hypersensitivity

There are multiple midge salivary gland proteins that are causative allergens in Insect Bite Hypersensitivity (Schaffartzik, Hamza, Janda, Crameri, Marti, & Rhyner, 2012). Research has identified causative allergens from *C. nubeculosus, C. sonorensis,* and *C. obsoletus* (Marti et. al., 2015), which have been tested using a protein microassay for detection of IgE antibodies in IBH-affected horses from 27 *Culicoides* salivary gland proteins (Novotny et al., 2021). Previous studies on the treatment of IBH have only used Whole Body Extracts (WBE) which

only contain a small fraction of the causative salivary gland proteins (Jonsdottir et al., 2019). This study used some of these antigens for the phase one clinical trial.

1.4 Equine IgG Subclasses

The horse has the most Immunoglobulin Gamma constant regions of any mammalian species studied so far (Lewis, Wagner, & Woof, 2008). Early studies demonstrated 5 different equine IgG subclasses: IgGa, IgGb, IgGc, IgG(T), and IgG(B) (Lewis, Wagner, & Woof, 2008). Sequencing of equine DNA identified seven Immunoglobulin heavy chain constant genes (IGHC) for IgG in genomic DNA of all horses and breeds (Wagner 2006), indicating that the IGHC regions of horses contain 7 immunoglobulin gamma heavy chain (IGHG) genes. In the horse genome, the heavy chain genes are arranged in order: IgM, IgD, IgG1, IgG2, IgG3, IgG7, IgG4, IgG6, IgG5, IgE, and IgA (Wagner, Miller, Lear & Antczak, 2004). IgGa has since become IgG1, IgGb reclassified into IgG4 and IgG7, as they have high sequence and functional similarity, indicating a recent gene duplication and are so referred to as IgG4/7 (Lewis, Wagner, & Woof, 2008). IgGc became IgG6, IgG(T) reclassifies as IgG3 and IgG5. Unlike IgG4/7, IgG3 and IgG5 are highly different in their sequence and so have different functional characteristics (Lewis, Wagner, & Woof, 2008). Finally, IgG(B) has been renamed as IgG2 (Lewis, Wagner, & Woof, 2008).

Overall IgG is the predominant antibody class in equine serum and colostrum, and compared to the sera of mares, IgG4/7, IgG1, IgG3, and IgG5 were found in high levels in colostrum (Sheoran, Timoney, Holmes, Karzenski & Crisman, 2000) (Wagner et. al., 2006). IgG4/7 are the predominant subclasses in colostrum followed by IgG1, IgG3, and IgG5, with IgG6 hardly detectable (Lewis, Wagner, & Woof, 2008). IgG is present in mucosal surfaces, being the most abundant in the urinary tract, lower respiratory tract, and the lungs (Butler, 1998). Only IgG1 and IgG4/7 have been detected in nasal wash samples from adult horses (Sheoran, Timoney, Holmes, Karzenski, & Crisman, 2000). IgG4 and 7 are the predominant classes in serum, followed by IgG3, IgG5, IgG1, and IgG6 (Lewis, Wagner, & Woof, 2008). IgG4 is suggested to play an essential role in protection against bacterial and viral infections (Wagner et. al., 2006). IgG2 is found in very low concentration in serum and there are currently no specific reagents to detect it.

The structure and function of individual equine IgG subclasses has not been well studied. However, IgG subclasses mediate effector function by interacting with FcγRIIAs and so help activate the complement pathway (Lewis, Wagner, & Woof, 2008). IgG1, 3, 4, 5, and 7 can

stimulate a strong respiratory burst from the production of reactive oxygen species by neutrophils mediated by the equine homologue of FcgRIIA (Lewis, Wagner, & Woof 2008) (Wang & Jönsson, 2019) indicating that these subclasses interact with FcγRIIA. However, IgG2 and 6 give little or no respiratory response, suggesting these subclasses do not efficiently interact with FcγRIIA (Lewis, Wagner, & Woof, 2008). IgG3 was found to be the best activator of the complement pathway followed by IgG1, IgG4, and IgG7, while IgG2, IgG5 and IgG6 fail to bind the first complement component C1q (Lewis, Wagner, & Woof, 2008). There is no available evidence demonstrating other IgG Fc receptors. The known functions of the IgG subclasses are listed below in *Table 1.1* (Wagner, 2006), (Lewis, Wagner, & Woof, 2008).

Table 1.1 known functions of the equine IgG subclasses.

Heavy Chain Constant Gene	Immunoglobulin Subclass	Activation of Complement Pathway	Activation of Respiratory Burst
IGHG1	IgG1	+	+
IGHG2	IgG2	-	-
IGHG3	IgG3	++	+
IGHG4	IgG4	+	+
IGHG5	IgG5	-	++
IGHG6	IgG6	-	-
IGHG7	IgG7	+	+

The CVS40 anti IgG(T), now IgG3 and 5 antibodies was shown to induce an immediate skin reaction when injected intradermally in healthy horses, which suggested that it has a role in the sensitisation of mast cells and basophils in adult horses, however evidence for this was not found in younger horses (Wagner, Miller, Erb, Paul Lunn, & Antczak, 2009). Other anti IgG3 and 5 antibodies did not induce any immediate skin reactions in adult horses, suggesting that the skin reactions that were induced by CVS40 may be mediated by other IgG-independent and/or indirect mechanisms. The CVS40 anti-antibody may have an indirect effect on mast cell degranulation via the formation of IgG(T)/anti-IgG(T) complexes which is then followed by binding to activating FcγRs (Wagner, Miller, Erb, Paul Lunn, & Antczak, 2009).

1.5 IgG in Human Allergy

In murine models and human subjects, oral immunotherapy of food allergen has been accompanied by the induction of allergen-specific IgG antibodies (Kanagaratham, Ansari, Lewis, & Oettgen, 2020). Mast cells and basophils are activated by IgE and are important sources of IL-4 that move the immune system from tolerance towards type 2 immunity by

promoting Th2 and supressing regulatory T cells, but this action is supressed when inhibitory signals are produced by IgG antibodies (specifically IgG4 in humans) and signalled via FcgRIIb (Kanagaratham, Ansari, Lewis, & Oettgen, 2020). There is increasing evidence demonstrating that IgG antibodies provide natural protection from allergic reactions to food allergen specific IgE, demonstrating the protective effects of Antigen Specific Immunotherapy (Kanagaratham, Ansari, Lewis, & Oettgen, 2020). Human IgG4 does not bind the complement system or bind to Fc receptors to cause a respiratory burst (Segawa et al., 2010). IgG4 is present at a higher concentration in serum than IgE, can recognise more epitopes, has a limited ability to form immune complexes and mediate effector function (Kanagaratham, Ansari, Lewis, & Oettgen, 2020). It has been suggested that the function of human IgG4 is that of a blocking antibody, preventing allergens from being engaged by FcϵRI-bound IgE on effector cells (Kanagaratham, Ansari, Lewis, & Oettgen, 2020). As well as this, IgG4 has the ability to exchange Fab-arms which is why IgG4 is unable to activate the complement pathway (Koneczny, 2018). These antibodies are bispecific and so are able to cross-link two different antigens rather than two of the same antigens (Koneczny, 2018). Equine IgG4 and 7 were demonstrated to have bands of a similar size to some lower molecular weight individual heavy chain fragments under non-reducing conditions, which may represent half molecules or light chain dimers (Lewis, Wagner, & Woof, 2008). However, there is no available evidence demonstrating the ability of equine IgG4 or 7 to exchange Fab-arms. It is not known if any of the equine IgG subclasses equals the role of human IgG4, however equine IgG2, IgG5, and IgG6 do not bind the complement system, with IgG2 and IgG6 failing to mediate a respiratory burst, but IgG5 may activate mast cells (Ziegler et. al., 2018).

In humans, tolerance to cow's milk can be achieved by decreased epitope binding derived from 5 major cow's milk proteins by IgE and subsequent increase of IgG4 epitope binding (Savilahti et. al., 2010). Maintenance of tolerance to cow's milk in atopic individuals is associated with higher levels of specific IgG4 in combination with low levels of specific IgE (Ruiter et. al., 2007). It is clear a rise in human IgG4 titre is associated with antigen specific immunotherapy success and so here we considered whether an equine equivalent exists despite there being a lack of information on equine IgG subclasses and their roles. Despite lack of information, Ziegler et. al., (2018) demonstrated that in Icelandic horses imported to continental Europe, allergen-specific IgG1 levels rise significantly, followed by significantly increasing IgG5, which is then followed by increased IgE levels and the onset of clinical signs in IBH-affected horses. However, IgG5 levels to certain allergens were already increased years before onset of

clinical signs due to cross-reactivity between *Culicoides spp.* and *Simulium* spp. as *Simulium vittatum* are present in Iceland (Ziegler et. al., 2018).

1.6 Antigen Specific Immunotherapy

Antigen Specific immunotherapy (AIT) is the only curative treatment for type I hypersensitivities, leading to a shift from a Th2 immune response to a regulatory immune response (Eckl-Dorna et. al., 2019). IgG antibodies are produced and block allergen specific IgE antibodies from binding to allergens (Eckl-Dorna et. al., 2019). Human IgG4 levels have been shown to decrease by 80% to 90% within a year of stopping AIT (Shamji & Durham, 2017). However, the inhibitory activity of human IgG against IgE continues for several years and accompanies long-term clinical efficacy which suggests that despite decreasing serum IgG4 antibody levels after discontinuation of immunotherapy, IgG4 retains a high affinity to inhaled allergens (Shamji & Durham, 2017).

1.7 Antibody Class Switching

Antibody producing B-cells can class switch more than once during cell division under the influence of different cytokines such as IL-4 and TGF-β which determines what subclass they switch into (Allen, 2022). Naïve B-cells encode the C regions of IgM and IgD and can only class-switch to genes that are further downstream as upstream genes encoding the previously expressed C regions are removed (Allen, 2022). For example, an IgM producing B-cell can switch to any IgG, IgE, or IgA, but an IgE B-cell can only class switch to IgA (Wagner, Miller, Lear & Antczak, 2004). IgE class switching is favoured by IL-4 or IL-13 which are classic Th2 cytokines, while IgA class switching is favoured by TGF-β which is produced at mucosal surfaces and regulatory T cells (Stavnezer, & Kang, 2009). In human hay fever patients, B-cells class switch from IgM and IgG to IgE in the nasal mucosa (Smurthwaite et. al., 2001). Allergen specific IgE synthesis is shown to continue between seasons despite no pollen production occurring (Smurthwaite et. al., 2001). This continuous production of allergen specific IgE is thought to provide immediate mucosal hypersensitivity and a rapid amplification of the allergic response upon allergen re-exposure (Smurthwaite et. al., 2001).

1.8 IgE

IgE antibody levels were assayed prior to this project focusing on IgG subclass antibodies commencing, but to provide context for the IgG results these will be presented.

1.9 Aims, objectives, & hypotheses

This study was conducted in Laboratory 256 In the Churchill Building at the University of Bristol Veterinary School in Langford. The principle aim of this study is to determine any changes in equine serum IgG subclass antibodies after Oral Immunotherapy of major allergens involved in Insect Bite Hypersensitivity. Another aim is to demonstrate safety to the administration of oral immunotherapy in increasing doses to demonstrate a phase II trial could be feasible. Human IgG4 significantly increases and serves as a blocking antibody after AIT in food allergy and research into an equine equivalent has not been conducted due to the lack of literature about the equine IgG subclasses. The objectives are to perform an indirect ELISA with 30 *Culicoides* salivary gland proteins using four equine IgG subclasses: IgG1, IgG4/7, IgG5, and IgG6 on 30 horses whose sera were collected before oral immunotherapy in the spring and after oral immunotherapy in early autumn to determine any changes and binding of IgG. The Null Hypothesis (H_0) is that oral immunotherapy of *Culicoides* salivary gland antigens has no effect on serum levels of subclass IgG antibodies, and the Alternative Hypothesis (H_1) is that oral immunotherapy of *Culicoides* salivary gland antigens will change the serum levels of subclass IgG antibodies.

2.0 Materials and Methods

2.1 Ethical Considerations

All relevant health and safety, COSSH, and BIOCOSHH forms were read and signed in the laboratory 256 health and safety folder. As the study was not performed on live animals, and only required the use of Genetically Modified *Escherichia coli*, no specific ethical considerations were required.

2.2 Experimental Background and Design

This study forms part of a phase 1 clinical trial of oral immunotherapy for the treatment of *Culicoides* hypersensitivity in horses. In work carried out over three years prior to this laboratory study conducted by Wilson et. al (Plos Biology submitted), 30 horses split into 2 cohorts were recruited to the study under a Home Office license. This trial was undertaken with rescue ponies so it was not possible to determine certain details about them. For example, breeds and ages were unknown, and sex and bodyweights were not specifically recorded. All ponies were reported to have IBH but were not demonstrating clinical signs at the time of the trial due to the preventative measures of dusk-dawn housing and the wearing of fly rugs being undertaken. However, three of the 30 ponies: Pixie, Flash, and William were later determined

to be healthy and so used as controls (*Appendix F*). Serum IgE antibodies against the 30 recombinant allergens in this study were measured by ELISA. The 12 most allergenic proteins were then selected for immunotherapy treatment. In order the strongest allergens were: CO23, CO120, CO112, CO110, CO167, CO142, Cul181, CP60, CP129, CO13, CO117, and CO37. (Refer to *Appendix B* for the full data). In year one (the first cohort) 10 horses were blood sampled in the autumn before commencing treatment. The treatment consisted of 1 nanomole (nMol) of each allergen dissolved in carboxymethyl cellulose gel with sugar and peppermint flavouring for palatability (N=5), and a placebo of peppermint gel only (N=5). The gels were fed bi-weekly for three months and a second blood sample was taken one month later in spring. During the second year of the trial, 20 horses (cohort 2) were divided into 2 groups – control (placebo) (N=5), and a treatment group of 2nMol (N=15) with the same 12 strongest allergens listed above. After one month of treatment, 10 horses receiving 2nMol moved up to a 4nMol dose (N=10), and after a further month of immunotherapy, 5 horses receiving a 4nMol dose moved up to an 8nMol dose (N=5) for one month, leaving 5 horses in each dosage group. Blood sampling was again performed in the autumn before treatment commencing and then again in the spring. *Figure 2.1* highlights the study procedure time frame.

Based on information from human trials, common adverse reactions from oral immunotherapy include itching sensations and tingling of the tongue and lips (Jutel et. al., 2015). In horses this is likely to manifest as lip smacking, chewing and tongue movements. Other reactions that may occur include swelling of the tongue and the occurrence of urticaria (hives). The most severe reaction to be measured would be anaphylactic shock. Oral administration of immunotherapy reduces the possibility of anaphylaxis occurring (Anagnostu, 2021) which is why it was selected and none of these adverse reactions were observed in this trial.

Power calculations are used to determine how many subjects are required in a study. When performing power calculations, preliminary data such as standard deviations and means from normally distributed data are required. Given the nature of this pilot study there was no preliminary data available. However, retrospectively using the data gathered in this study to provide an estimate of means and variance, a sample size of 5 per dosage group would detect an 80% improvement with an alpha of 0.5, and a power of 29.9%. This provides us with information in which we can use in the design of any future experiments, e.g., for 60 subjects (number allowed under an animal test certificate issued by the veterinary medicines agency) divided into 20 controls and 40 test samples, the power of the experiment would be raised to 84.2%.

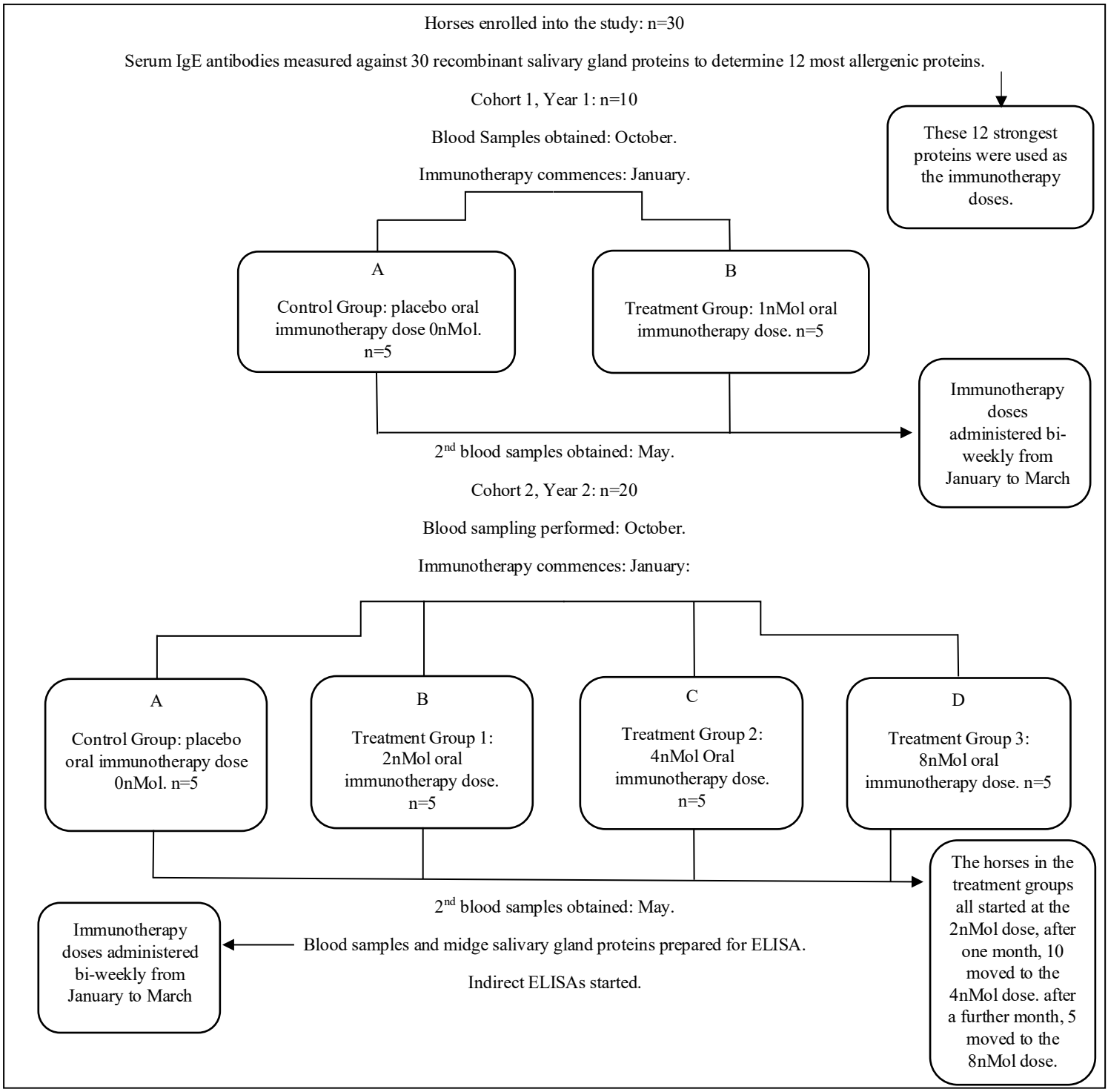

Figure 2.1: Study procedure timeframe

2.3 Recombinant Protein Purification

A total of thirty recombinant proteins derived from salivary gland cDNA were used in this trial. Both *Culicoides obsoletus* (CO), and *Culicoides pulicaris* (CP) derived allergens were used. They are listed below in *Table 2.1.*

Table 2.1: the 30 recombinant midge salivary gland proteins used in this study.

Allergen	*C. obsoletus*	kDa	Allergen	*C. pulicaris*	kDa
CO13	D7	16.5	CP1	Antigen 5	30.3
CO15	LRR Domain	35.7	CP14	D7	16.5
CO23	Kunitz	23.4	CP47	Unknown	78.9
CO37	Unknown	18.4	CP52	Unknown	16.4
CO60	Chymotrypsin	30.9	CP56	D7	15.9
CO110	D7	17.5	CP60	Trypsin	29.4
CO112	D7	16.9	CP113	LRR Domain	44.4
CO117	Maltase	68.7	CP129	WSC Domain	17.7
CO120	WSC Domain	17.6	CP140	Unknown	18.8
CO142	Antigen 5	29.9	Cul180	Unknown	73.0
CO145	Tissue Factor Inhibitor	19.3	Cul185	Trypsin	41.9
CO147	Apyrase	62.1			
CO149	Hyaluronidase	44.7			
CO167	Collagen-like	39.3			
Cul181	Kunitz	21.5			
Cul182	Antigen5	25.3			
Cul183	Unknown	20.7			
Cul184	Kunitz	25.1			
Cul186	Collagen-like	51.7			

Most recombinant proteins had been expressed in *E. coli* using a PET 100 vectors containing a synthetic DNA sequence optimised for expression in *E. coli* DNA ("Gene Cloning & Subcloning | Custom DNA Cloning Services", 2022). Not all of the proteins could be expressed optimally in standard BL21 DE3 cells, several were shown to give improved yield in Shuffle® T7 express *E. coli* ("Shuffle® T7 Express Competent E. coli". New England Bio Labs, 2022). In this project CO15, an LRR domain protein and CO117, a maltase protein were cloned and expressed using this strain. All other proteins for this study were made and stored previously.

2.3.1 Transfection

Plasmids containing the required protein were removed from a -20°C freezer, Shuffle® T7 express competent *Escherichia coli* was removed from the -70˚C freezer and both were placed on ice. 1µl of plasmid DNA was mixed into 20µl of Shuffle® cells in a microcentrifuge tube, gently mixed, and left on the ice for 30 minutes.

The bottom halves of the microcentrifuge tubes were gently swirled in a water bath at 42°C for 30 seconds then immediately placed back onto the ice for 2 minutes before pipetting 250µl Super Optimal Broth (SOC) media ("SOC Medium For use in transformation | Sigma-Aldrich", 2022) into the cells and placed into a shaking incubator at 37°C for 45 minutes.

2.3.2 Making LB Agar

Six empty agar plates were labelled, 4g powder LB agar ("Formedium Powdered Media & Liquids | UK Research & Microbiology", 2022) was weighed and added to a sterile 500ml glass bottle and 100ml distilled water was added. The bottle was shaken to disperse the agar, and then microwaved in short bursts until boiling point was reached and all agar had dissolved, then allowed to cool slightly ready for pouring. 100µl of a 100mg/ml solution of carbenicillin was added to give a final concentration of 100µg/ml, and the media poured onto the six plates and left to set in a biological safety cabinet. Once set, 100µl, 50µl, and 25µl of each SOC media containing *E. coli* was pipetted onto each plate and incubated overnight in a standard incubator at 37°C.

2.3.3 Culture in MDAG Media

The plates were removed from the incubator and assessed for growth. 5ml MDAG media was used as it is non-inducing to allow optimal growth and retention of plasmids (Studier, 2005). 5ml was pipetted into two universal containers, and 5µl ampicillin added. A single colony from the 25µl SOC media plates for both allergens was picked off aseptically and placed into the MDAG media. The cultures were incubated overnight in a shaking incubator at 37°C.

2.3.4 Preparing Bulk Cultures

In a biological safety cabinet, 100ml Cinnabar media was pipetted into sterile plastic conical flasks with baffles. 0.4% glucose and 25mMol IPTG was added to ensure autoinduction when the glucose was used up. 5µl antifoam 204 ("Sigma-Aldrich", 2022) was added, along with 100µl of 100ug/ml ampicillin antibiotic. 1ml of MDAG culture was added to the flasks after confirming bacterial growth, and the flasks placed in a shaking incubator at 37°C at 150RPM overnight. The pH and the O.D of the cultures was taken once removed from the incubator prior to harvesting the cells.

2.3.5 Cell Harvesting

To harvest the cells, the cinnabar media was removed from the conical flasks 25ml at a time into a weighed 30ml Oakridge centrifuge tube where it was spun at 8000G for 5 minutes until no remaining media was left in the flasks, leaving the cell pellet in the centrifuge tubes. The cell pellets were resuspended and washed in 20ml Tris pH8 and spun at 8000G for 5 minutes then repeated. The tube was reweighed to obtain the cell pellet weight and then frozen at -20°C overnight.

The following day, the pellet was resuspended and lysed in 8ml/g (20ml) NZY Bacterial Cell Lysis Buffer, 800µl protease inhibitors, 2µl/ml (40µl) lysozyme, and 2µl/ml (40µl) Dnase ("NZYTech.com", 2022). The cells were incubated on a rocker for 20 minutes at room temperature to allow the cells to lyse and leave behind the inclusion bodies. The inclusion bodies were then centrifuged at 16000G for 10 minutes at 4°C and the resulting pellet was resuspended and washed in 20ml 50mM Tris (pH8), 500mM NaCl, 1% Triton X114, 1mM EDTA solution at 16000G for 10 minutes. Next, the pellet was resuspended and washed in 50mM Tris (pH8), 50mM NaCl, 1% Triton X114, 1mM EDTA solution, and span at 16000G for 10 minutes. The lysate was then twice resuspended and washed in distilled water at 16000G for 10 minutes.

Finally, the lysate was resuspended in 13ml buffer made up of 1ml 1M Tris, 5ml 2M NaCl, 400µl 1M Imidazole, and 6.6ml distilled water. Then, 9.6g Urea (to make 8M) and 28mg of Tris(2-carboxyethyl)phosphine (TCEP) was added. The lysate was then placed on a rocker at room temperature overnight to dissolve before being refrigerated at 4˚C.

2.3.6 Purification using His-Tag column

The lysate in urea was passed through a syringe filter to remove any large impurities. The soluble recombinant proteins were purified using a prepared 1ml nickel ion His-Tag column. The column was washed in ethanol, followed by distilled water before loading the filtered lysate onto the column and allowing it to run through into a labelled flow through pot to monitor efficiency of target protein binding and run through.

Next, two column washes of 20mMol imidazole buffer were loaded onto the column (38g Urea, 5ml Tris, 25ml NaCl, 2ml imidazole, and 68ml distilled water) and were ran into the waste pot to remove contaminant non-specific binding proteins.

For a 1ml column, an initial volume of 600µl of elution buffer was loaded and allowed to run through to waste.

To collect protein, 2ml of elution buffer was loaded onto the column and collected into a labelled bijou containing 2ml of 400mMol Arginine refolding buffer. Once all elution buffer had run through, 0.5mM oxidised glutathione (6.12mg/10ml) and 5mM reduced glutathione (15.4mg/10ml) was immediately added and refrigerated at 4˚C.

To collect eluate two, a further 1ml of elution buffer was loaded onto the column and ran through into a bijou labelled eluate 2 which contained 1ml Arginine buffer. Once collected it was immediately refrigerated at 4˚C.

This process allows for high affinity binding of target protein to increase the chances of successful protein purification and refolding.

2.3.7 Gel Electrophoresis

To determine if His-Tag purification had been successful, the proteins were separated using Nu-page SDS gel electrophoresis ("NuPAGE™ 4 to 12%, Bis-Tris, 1.0–1.5 mm, Mini Protein Gels", 2022) to establish their purity and molecular weights. LDS sample buffer, sample reducing agent, and water were made up into a master mix to create enough volume for 15 samples (37.5µl buffer, 15µl reducing agent, and 22.5µl water). 5µl was added into 11 microcentrifuge tubes, and 5µl of each protein sample was added and mixed. The protein weight markers were placed into a 12th microcentrifuge tube. The samples were then placed on a heating block set at 80°C for 10 minutes to denature the proteins then centrifuged for 10 seconds at a low speed to push any condensation back into the mixture. The samples and the markers were then loaded onto precast gels, MES buffer added into the buffer tank, and the gel ran at 200V for 35 minutes.

To view the bands on the gel, the gel was removed from the buffer tank, placed into a square petri dish, and washed in distilled water for 5 minutes to remove any remaining MES buffer. The water was then replaced with a stain fix for 15 minutes with the petri dish on a rotary plate before being removed and then placed in Coomassie stain for 30 minutes on the rotary plate. De-stain was then used to remove the excess staining from the gel, leaving the bands. If any bands were present, they were viewed on top of a lighting plate and their molecular weights compared against the markers lane and the documented weight of the 30 proteins. If the molecular weights matched, the protein stock was aliquoted to be used in an ELISA and the rest stored at -70°C. The protein concentration of the samples was measured by Micro Bradford assay ("BIO-RAD", 2022).

2.4 Experimental Study of IgG1, IgG4/7, IgG5, & IgG6

2.4.1 ELISA Start

This ELISA protocol was based on the IgE ELISA that was undertaken prior to this study (Wilson et. al., PLOS Biology submitted), but preliminary experiments using IgG1 and IgG4/7

to determine the optimal dilution and duration of incubation for IgG was carried out. The allergens CO145 which provided strong binding during the IgE trial and Cul183 which provided weak binding during the IgE trial were used (*Appendix B*). This initial study demonstrated that a 1:200 dilution of horse sera and shorter incubation periods were required to develop the final enzyme reaction. The two antibody subclasses were purchased from Bio-Rad ("Anti Horse IgGa Antibody, clone CVS48 | Bio-Rad", 2022) ("Anti Horse IgGb Antibody, clone CVS39 | Bio-Rad", 2022).

Day One: To test the 30 allergens, four were selected each week. This was to ensure all stages could be done in time. ELISA plates were labelled with which allergen they were being coated with and which primary antibody was being detected, this gave 8 plates in total (2 for each allergen, one anti-IgG1, and one anti-IgG4/7). The allergens were each pipetted into 6.5ml of carbonate buffer (pH 9.6) according to their individual protein concentrations. The universal containers were then inverted several times to ensure even mixing. 100µl of buffer was pipetted individually into 60 wells of a 96 well high binding microtiter ELISA plate ("Microplate, 96 well, ps, f-bottom, clear | Greiner.com, 2023") in the same layout as the IgE study (*Appendix C*). The plates had plate lids placed over them, put into plastic boxes with damp paper tissue on the bottom, sealed with lids, and refrigerated overnight.

Day Two: the plates were removed from the fridge and washed using a washer on a programme set to six washes, and the plates were immediately coated with 150µl blocking buffer (1:20 dilution) and subsequently left to incubate for 1 hour at room temperature. The blocking buffer was knocked out and 90µl blocking buffer was immediately added, followed by 10µl of a 1:20 dilution of horse sera to give a final concentration of 1:200 (30 pre-treatment samples and 30 post-treatment samples). The wells were mixed to evenly distribute the horse sera and left to incubate for 2 hours at room temperature before being rewashed and adding 100µl of either monoclonal anti-IgG1 primary antibody (clone number CVS48) or monoclonal anti-IgG4/7 primary antibody (clone number CVS39) (1:2500 dilution). The plates were then refrigerated overnight. This allowed the plates to reach an equilibrium and as numerous plates were done at the same time this helps to minimise differences in the incubation times.

Day Three: The plates were removed from the fridge and washed using the same protocol as day two. 100µl of Donkey anti-mouse alkaline phosphatase minimal cross reactivity enzyme conjugate (secondary antibody) at a dilution of 1:5000 ("Secondary Antibodies – Jackson ImmunoResearch", 2022) was immediately added, and all plates were left to incubate for 2

hours at room temperature in the plastic boxes. The plates were rewashed and 100µl of alkaline phosphatase substrate (1mg PNPP/ml coating buffer) was added to all plates. 100µl ELISA standard solution (alkaline phosphatase substrate with fully developed colour) was serially diluted in 100µl coating buffer in rows six and seven to establish the standard curve. The plates were incubated for ninety minutes at room temperature before being read using a plate reader at 405nm and 492nm and results recorded.

2.4.2 Sourcing and experimental study of IgG5 and IgG6 monoclonal antibodies

The IgG5 and IgG6 monoclonal antibody mouse cell line clones were sourced from The University of Cambridge. Clones CVS40 IgG5 and CVS53 IgG6 were used for the ELISA. These mouse cell lines were revived and subcloned to check that they were stable and producing monoclonal antibodies using Advanced Reduced Serum Media (RPMI) ("Invitrogen | Thermo Fisher Scientific – UK", 2022). The thawed cells were suspended in 10ml media then spun down to remove any Dimethyl Sulphoxide (DMSO) before being incubated in a 10ml flask using RPMI media for 48 hours at 37°C. Their growth was then examined under a microscope before cloning. To determine the dilution factor, the viable cells were counted using trypan blue. A sterile 96 well cloning plate was used. 3.3ml of RPMI media with plasmocin – a mycoplasma antibiotic was added to a bijou and 300µl cell culture added. 100µl was pipetted into rows 1-3 of the cloning plate, before adding 2.4ml media back into the bijou. 100µl was pipetted into rows 4-6, then 2.4ml media added back into the bijou. 100µl was pipetted into rows 7-9, and 2.4ml media added back. 100µl was pipetted into rows 10-12. Diluting the cells in media allows at between 100 cells per well and <1 cell per well (limiting dilution) to grow. The cloning plates were placed into a 37°C incubator for 8 days.

Each well containing a single colony of cells was tested for antibody production by ELISA using IgG fractions which were prepared by precipitation in 50% saturated ammonium sulphate. The precipitate was redissolved in PBS before being fractionated using protein G. A plate was coated with the following protein G sub-fractions: flow through, first pass eluate 1, second pass eluate 1, second pass eluate 2 at 5µg/ml and incubated at room temperature for 1 hour. The plate was washed, 100µl blocking buffer immediately added, and incubated for 1 hour. The blocking buffer was knocked out and 100µl immediately readded, with 150µl in the top row. 50µl of the three cell line supernatants suspected of being the target antibodies were added to the plate and doubly diluted leaving the final row blank and incubated for 1 hour. 100µl enzyme conjugate was added after washing, then incubated for 1 hour. 100µl alkaline

phosphatase substrate was added after washing and incubated for 30 minutes before being read at 405nm and 492nm.

The flow through was enriched for IgG5, with the remaining IgG subclasses being in the bound eluate. The IgG6 monoclonal verified by Lewis, Wagner & Woof (2008) reacted with the bound eluate but not the flow through, the IgG(T) CVS38 monoclonal reacted with both IgG3 and IgG5, and so bound to both the flow through and the eluted fractions as expected (Lewis, Wagner, & Woof, 2008). The final IgG antibody reacted only with the flow through which indicates that it is IgG5 specific (Lewis, Wagner, & Woof, 2008). (Refer to *Appendix D* for the image and *E* for the plate layout). The two other IgG(T) monoclonals did not yield any viable antibody clones.

Representative positive wells were expanded to 10ml volume cultures so that they could be frozen down for storage. To freeze, the cell line flasks were checked for growth and counted to determine how much media to remove and add back in for the cells to reach approximately 10^5 cells per ml. The removed supernatants were span at 600g for 5 minutes. After spinning, the supernatant was removed, and the pellet resuspended in 6.5ml of cryomedia. 1ml of supernatant was added into 6 cryotubes and placed into a 'Mr Frosty' ready for freezing at -70°C.

The remaining supernatant was used in an ELISA to determine antibody recognition. ELISA plates were coated with 100µl original horse precipitate that forms after mixing 1ml of plasma with 1ml saturated ammonium sulphate. This selectively precipitates immunoglobulins leaving the albumins in solution (1µl/ml coating buffer). One half of the plate was at a 1:1000 dilution, and the other half at a 1:500 dilution. They were incubated for 1 hour at room temperature, before being washed and 100µl blocking buffer added, and incubated for 1 hour. The blocking buffer was knocked out before 100µl blocking buffer was readded with 150µl in the top row. 50µl cell line supernatant was added to the 150µl blocking buffer (1:4 dilution) and was doubly diluted down the plate leaving the last row blank. The plates were incubated for 1 hour then washed. 100µl enzyme conjugate was immediately added and incubated at room temperature for 1 hour. The plates were washed and 100µl alkaline phosphatase substrate added. The plates were incubated for 30 minutes before being read at 405nm and 492nm.

To screen the monoclonal antibodies, 5 ELISA plates were coated with the same original horse 50% ammonium sulphate precipitate (1µg/ml) as above and refrigerated overnight. The clone plates were removed from the incubator and checked for any monoclonal growth and placed

back into the incubator until needed. The coated plates were removed from the fridge and washed before immediately adding 100µl blocking buffer. To prevent contamination when adding the antibodies, the plates were taken to a biological safety cabinet and incubated at room temperature for 1 hour. The clone plates were removed from the incubator and taken to the safety cabinet where 100µl was removed and added to the ELISA plates without removing the blocking buffer and incubated for 1 hour. 100µl RPMI media was placed back into the clone plates before being returned to the incubator. The ELISA plates were washed before adding 100µl enzyme conjugate and incubated for 1 hour. Finally, 100µl Alkaline Phosphatase Substrate was added after washing and incubated for 30 minutes before being read at 405nm and 492nm. This process allowed the confirmation that monoclonal antibodies had been grown and what IgG subclass they were.

The protocol was adjusted from IgG1 and IgG4/7 for testing of IgG5 and IgG6 as initial results with the same protocol gave low OD values. As a result, the duration of the serum incubation was increased to overnight. Day 1 remained the same.

Day Three: the plates were removed from the fridge, washed using the same protocol as IgG1 and IgG4/7, and then 100µl IgG5 (1:50) or IgG6 (1:25) was added. The plates were incubated for 2 hours at room temperature, then rewashed and 100µl enzyme conjugate added. The plates were refrigerated overnight.

Day Four: the plates were washed as before, then 100µl Alkaline Phosphatase Substrate was added. The plates were incubated at room temperature for 40 minutes before being read using a plate reader at 405nm and 492nm and results recorded.

2.5 Statistical Analysis

Statistical analysis of results was performed using StatView using a significance level of 0.05, and *P* values less than this were considered significant. To compare pre-treatment sera against post-treatment sera, each horse's individual score (the OD) for each allergen before oral immunotherapy and post-oral immunotherapy were totalled. Healthy horses and IBH horses results were also combined. The ratio of the two samples was calculated by dividing the post-treatment score by the pre-treatment score. This simplifies the data to a single value for each horse. If there is no difference then the value will be equal to one, where antibody levels have risen after treatment the score will be greater than one, and if antibody levels have dropped after treatment the score will be less than one. A Kruskall-Wallace Test and a Mann-Whitney U test was performed to establish any significant differences between treatment groups for

Year 1 and Year 2. These were chosen because the measurements in this study are unrelated, so a non-parametric test was required. The Mann-Whitney U was used to compare between Year 1 horses and the Kruskall-Wallace was used to compare between Year 2 horses.

3.0 Results

To determine if sub-lingual antigen administration was related to changes in serum IgG subclass and IgE levels between pre- and post-treatment sera, the total post-treatment score for each horse was divided from the total pre-treatment score for each horse after running an indirect ELISA on 30 *Culicoides spp.* salivary gland antigens. To view the original IgG results file, refer to *appendix [F]*. No change in total post-treatment serum/total pre-treatment serum gave a score =1, an increase gave a score of >1, and a decrease in antibody titre gave a score of <1.

Shown below are graphs showing the ratio of post-treatment serum/pre-treatment serum for IgG subclasses 1, 4/7, 5, & 6 for year 1 horses and year 2 horses.

IgG1

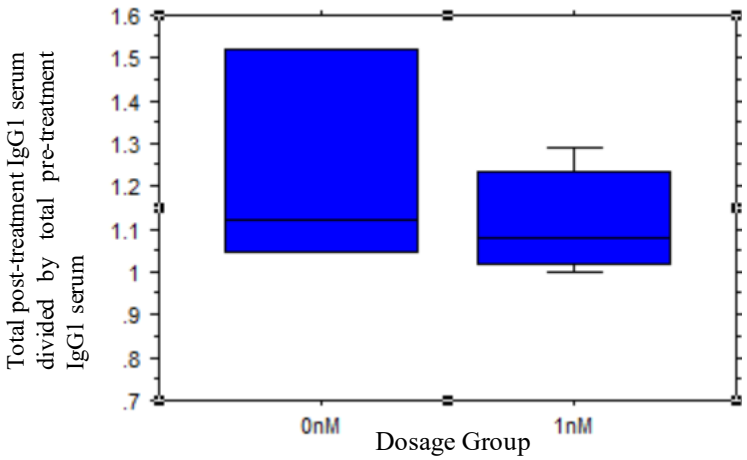

Figure 3.1: the ratio of total post-treatment serum IgG1 levels divided by total pre-treatment serum IgG1 levels compared between Year 1 horses. There was no significant difference between control horses (N=5) and those who received a 1nMol dose of allergen (N=5) (Mann Whitney U P>0.05).

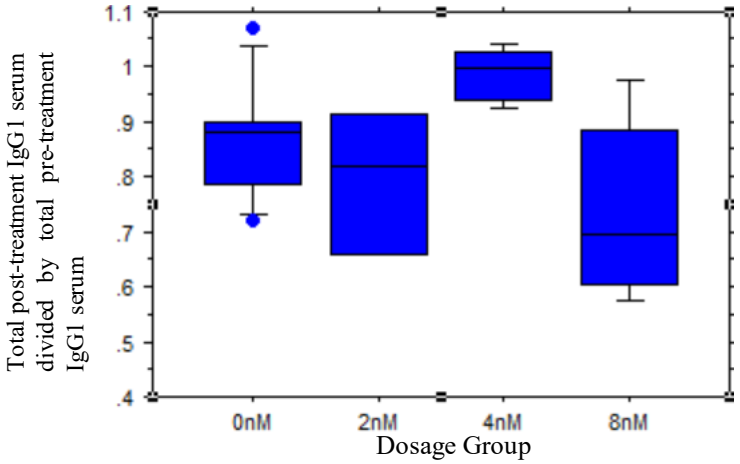

Figure 3.2: the ratio of total post-treatment serum IgG1 levels divided by total pre-treatment serum IgG1 levels compared between Year 2 horses. There was no significant difference between the groups (N=5 per dosage group) (Kruskall Wallace P>0.05).

Year 1 horses (*Fig 3.1*) showed no statistically significant differences between treatments (*P*=0.6242, Mann Whitney U). Similarly, the differences between dosages for year 2 horses (*Fig 3.2*) showed an interesting pattern but did not quite reach overall statistical significance (H=7.611, *P*=0.0548, Kruskall Wallace ANOVA).

IgG4/7

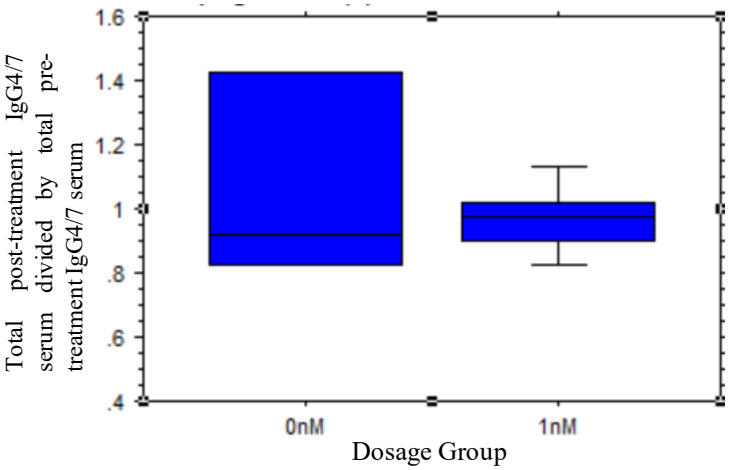

Figure 3.3: the ratio of total post-treatment serum IgG4/7 levels divided by total pre-treatment serum IgG4/7 levels compared between Year 1 horses. There was no significant difference between control horses (N=5) and those who received a 1nMol dose of allergen (N=5) (Mann Whitney U P>0.05).

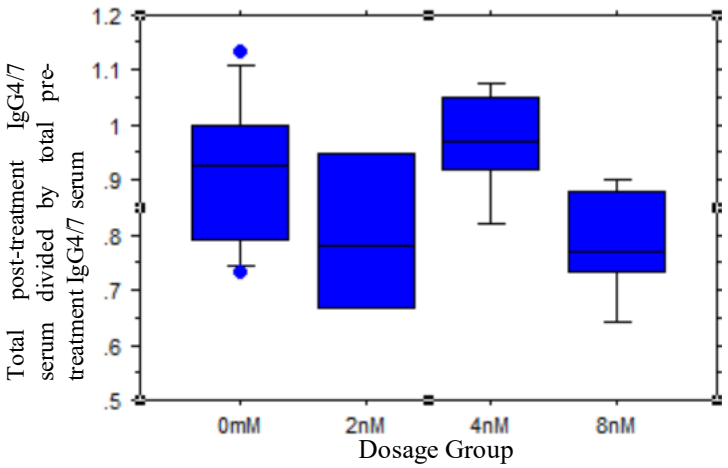

Figure 3.4: the ratio of total post-treatment serum IgG4/7 levels divided by total pre-treatment serum IgG4/7 levels compared between Year 2 horses. There was no significant differences between control horses and the 3 dosage group (N=5 per group) (Kruskall Wallace P>0.05).

Year 1 horses did not show any statistically significant differences between receiving no dose or a dose of 1nMol (*P*=0.9999, Mann Whitney U). Year 2 horses showed the same pattern of results but also did not demonstrate any significant differences between dosages (H=5.053, *P*=0.1680, Kruskall Wallace ANOVA). This demonstrates that no immunotherapy dose given in this study had any effect on total serum IgG4/7 over the period of this trial (*Fig 3.3, & 3.4*).

IgG5

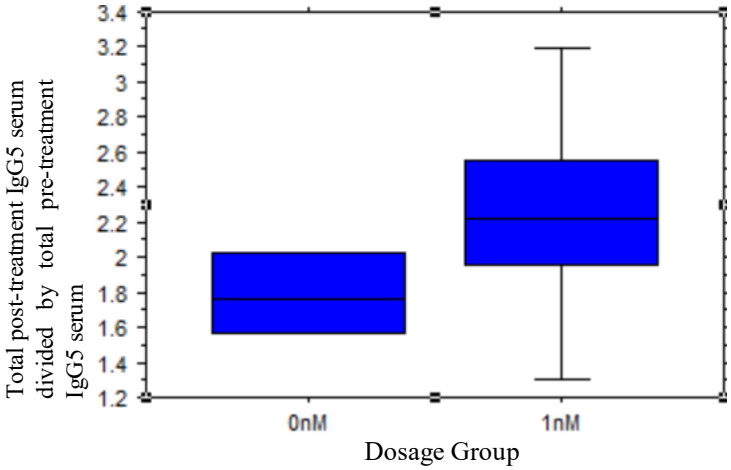

Figure 3.5: the ratio of total post-treatment serum IgG5 levels divided by total pre-treatment serum IgG5 levels compared between Year 1 horses. There was no significant difference between control horses (N=5) and those receiving a 1nMol dose of allergen (N=5) (Mann Whitney U P>0.05).

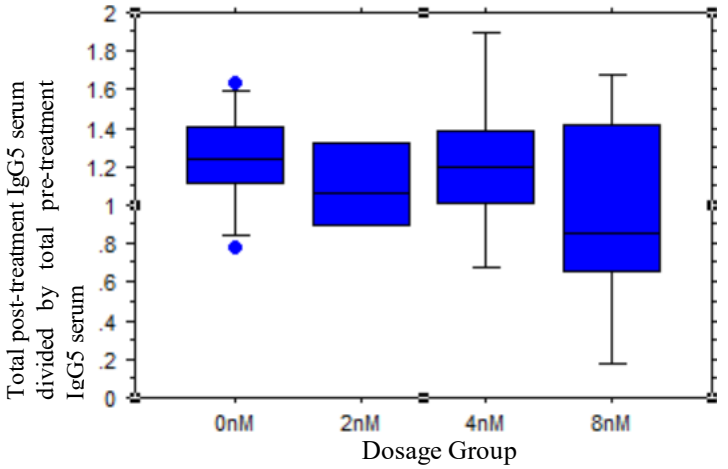

Figure 3.6: the ratio of total post-treatment serum IgG5 levels divided by total pre-treatment serum IgG5 levels compared between Year 2 horses. There was no significant differences between dosage groups (N=5 per dosage group) (Kruskall Wallace P>0.05).

Year 1 horses demonstrated no significant differences between the two treatments (*P*=0.3272, Mann Whitney U). Similarly, Year 2 horses showed no significant differences (H=0.768, *P*=0.8571, Kruskall Wallace ANOVA). This demonstrates that no immunotherapy dose given in this study had any effect on total IgG5 serum levels over the 2 years (*Fig 3.5, Fig 3.6*).

IgG6

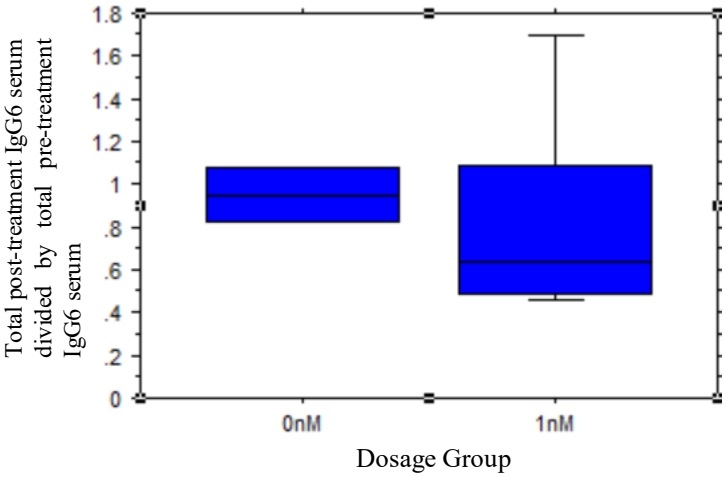

Figure 3.7: the ratio of total post-treatment serum IgG6 levels divided by total pre-treatment serum IgG6 levels compared between Year 1 horses (no dose or dose of 1). There was no significant difference between the control group (N=5) and those who received 1nMol allergen (N=5) (Mann Whitney U P>0.05).

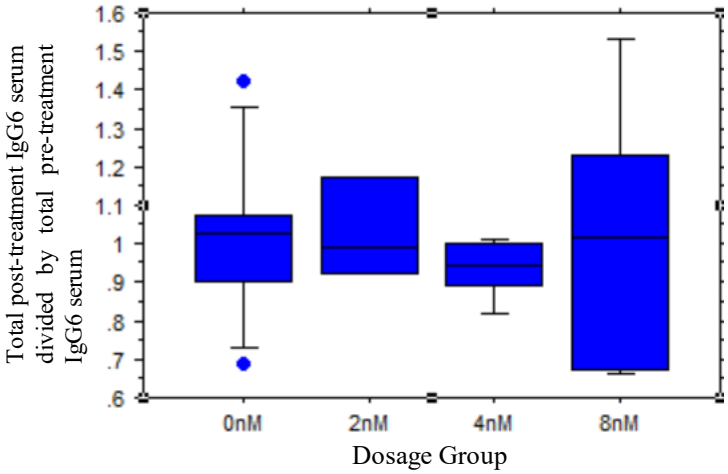

Figure 3.8: the ratio of total post-treatment serum IgG6 levels compared between Year 2 horses. No significant difference was found between the control (N=5) and the 3 dosage groups (N=5 per dosage group) (Kruskall Wallace P>0.05).

Year 1 horses showed no statistically significant differences between treatments (*P*=0.2207, Mann Whitney U). Year 2 horses also showed no significant differences (H=0.897, *P*=0.8261, Kruskall Wallace ANOVA). This shows that no immunotherapy dose given in this study had any effect on total serum IgG6 levels for year 1 or year 2 (*Fig 3.7, Fig 3.8*).

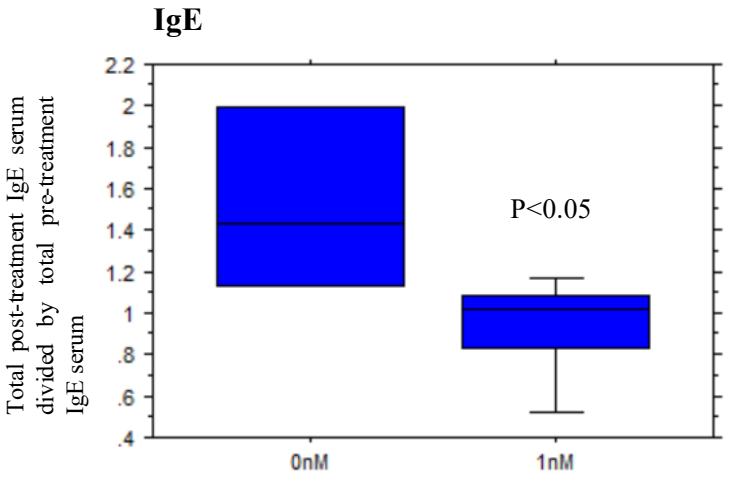

Figure 3.9: the ratio of total post-treatment serum IgE levels divided by total pre-treatment serum IgE levels compared between Year 1 horses (no dose or dose of 1). There was a significant difference between the control group (N=5) and those who received a 1nMol dose (N=5) (Mann Whitney U P<0.05).

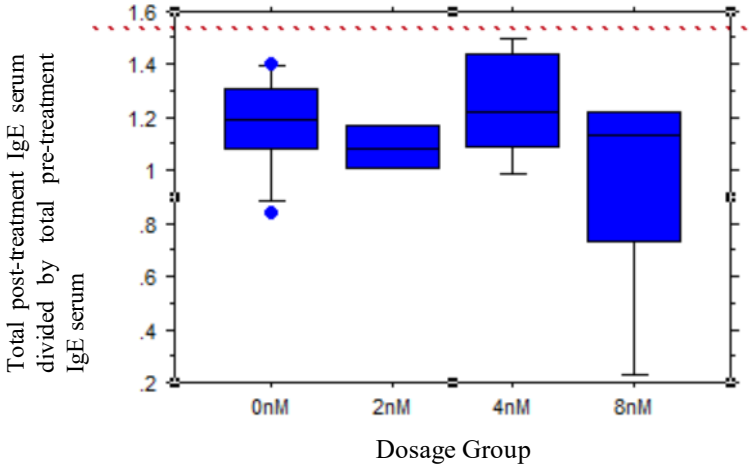

Figure 3.10: the ratio of total post-treatment serum IgE levels compared between Year 2 horses. There were no significant differences between the control group (N=5) and the 3 doses (N=5 per group) (Kruskall Wallace P>0.5351).

Year 1 horses demonstrated a statistically significant difference between no dose and a dose of 1nMol (*P*=0.05, Mann Whitney U) (*Fig 3.9*). Year 2 horses did not show any significant differences (*P*=0.5351, Kruskall Wallace ANOVA) (*Fig 3.10*). A dose of 1nMol had an effect on total serum IgE levels when compared to horses that received no dose, while no immunotherapy dose for year 2 horses had an effect on total serum IgE levels compared to the control dose.

4.0 Discussion

This project forms part of a larger study, the overall aim of which was to conduct a phase 1 trial of oral immunotherapy for equine Insect Bite Hypersensitivity (IBH). During the development of therapeutic medications, the process of clinical trials must follow a series of defined steps as described in "What is a clinical trial? | MRC Clinical Trials Unit at UCL", (2022). Phase I evaluates the safety of the potential treatment to determine any side effects and therefore only a small sample of patients are used, thus making any statistically significant results unlikely. Phase II tests the treatment to determine any effect on the condition it is targeting and a potential dose that it is effective at. Phase III assesses how effective the treatment is against a placebo or the current available treatment, which involves a large number of randomised patients. These take longer than phase I and II trials. Finally, phase IV trials are done once regulatory authorities have approved a drug. These are to determine any effects in different populations and if any side effects are experienced with long-term use. As a

predominantly phase I study, the administration of midge allergen oral immunotherapy was proved safe, as there were no side-effects to any of the given doses which is the role of a phase I study. However, as no side effects may simply reflect an insufficient dose of allergen, there are aspects of a phase II trial as different increasing doses have been tested. Side effects would most likely be seen in the allergic horses used in the trial, which is a change from a typical phase I trial where healthy volunteers are used ("What is a clinical trial? | MRC Clinical Trials Unit at UCL", 2022). As there were no side-effects, it is possible that the oral immunotherapy given was below a threshold dose (Hendry, 2015). Therefore, increasing doses of antigen were used, and in addition to assessing IgE antibodies, IgG subclasses were measured in both IBH horses and healthy controls to look for changes in immune response that would indicate an effect of treatment.

There is little known about how antibody levels would change in horses during allergen immunotherapy. But in large scale human immunotherapy trials, allergen specific IgE has been well-documented to rise during the first few months of oral immunotherapy before falling again (Schoos et. al., 2020). In a study of 161 children, the ratio of allergen specific IgE to total IgE was analysed to predict oral food challenge (OFC) outcome (Gupta, Lau, Hamilton, Donnell, & Newhall, 2014). Ones that failed OFC had higher ratios of allergen specific IgE to total IgE (Gupta, Lau, Hamilton, Donnell, & Newhall, 2014). When studying total IgE levels, Meglio, Bartone, Plantamura, Arabito, & Giampietro (2004) and Staden et. al., (2007) demonstrated no changes during oral immunotherapy. As well as IgE, human IgG4 has been demonstrated to rise during immunotherapy and to have potential protective properties against allergies as it does not activate the complement pathway (Schoos et. al., 2020), inhibits mast cell degranulation (Davies & Sutton, 2015), and can activate a respiratory burst (Zhang, Voice, & Lachmann, 1995), and so is considered an anti-inflammatory antibody (Schoos et. al., 2020). IgG4 may inhibit immune complex formation of other antibody isotypes (Schoos et. al., 2020). Allergen specific IgG4 is strongly induced after prolonged exposure to protein antigens (Aalberse, van der Gaag, & van Leeuwen, 1983). Bedoret et. al., (2012), Chinthrajah et. al., (2019), and Itoh-Nagato et. al., (2018) all demonstrate an increase of specific IgG4 during and after oral immunotherapy. These increases are associated with a successfully consumed dose of oral immunotherapy and immunotherapy induced sustained unresponsiveness (Schoos et. al., 2020). The decrease in IgE activity and production is therefore due to the blocking activity of IgG4 (van Neerven et al., 1999). IgG4 can block IgE by inhibiting CD23-dependent IgE

activity from either directly competing for allergen with receptor-bound IgE, and/or binding of IgG4 to inhibitory FcγRIIb (James, & Till, 2016).

4.1 Discussion of Results

4.1.2 Equine IgE

Equine Insect Bite Hypersensitivity (IBH) has been characterised as an IgE mediated Type I or IV hypersensitivity dermatitis (Birras et. al., 2021). IgE is a well-documented mediator of allergy (Hamza et. al., 2008), and in this study IgE levels significantly decreased for year 1 horses (P=0.05), which demonstrates that oral immunotherapy of midge salivary gland proteins affected IgE production and caused a decrease with a dose of 1nMol compared to no dose (*Fig 3.9*). Although there was no statistically significant difference for year 2 horses (P=0.5351), there is an apparent dose effect with a rise in serum IgE after administration of a 4nM dose followed by a fall in serum IgE after an 8nM dose (*Fig 3.10*). This is counterintuitive as an 8nM dose might be expected to stimulate an even larger rise, unless it is considered that the possibility of the reduced IgE antibody levels at the higher 8nM dose are beginning to show the first stages of IgE suppression associated with immunotherapy.

4.1.3 Equine IgG

There are seven equine IgG subclasses, with their functions not well-studied (Lewis, Wagner, & Woof, 2008). Following studies of human IgG4 after immunotherapy (Kanagaratham, El Ansari, Lewis & Oettgen, 2020), this study aimed to identify the possibilities of determining an equine IgG subclass equivalent for use in Antigen Specific Immunotherapy in Insect Bite Hypersensitivity. It is unknown if any equine IgG subclass is a direct homologue of human IgG4, however IgG2 and IgG6 lack inflammatory properties like human IgG4 does (Lewis, Wagner, & Woof, 2008) (Koneczny, 2018). There is evidence that an increase in equine IgG5 can predict the later onset of IBH, play an active role in the pathology of the disease, and may activate mast cells (Ziegler et. al., 2018).

The antibodies IgG1, IgG4/7, and IgG5 followed the pattern of IgE. No dosage group gave any significant differences to serum antibody levels, however IgG1 provided a borderline figure (P=0.0548) and showed the same pattern of an apparent rise for the 4nMol dose which fell back towards the baseline with the higher 8nMol dose. This indicates that no dose or a dose of 1nMol had no effect on serum levels of IgG1 (*Fig 3.1*), as does a dose of 2nMol, 4nMol, or 8nMol, but is borderline. (*Fig 3.2*). No other antibody subclass demonstrated any borderline

significance, but the pattern of results was similar in IgG4/7. It is necessary for phase I trials to be small-scale with low power, but also in this case doses were not fed for long enough for the effects of the treatment to be seen. But as side effects in human trials are seen early in the treatment regimen, it did demonstrate no allergic side effects to the oral immunotherapy at the doses given.

The total antibody serum levels for IgG5 stay above 1 which indicates that IgG5 is higher in the spring blood samples compared to the early autumn samples. This may be because IgG5 levels are already increased prior to the first clinical signs of IBH appearing (Ziegler et. al., 2018). This could be due to the return of midges which stimulates a response after the winter remission and therefore, it could be suggested that the higher levels of specific IgG5 are a rebound in the spring when midges return after the winter absence and is unaffected by oral immunotherapy. The presence of midges – the cause of the allergy, during an immunotherapy trial is also unusual as many human allergy trials are conducted alongside strict avoidance of allergen exposure.

IgG6 was the only subclass antibody to not follow the pattern of IgE where an immunotherapy dose of 4nMol caused an increase in serum antibody levels before falling with an immunotherapy dose of 8nMol. In the treatment of human allergy, this increase followed by a decrease is then accompanied by a gradual increase in allergen specific IgG4 and is one of the effects that Antigen Specific Immunotherapy (AIT) has on the immune system (Schoos et. al., 2020). IgG6 is hardly detectable in colostrum, is the least predominant IgG subclass in serum, gives little or no respiratory burst, and does not bind the first complement component (Lewis, Wagner, & Woof, 2008). Its precise function is unknown, but the lack of change in serum IgG6 antibody levels for year 1 or year 2 horses for any dosage group along with its lack of presence in serum, indicates that IgG6 may not act as a blocking antibody in IBH and so does not support that it is an equine IgG subclass directly equivalent to human IgG4 at this time.

The pattern of lower antibody levels seen in the 8nMol dose compared to the 4nMol dose may be due to a rapid suppression of the immune response with the 2nMol dose being too low to stimulate an antibody increase or the subsequent fall. A future phase II study would allow an 8nMol dose to be extensively trialled to resolve this question, as well as using repeat blood sampling to track the immune response and determine if this antibody increase followed by a decrease is a temporary effect or a permanent change. This would also provide more detailed studies on the clinical efficacy of this immunotherapy.

4.2 Limitations of Study

This study was only conducted using 30 horses and so only a limited amount of data has been generated. This means that any outlying individual differences between horses within dosage groups will significantly skew the data points. Any future studies should involve a larger number of horses to prevent any major value differences from significantly skewing the data. However, this was a phase I trial which are traditionally small-scale. As well as this, the horses in this study were provided from a charity and so there would only be a certain number present with Insect Bite Hypersensitivity. Any future studies could involve individual horse owners as well as charities to increase participation. Owners of horses with IBH take precautions to prevent symptoms from occurring such as rugging, avoiding turnout during peak midge feeding times, and use repellent sprays so welfare is not compromised (World Horse Welfare, 2022). As a result, it can be difficult to determine if a horse has IBH or not. It would not be possible for owners to remove preventative measures since that would compromise welfare, so the word of the owner has to be taken as to whether the horse genuinely has IBH or is unaffected.

Another limitation of this study is that the temperature of the laboratory may not have been at a constant temperature during the incubation periods of the ELISA plates. Temperature can affect the length of time it takes for ELISA components to bind ("Factors that affect ELISA Results – ELISAKit.net", 2022). Cooler temperatures increase the time it takes for substrate binding while warmer temperatures decrease the time it takes for substrate binding to occur ("Factors that affect ELISA Results – ELISAKit.net", 2022). This could be resolved by incubating the plates in an incubator set at standard room temperature (21˚C) in future studies. However, the decision was taken in this study to not use an incubator as it was started without one, so consistency was kept. Also, a suitable serum standard was unable to be provided due to the sheer number of allergens being tested and some only providing trace readings. This means that the standard used was based on a dilution of substrate that had maximally reacted to give a strong yellow colour. This standard is useful as an indication that the ELISA has worked, but it does not provide a way of directly comparing the responses to different allergens other than to broadly say which were strong or weak allergens. However, as all 60 samples for each allergen were run on a single plate, comparing the results of pre and post treatment samples is valid.

4.3 Conclusions

This study demonstrated oral immunotherapy of multiple midge salivary gland proteins to horses with IBH as well as healthy controls gave no side-effects and that the higher doses of 4nMol and 8nMol may influence subclass IgG levels. This allows a phase II study of oral immunotherapy on a larger group of horses with Insect Bite Hypersensitivity using higher doses to be conducted. Therefore, a potential effective dose at which the symptoms of IBH may lessen and resolve can be researched. As well as this, a larger study group allows any significant changes in IgG subclass antibody levels to be observed, and a direct homologue if any, to human IgG4 to be identified. Overall, this study demonstrated its primary aim of confirming the safety of administering oral immunotherapy of *Culicoides spp.* salivary gland proteins in increasing doses, and that immunotherapy may have some influence over IgG subclass levels and does change IgE antibody levels.

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Appendices

Appendix A

Differences between Th1 and Th2 responses

	T helper 1 (Th1) response	**T helper 2 (Th2) response**
CD4+ T cell	yes	yes
Cytokines produced	Il-2, IFN-γ	IL-4, IL-5, IL-9, IL-13
Stimulates	Cytotoxic effector cells	Differentiation to plasma cells
Immune response	Cell-mediated but can help B cells to produce specific IgG subclass antibodies involved in cytotoxic responses	Humoral – differentiation to plasma cells to secrete antibody (usually IgG, IgA, or IgE)

Appendix B

Excel file showing original IgE ELISA screen determining the most allergenic proteins

Initial IgE ELISA screen

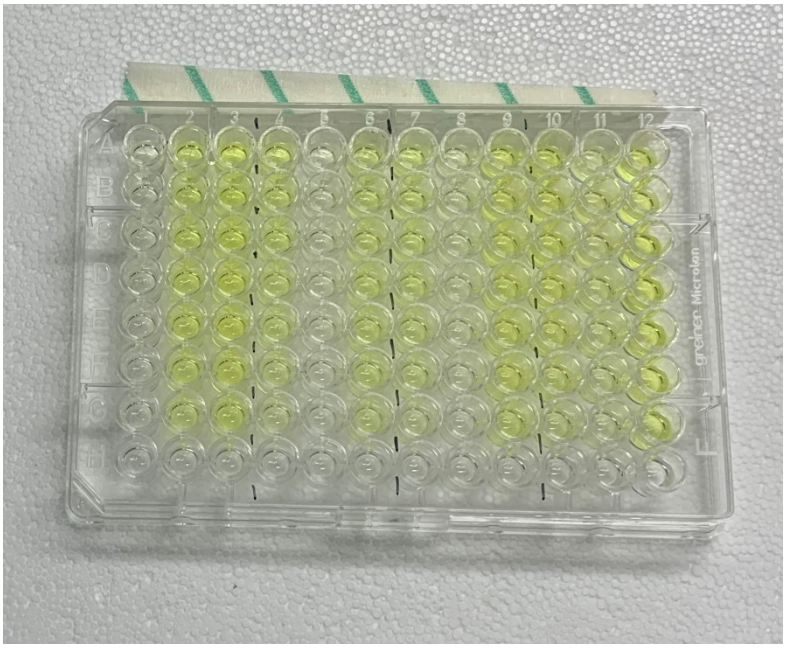
Appendix C

ELISA Plate Layout for experimental study of IgG subclass antibodies

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	100	100	Blank	Blank	Blank	Blank	Blank
B	Frisky 1	Angharad 1	Zahlee 1	Yori 1	Pebbles2 1	50	50	Frisky 2	Angharad 2	Zahlee 2	Yori 2	Pebbles2 2
C	Neddy 1	Alfie 1	Flo 1	Tavy2 1	Autumn 1	25	25	Neddy 2	Alfie 2	Flo 2	Tavy2 2	Autumn 2
D	Harry 1	Gracie 1	Oaky 1	Naji 1	Pixie 1	12.5	12.5	Harry 2	Gracie 2	Oaky 2	Naji 2	Pixie 2
E	Frolic 1	Iona 1	Daisy 1	May 1	Flash 1	6.25	6.25	Frolic 2	Iona 2	Daisy 2	May 2	Flash 2
F	Nettie 1	Romany 1	Revelation 1	Buttermilk 1	William 1	3.125	3.125	Nettie 2	Romany 2	Revelation 2	Buttermilk 2	William 2
G	Wellow 1	Fizzy 1	Bonny 1	Breeze 1	Diamond 1	1.5625	1.5625	Wellow 2	Fizzy 2	Bonny 2	Breeze 2	Diamond 2
H	Blank	Blank	Blank	Blank	Blank	0	0	Blank	Blank	Blank	Blank	Blank

Appendix D

Image of IgG subclass antibody Mouse Clones antibody production test by ELISA



Appendix E

Layout of IgG Subclass antibody Mouse Clones Antibody Production Test ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
	Protein G Flow Through	Protein G Flow Through	Protein G Flow Through	Protein G First Pass Eluate 1	Protein G First Pass Eluate 1	Protein G First Pass Eluate 1	Protein G 2nd Pass Eluate 1	Protein G 2nd Pass Eluate 1	Protein G 2nd Pass Eluate 1	Protein G 2nd Pass Eluate 2	Protein G 2nd Pass Eluate 2	Protein G 2nd Pass Eluate 2
A	CVS53 (IgG6)	CVS40 (IgG5)	CVS38 (IgG3+5)	CVS53 (IgG6)	CVS40 (IgG5)	CVS38 (IgG3+5)	CVS53 (IgG6)	CVS40 (IgG5)	CVS38 (IgG3+5)	CVS53 (IgG6)	CVS40 (IgG5)	CVS38 (IgG3+5)
B	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,
C	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,
D	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,
E	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,
F	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,
G	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,	,,,,
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Appendix F

Original Results File for ELISA of IgG subclasses

IgG ELISA Results 1