Antigenic and immunological determinants of acute allergic susceptibility to meat in a uniquely defined cohort in the Eastern Cape

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Acknowledgements

Science is an art to those with creative minds.

~ sibuco

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I dedicate this thesis to my father (Mr Innocent Patrick Murangi), mother (Mrs Charity Murangi), and siblings (Mai Israel, Tinashe, Tanaka). Indeed *"I am confident of this, that the one who began a good work among you will bring it to completion by the day of Jesus Christ" (Phil. 1:6)*

Personal funders:

Abbreviations

Acquired tick resistance	ATR
Advanced glycation end products receptor	RAGE
Alpha-gal Knockout mice	AGKO
Alternatively activated macrophages	AAM
Antigen-presenting cells	APCs
Atopic dermatitis	AD
Cross-reactive carbohydrate determinants	CCDs
Dendritic cells	DCs
Forkhead box P3 protein	FOXP3
Galactose alpha 1,3 galactose	alpha-gal
Gastrointestinal tract	GI tract
Goblet cell antigen passages	GAPs
Granulocyte-macrophage colony-stimulating factor	GM-CSF
High Molecular Group Box 1	HMGB1
House dust mite	HDM
IgE receptor	FcεR
Immunoglobulin	Ig
Innate Lymphoid Cell	ILC
Interleukin	IL
Lipopolysaccharide	LPS
Major Histocompatibility Complex	MHC
Mesenteric lymph nodes	MLN
Prostaglandin E2	PGE2
Retinoic acid	RA
Secretory epithelial cell antigen passages	SAPs
T helper type 2 immune response	Th2
T regulatory cells	Tregs
Thymic stromal lymphopoietin	TSLP
Tick salivary gland extract	TSGE
Transforming growth factor	TGF-β
Tumor necrosis factor-alpha	TNF-α

Abstract

Allergic sensitization can occur after allergen exposure through the oral-mucosal or cutaneous route. Allergic remission is associated with a decrease in total and specific IgE levels to allergens. *Ascaris lumbricoides* is a potent inducer of IgE through the establishment of a strong Th2 environment. IgE induction following *A. lumbricoides* infection is a risk for allergic sensitization. Tick disruption of host skin during feeding has a systemic effect resulting in the induction of a Th2 phenotype with elevated IgE production. Raised IgE can be driven by exposure to parasite proteins and lipids with complex glycosylation patterns. Our study demonstrates the presence of alpha-gal in both adult and larval developmental stages of *A. lumbricoides, Amblyomma hebraeum* and *Rhipicephalus evertsi*. Alpha-gal glycosylation was prominent on 100kDa and 130-250kDa protein bands. *A. hebraeum* and *R. evertsi* showed differential expression of alpha-gal glycosylated proteins during feeding with band intensity increasing proportionally to an increase in feeding time in the salivary glands. Immunolocalization of alpha-gal in *A. lumbricoides* adult worms showed staining in the lining of the gastrointestinal tract while in *A. hebraeum* and *R. evertsi,* staining was prominent in the salivary glands. Screening for IgE demonstrated elevated IgE to *A. lumbricoides* in human research participants with challenge-proven alpha-gal allergy which positively correlated to alpha-gal IgE. Furthermore, non-alpha gal glycosylated *A. lumbricoides* antigens caused significant activation of a humanized rat basophil RS-ATL8 IgE reporter cell system after incubation with sera from alpha-gal allergic individuals. Interestingly, serum IgG4 from alpha-gal allergic individuals showed surface labelling of *A. lumbricoides* larvae *invitro*. Alpha-gal positive participants also demonstrated raised IgE and IgG4 towards *A. hebraeum* proteins. Proteomic analysis suggests alpha-gal glycosylation to be present on alpha-2-macroglobulin found in lysates from both *A. lumbricoides* and *A. hebraeum*. These findings present *A. lumbricoides, A. hebraeum* and *R. evertsi* as potential sources of sensitization to alpha-gal and hypersensitivity reactions including anaphylaxis in humans after the consumption of red meat or use of pharmaceutical products from a mammalian source.

CHAPTER 1: THESIS INTRODUCTION AND LITERATURE REVIEW

This thesis investigates the role of parasitic exposure in the development of alpha-gal allergy. The introduction and literature review will include situating alpha-gal syndrome within the broader context of food allergy, the role of the immune system in allergy, and the role of parasitic infections in causing allergic sensitization.

1.1. Immune and non-immune mediated reactions to food

Ingestion of food can cause reactions which can be classified based on whether all individuals are affected (toxic reactions) or whether there is an individual hypersensitivity reaction. Hypersensitivity reactions are further subdivided according to whether there is involvement of the immune system or not [1–4] **(Figure 1.1)**. Food intolerance is a non-immune system related reaction to food which occurs as a result of increased susceptibility to an inherent characteristic of the food or an abnormal non-immune response by the host. These inherent food properties include pharmacologic active compounds such as caffeine which causes jitteriness. On the other hand, when food intolerance is caused by abnormal host responses this can be due to a lack of specific metabolic enzymes such as lactase which results in lactose intolerance. Food intolerances are often dose-dependent and not consistently reproducible [5]. Studies have shown most self-reported food allergies to be in fact food intolerances [5–7].

Food allergy is an adverse food reaction which is driven by an immune response to food proteins [8] and usually occurs consistently on every exposure. Food allergies can be categorized into three types based on the underlying immunological mechanism they involve [9]:

a) IgE-mediated, are the most well-known and well-characterized food allergic reactions, involving antibodies known as Immunoglobulin E (IgE). These usually (apart from the case of alpha-gal syndrome) occur rapidly and exhibit clinical symptoms within minutes to a few hours after ingestion. Avoidance strategies are usually necessary to prevent reactions.

b) Non-IgE mediated, occurs when the cell compartment of the immune system is responsible for the food allergy and specific IgE to foods is not responsible for pathology. These can lead to chronic disease and are more difficult to diagnose and manage, and are beyond the scope of this thesis.

c) Mixed IgE cell-mediated, involves both IgE and immune cells in the allergic reaction.

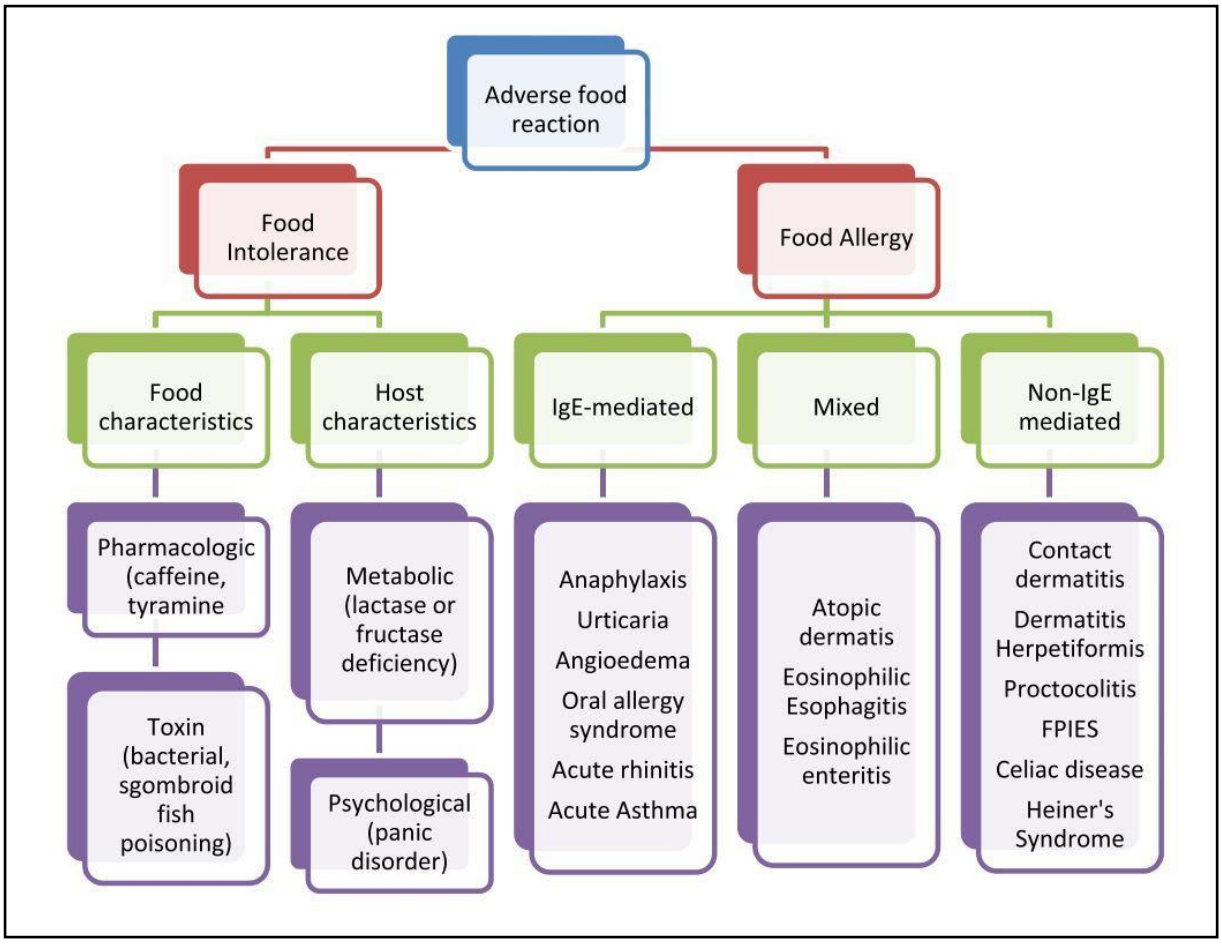


Figure 1.1: Understanding and categorizing immune and non-immune mediated reactions after the consumption of food. Adapted from [10]

1.1.1. IgE-mediated food allergy

The main function of the immune system is to safeguard living organisms against a variety of invasive pathogens (parasites, bacteria, viruses, fungi) and to minimize the damage they may cause [11]. When a foreign organism is detected within the host's body, a sequence of events are initiated against the pathogenic antigens. This causes the innate and adaptive arms of the immune system to combine, coordinate, and respond in a way that efficiently handles the pathogen [12,13]. In some cases, an immune response is elicited against inherently harmless environmental and food antigens.

The development of IgE antibodies to harmless food antigens causes IgE-mediated food allergy. These reactions are reproducible and can be confirmed by detecting food-specific IgE in the serum of affected individuals [14,15]. This misdirected IgE response may have originated as a response to either toxins and venoms (toxin hypothesis) [16–19] or metazoan parasites (worms and parasitic arthropods) [20] **(Figure 1.2)**.

The toxin hypothesis argues that the IgE neutralization of toxins and venoms is immediate and elicits unpleasant allergic symptoms which promote toxin avoidance behaviour [18]. On the other hand, the parasite hypothesis argues that IgE developed later in mammals to counter large metazoan parasites that cannot be phagocytosed. Hence IgE acts to eliminate these parasites via other mechanisms as demonstrated by the IgE-mediated trapping of *Nippostrongylus brasiliensis* larvae in the skin of mice [21] IgE responses against food antigens may result from aberrant sensitization and loss of tolerance or via cross-reactivity among food and environmental antigens, possibly including helminths [22].

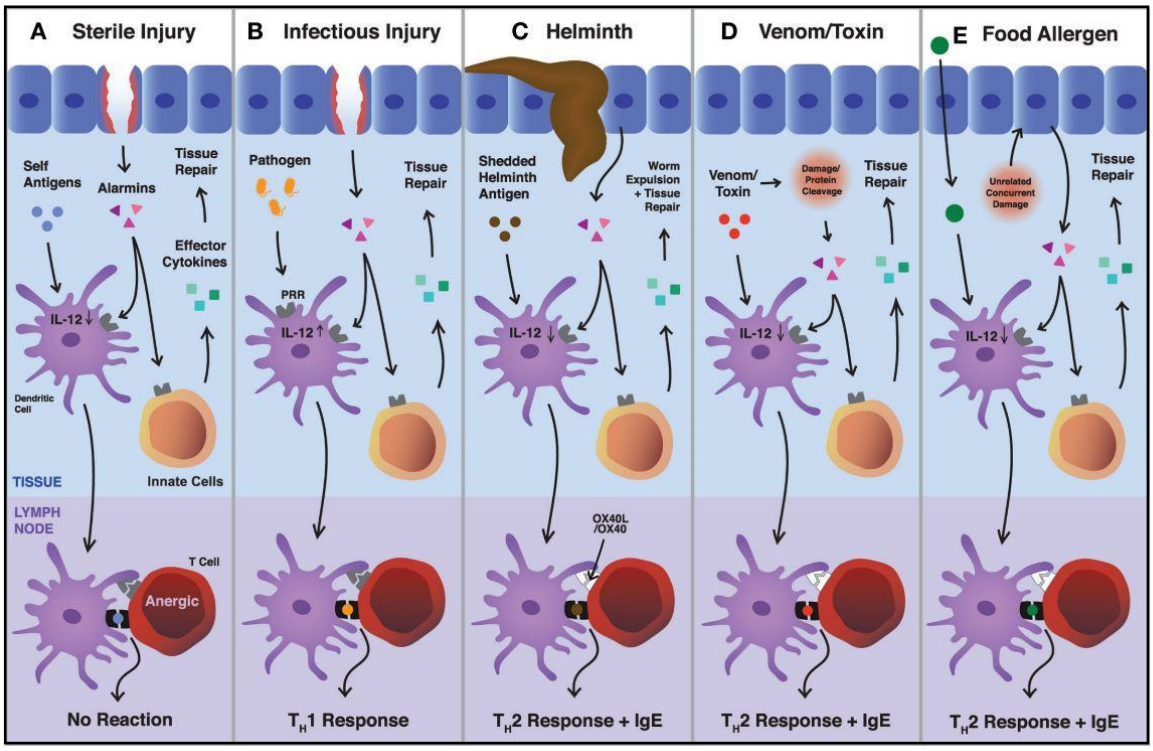


Figure 1.2: Mechanism of IgE production. (A-D) Tissue damage induces epithelial cells to release alarmins such as IL-33, IL-25, and TSLP. These interact with specific receptors on innate immune cells which proliferate and produce effector cytokines, such as IL-5, IL-13, and amphiregulin. Effector cytokines can directly influence tissue repair and also act to recruit other cells required for re-epithelization and extracellular matrix production. Antigens at the site of injury are taken up by dendritic cells which migrate to the draining lymph nodes where they cause naïve T-cell differentiation into either Th1 or Th2 cells. **(A)** At the site of a sterile injury, only self-antigens are present and when DCs present these to naïve T cells, no reaction occurs due to tolerance. **(B)** Stimulation of DCs via pathogen recognition receptors by infectious pathogens induces upregulation of IL-12 by DCs. This facilitates the differentiation of naïve T cells to Th1 response. **(C-D)** Epithelia disruption by invading helminths and proteases from venoms downregulates IL-12 production by DCs which induce naïve T-cells to differentiate

into Th2 cells. These drive B cells to produce IgE. **(E)** Uptake of food allergens via a disrupted epithelium or in the presence of adjuvants (cholera toxin) initiates a Th2 response which results in IgE production against the food allergen. Adapted from [23]

1.2. The immune system and allergy

When a healthy individual consumes food, it leads to a state of immune tolerance in the gastrointestinal (GI) tract. This tolerance prevents a harmful immune response upon subsequent exposure to the food [24–27]. Oral tolerance to food is an active process that involves complex interactions between components of the innate and acquired immune systems. In this state, the immune system is unresponsive to ingested antigens [28]. Regulatory T cells (Tregs) hold a pivotal role in establishing oral tolerance to food allergens [29]. Tregs represent a specific subset of T cells responsible for dampening immune responses and upholding immune equilibrium. In the context of children, it's important to note that Tregs are both less numerous and less effective compared to their adult counterparts, and this discrepancy may contribute to the elevated incidence of food allergies observed in children [29].

Food allergy occurs due to the inability to establish or maintain oral tolerance to certain food proteins **(Figure 1.3)**. This may lead to IgE-mediated responses that range from mild to life-threatening after ingestion of these foods. The way food is processed can have a significant impact on how allergenic proteins are broken down or transferred, potentially rendering specific foods safe for individuals with allergies [30]. Some processing techniques can also have immune-modulating effects, enhancing an individual's tolerance to allergenic foods. For instance, clinical trials have shown that children with egg allergies who can tolerate baked egg products are more likely to develop tolerance to other forms of egg consumption [30].

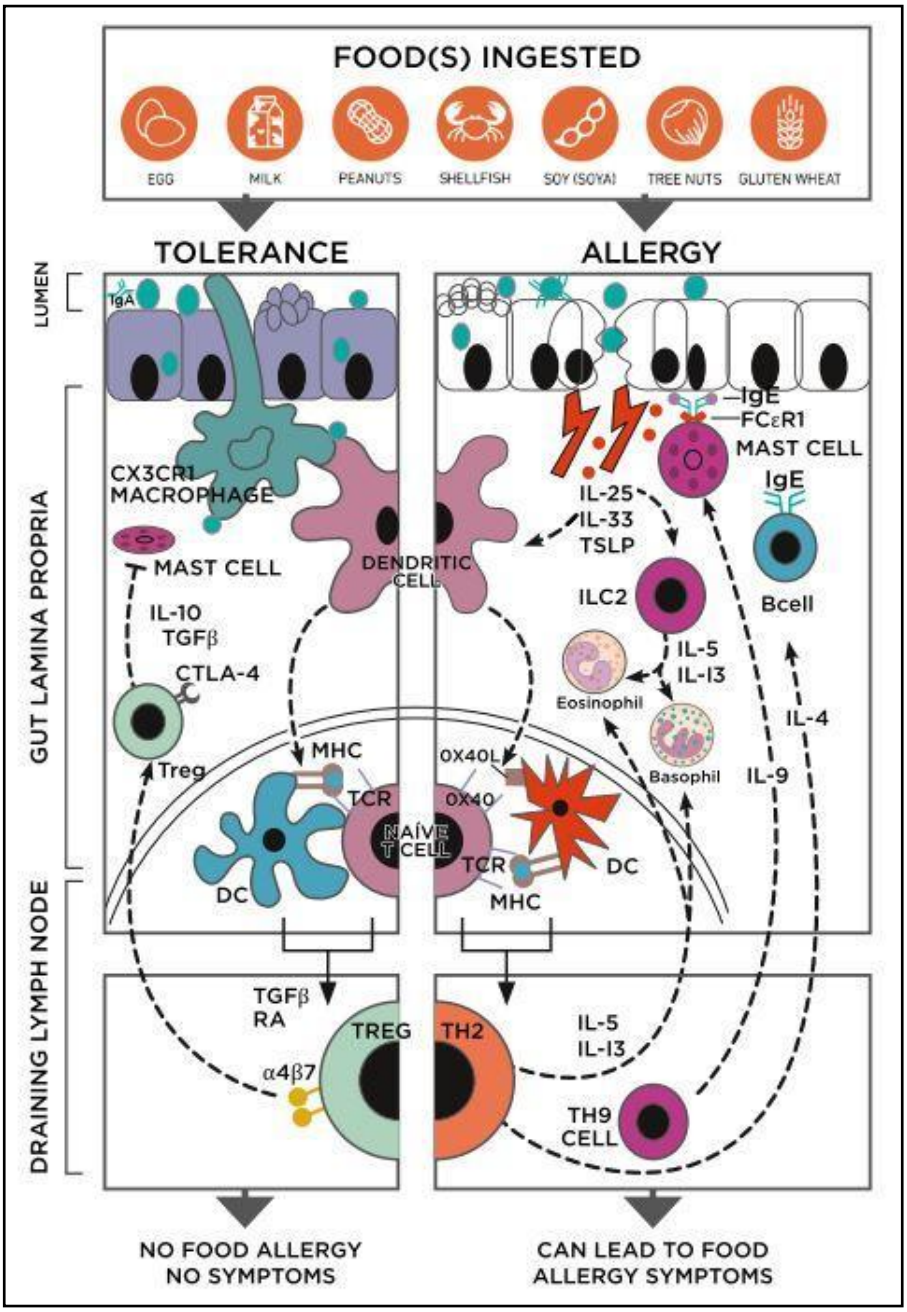


Figure 1.3: A comparison between tolerance and an allergic response to food. Tolerance *(on the left side)* Sampling of food antigens from the lamina propria in the presence of non-inflammatory cytokines such as transforming growth factor-beta (TFG-β) and retinoic acid (RA) results in the presentation of food antigens by DCs via the major histocompatibility complex to naïve T cells via the T cell receptor. In the absence of a co-stimulatory ligand, naïve T cells differentiate into T regulatory cells which migrate to the lamina propria where they maintain tolerance to food antigens by expressing CTLA-4 and production of IL-10 and TFG-β. **Allergic response** *(on the right)* Alarmin release prior to gut damage promotes ILC2 expansion and DCs activation. In the presence of pro-inflammatory cytokines DCs express OX40L which binds to OX40 on naïve T cells during antigen presentation. This induces naïve

T cells to differentiate into Th2 cells which produce IL-4, IL-5, and IL-13. Production of allergen IgE is driven by IL-4 mediated B cell class switching. IL-5 and IL-13 promote the recruitment of eosinophils and basophils to the lamina propria. Th9 cells also secrete IL-9 which recruits mast cells. Allergen-specific IgE binds to basophils and mast cells which thus causes allergic sensitization. Adapted from [9]

The dysregulated Th2 immune response which leads to food allergy occurs in two phases namely sensitization and effector phase.

1.2.1. Sensitization phase

Food antigens which induce IgE development after ingestion by a host are called allergens. Allergic sensitization is the primary development of IgE antibodies against food allergens [31] **(Figure 1.4)**. This is orchestrated by both innate and adaptive immune responses as follows:

1.2.1.1. Epithelial barrier dysfunction

The GI tract epithelial barrier is constantly exposed to a wide range of invasive pathogens as well as harmless food proteins and commensal bacteria. Its main function is to prevent the unnecessary entry of antigens. When intact, the epithelial barrier maintains immune tolerance and mediates antigen transfer through paracellular diffusion, microfold (M) cells [32–34], active transport through enterocytes, and specialized macrophages projecting through epithelial lining [35]. Epithelial barrier function is enhanced by the production of mucins by goblet cells. This provides both physical and chemical protection against enteric bacterial penetration in the mucosa [35–38]. In the absence of pro-inflammatory cytokines, the release of immune mediators such as interleukin 10 (IL-10) and/ or transforming growth factor beta (TGF-β), promotes the development of regulatory T cells [39–41] which further creates a tolerogenic environment.

When damage is inflicted on the epithelial barrier, epithelial cells produce alarmins namely thymic stromal lymphopoietin (TSLP), IL-33, IL-25, and High molecular group box 1 (HMGB1). Studies by Hammad et al. (2009) show initiation of a house dust mite (HDM) specific Th2 response after activation of lung epithelium cells via the toll-like receptor 4 by lipopolysaccharide (LPS) contaminants in HDM aeroallergens which induce TSLP, GM-CSF (Granulocyte-macrophage colony-stimulating factor), IL-25, and IL-33 [42] TSLP stimulates dendritic cells (DCs) to release Th2 chemokines (CCL17 and CCL22) and prime naïve T-cells to differentiate into Th2 cells producing IL-4, IL-5, and IL-13. Studies in human subjects show the ability of TSLP to act as an adjuvant for the stimulation of Th2 immune responses in the same manner as cholera toxin in food allergy murine models [43] and upon epithelial barrier injury there is an overexpression of TLSP in the gut and skin epithelium which has systemic

effects [44]. In a murine model, Blazquez and colleagues demonstrated the need for TLSP in the development of food allergy, and not in oral tolerance [45]. Epithelial barrier injury also results in IL-33 directed mast cell expansion in the small intestines capable of driving an anaphylactic reaction upon oral challenge [46]. Asthmatic patients express elevated IL-33 levels which induce IL-5 and IL-13 in mast cells and IL-4, IL-5, IL-6, and IL-13 in basophils, resulting in allergic symptoms [47].

Production of HMGB1 archetypal alarmin during epithelial injury activates nuclear factor (NF) kappa beta and mitogen-activated protein kinase pathway cell signalling and induces inflammatory cytokines via the receptor for advanced glycation end-products (RAGE) [48–51]. RAGE expression in skin, GI tract and lung epithelium is associated with acute inflammation [52–54] and barrier dysfunction [55,56] which allows for increased barrier permeability with food antigen hence increasing the risk for food allergy especially in a host with low Vitamin D levels [57–60]. In a house dust mite-induced animal model of allergic asthma, the lack of RAGE resulted in the elimination of most of the evaluated disease measures, such as airway hypersensitivity, eosinophilic inflammation, and airway remodelling. However, IL-4 secretion, isotype class switching, and antigen recognition were unaffected [61].

The expression of major histocompatibility class I and II (MHC-I and MHC-II) on intestinal epithelial cells [62] or the secretion of intestinal epithelial cells exosomes containing MHC-II/ peptide complexes [63–65] allow for direct and/or indirect presentation of food antigens to immune cells. Intestinal epithelial cells express low [66,67] and high affinity [68] IgE receptors. Subepithelial immune cells can be stimulated by the transportation of intact IgE-allergen complexes from the luminal surface of the gastrointestinal (GI) tract to the basolateral surface through these receptors. [69,70].

Epithelial barrier dysfunction can arise due to various factors, including genetic predispositions, exposure to allergens, and environmental triggers [71–75]. Genetic anomalies related to barrier components or exposure to agents that damage the epithelial barrier can disrupt the integrity of both skin and mucosal tight junctions [72]. Allergens may also contain proteases capable of interfering with the mucosal epithelial barrier [73]. Dysregulation of the gut microbiota has been linked to heightened gut barrier permeability and the translocation of microbial and dietary antigens [74]. In conditions such as allergic lung disease and asthma, epithelial barrier dysfunction is attributed to disrupted tight junction formation. These junctions, consisting of multi-protein subunits, play a crucial role in fostering cell-to-cell adhesion and maintaining barrier integrity [76]. However, not everyone who experiences epithelial barrier disruptions develops food allergies. Studies suggest a combination of factors to independently increase or decrease the risk of some people developing food allergy [77,78]. A combination of

factors such as hygiene and lack of exposure to microbial factors, composition of the intestinal microbiota, diet, obesity, and lack of Vitamin D, may increase the risk of food allergy development [57–60,77,78]. The unequal susceptibility to these factors may thus be responsible for variations in allergy among individuals.

1.2.1.2. Antigen presentation

Communication between the innate and adaptive arms of the immune system is facilitated by antigen-presenting cells (APCs). DCs were first observed as adherent cells from murine spleens which have a large contact surface area in comparison to its overall cell volume [79]. GI tract-associated CD103+ DCs sample antigens from the gut lumen directly or via transfer from specialised macrophages [35,80] and present them to naïve T cells in the Peyer's patch or the gut-draining mesenteric lymph nodes (MLNs) [81,82].

DCs induce the production of retinoic acid (RA) and tumour growth factor β (TGF-β) which prime naïve T-cells to express the transcription factor fork-head box protein 3 (FoxP3) [40,41,83–85]. In addition, DCs release IL-10 [86,87] which has immunosuppressive properties and together with their induction of FoxP3+ T regulatory cells result in oral tolerance to food antigens.

However, in the presence of IL-25, IL-33, and TSLP, DCs are activated to express tumour necrosis factor ligand superfamily member 4 (TNFSF4, commonly known as surface OX40L). Upon presentation of food antigens on DC MHC, the interaction between DC OX40L and naïve T-cells OX40 receptor induces naïve T-cell differentiation into Th2 cells. The induction of this Th2 phenotype promotes an allergic state [88,89].

1.2.1.3. T cell activation

Naïve CD4+ T cells are activated by APCs; they differentiate into various types of effector cells depending on the pathogen and cytokine environment. The differentiation process is directed by T cell receptors (TCRs), co-stimulatory ligands, and cytokine receptors, which initiate intracellular signalling pathways and selective gene expression. The different types of CD4+ T cells that can form include Th1, Th2, and Treg cells, each with a specific cytokine and effector cell profile [90–92].

In the presence of TGF-β, retinoic acid and IL-10 from APCs, naïve T cells differentiate into FoxP3+ T-regs. These express gut-homing receptor α4β7 which allows for their translocation to the lamina propria where they induce immunosuppression via CTLA-4 binding to basophils and mast cells [93,94]. T-regs also secrete IL-10 and TGF-β ensuring a tolerogenic environment is maintained [95].

When epithelial injury occurs, DCs become activated and produce pro-inflammatory cytokines. In a pro-inflammatory cytokine environment, naïve T cells differentiate into Th2 cells. The secretion of a range of cytokines, including IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, by these cells promotes B cell class switching. This process leads to the production of IgE antibodies, establishing sensitization and triggering an allergic response to food [96–98]. Studies in humans [99,100] and murine models [101–103] show elevation of these cytokines in response to food allergens. In addition to Th2 involvement in causing sensitization and an allergic state, studies by Sehra et al. (2015) have shown naïve T cell differentiation to Th9 cells to aid in sensitization by secreting IL-9 which induces accumulation of mast cells in tissue [104].

1.2.1.4. IgE production

IgA production by plasma B cells acts as the first line of defence by preventing the absorption of intact proteins and inhibiting the adhesion of pathogens. IgA is the most abundant immunoglobulin isotype along mucosal surfaces and induces immunological tolerance by preventing the development of inflammation [105–107]. IgA production by B plasma cells is maintained by TGF-β and IL-10 from Tregs. Low levels of IgA and its deficiency have been shown to predispose individuals to the development of food allergy [108–110]. On the other hand, elevated food-specific IgA is associated with desensitization [111,112].

Upon naïve T cell activation into Th2 cells, IL-4 and IL-13 cytokine secretion and interaction of CD40 on B cells and CD40-ligand on Th2 cells induce B cells immunoglobulin isotype class switching to IgE [113,114]. IgE antibodies bind to IgE receptors on mast cells, basophils, and eosinophils. This creates a sensitized state which results in allergic reactions on secondary exposure to the food antigens.

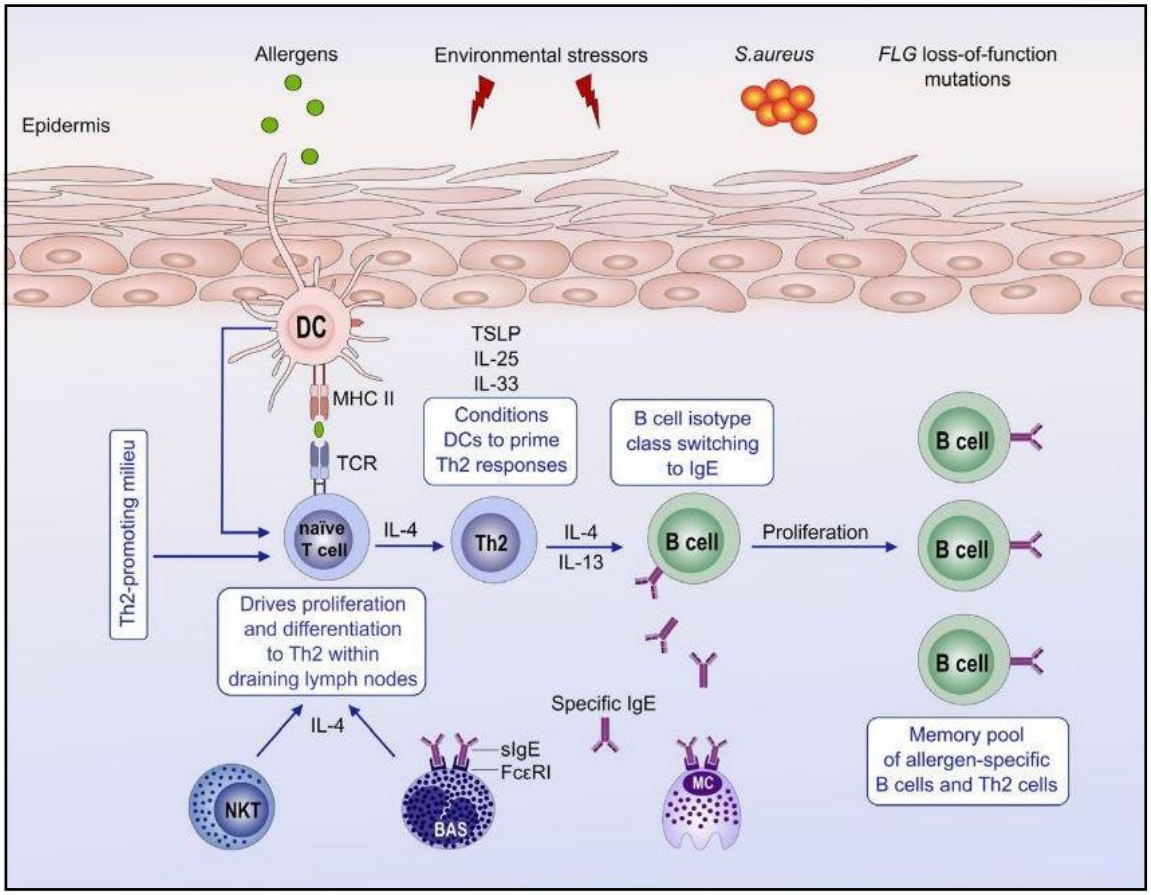


Figure 1.4: The sensitization phase of food allergy. Disruption of the epithelial barrier causes a release of alarmins which activate DCs. When activated, DCs migrate to draining lymph nodes where they present food antigens to naïve T cells. These differentiate into IL-4 and IL-13 producing Th2 cells. These cytokines induce class switching by B-cells into allergen-specific IgE-secreting plasma cells. IgE produced binds to the high-affinity IgE receptor (FcεRI) on mast cells and basophils. Production of memory B cells and Th2 cells also occurs. Adapted from [115].

1.2.2. Effector phase

The first exposure to a food allergen causes the production of IgE which binds to mast cells and basophils. Crosslinking between bound IgE and food allergens on secondary exposure results in the induction of an allergic reaction with clinical manifestations **(Figure 1.5)**.

1.2.2.1. Mast cells and basophils

Mast cells originate from the bone marrow, and migrate to peripheral tissues [116,117] while basophils remain circulating in blood [116,118,119]. Both express high-affinity IgE receptors (FcεRI) [120] and contain granules in their cytoplasm. Food antigen crosslinking with food-specific IgE bound to mast cells and basophils leads to receptor aggregation, activation of signalling

pathways, and degranulation of these effector cells. This results in the release of preformed mediators such as histamine, tryptase, platelet-activating factor, prostaglandins, and leukotrienes into the bloodstream [112]. These mediators are responsible for the clinical manifestations of an allergic reaction.

Histamine induces vasodilation, enhances vascular permeability, elevates heart rate, increases cardiac contraction, and stimulates mucus secretion. Its release and action is rapid and is often associated with anaphylaxis. In a murine model of chronic allergic contact dermatitis, histamine was shown to suppress T reg expansion [121]. Tryptase is responsible for angioedema, and its levels peak 60–90 min after the onset of an allergic reaction [122,123]. Platelet-activating factor is involved in several key aspects of anaphylaxis. It causes heightened vascular permeability, chemotaxis, and degranulation of eosinophils and neutrophils. Additionally, it contributes to bronchoconstriction, making it a significant contributor to the development of anaphylaxis. Prostaglandin causes bronchoconstriction, vasodilation, coronary and pulmonary artery vasoconstriction during anaphylaxis. This also acts to recruit more basophils, eosinophils, dendritic cells, and Th2 cells to the reaction site which in turn creates a positive loop for allergic reaction exacerbation. Lastly, Leukotrienes play a role in increasing vascular permeability, promoting smooth muscle contraction, and enhancing mucus secretion.

Activation of mast cells has been shown to drive transcription of cytokine genes namely IL-4, IL-6, IL-9 and IL-13 [124]. This induces the expansion of Th2 cells, and IgE-producing plasma cells and enhances mast cell proliferation, survival, and expression of FcεRI [104,125–136]. In addition to IgE-mediated mast cell activation, mast cells may degranulate in an IgE-independent manner via (amongst others) the Mas-related G protein-coupled receptor (Mrgprx2) [137,138]. Sensitization to *Dermatophagoides farinae* has been shown to be driven by mast cell activation in a non-IgE mediated manner [139]. Induction of Th2 responses by basophils acting as APCs has also been demonstrated in response to papain [140], ovalbumin, [141,142] and helminths. [142].

1.2.2.2. Type 2 innate lymphoid cells (ILC2s)

Innate lymphoid cells (ILC2s) are tissue-resident cells that are predominantly distributed in mucosal tissues such as lung, small intestine, skin, and adipose tissue. Despite their low levels ILC2s play essential roles in allergic diseases through enhancement of type 2 inflammation [143–149]. This is through the release of IL-5, IL-13, IL-4, IL-25, and IL-9 [150]. These cytokines promote allergic phenotypes and suppress allergen-specific T regs induction [144,151,152]. Studies by Rivas et al. (2016) demonstrate the blockade of allergen-specific Treg cells generation by IL-33 stimulated ILC2s through the production of IL-4 thus favouring food allergy [144]. Likewise,

Gurrametal et al. (2023) shows IL-33-mediated ILC2s activation in murine models to result in Th2 responses to papain [145]. Apart from IL-33 stimulation, Gour et al. (2018) implicated ILC2s recruitment to be C3a-dependent [146]. In their study they showed that ILC2s exhibit a direct response to C3a, a response which in turn promotes type 2 immune responses favouring food allergy. Specifically, this response involves two critical mechanisms: firstly, ILC2s under the influence of C3a increase the production of IL-13 and granulocyte-macrophage colony-stimulating factor while simultaneously inhibiting the production of IL-10 by ILC2s themselves. Secondly, this interaction enhances the antigen-presenting capacity of ILC2s, particularly during their crosstalk with T-cells [146]. Their expansion in the intestines is initiated by IgE-activated mast cells and in the absence of IgE and mast cells this expansion is lost [152].

During helminth infection, ILC2s interact with CD4 T cells and augment Th2 polarization [150]. Individuals with acute exacerbation of chronic obstructive pulmonary disease, have also demonstrated the ability of ILC2s to promote Th2 cell differentiation via an activated Notch-GATA3 signal pathway [153]. Therefore, ILC2s play a pivotal role in enhancement of Th2 immune responses and hinderance of the T regulatory network which may result in the development food allergy.

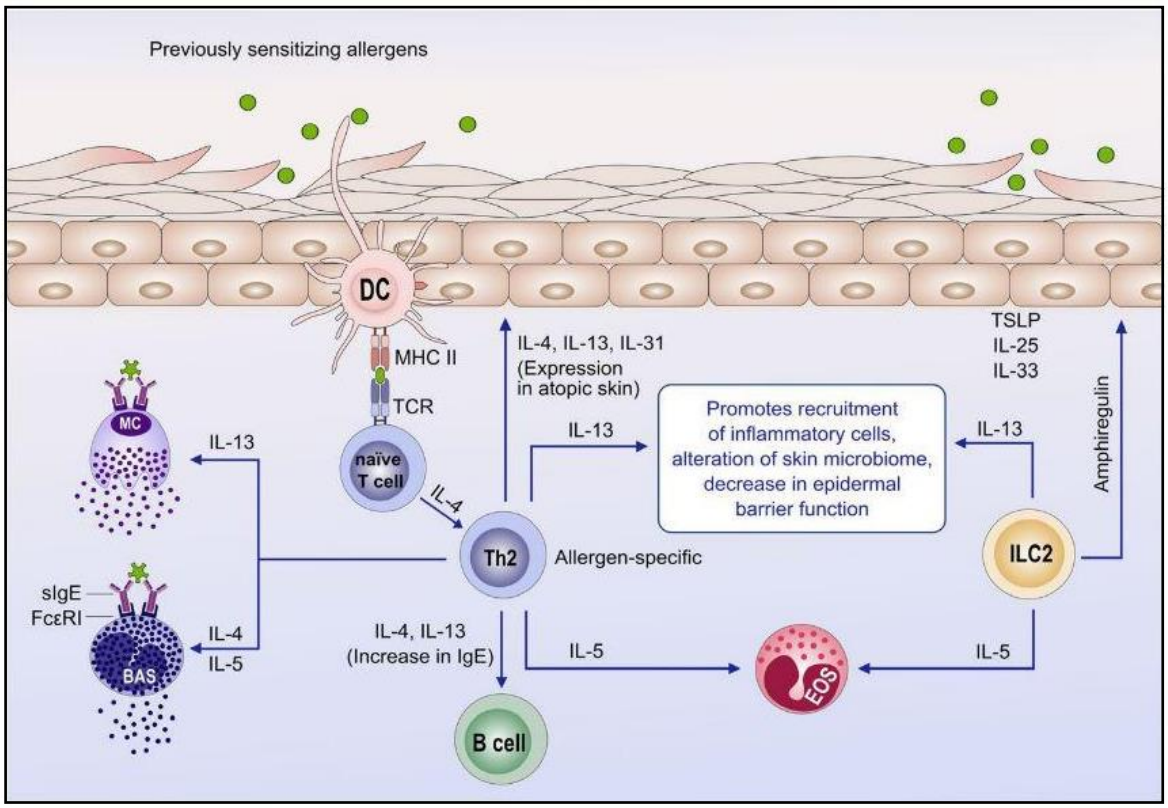


Figure 1.5: The Effector Phase of food allergy. Secondary exposure to previous sensitizing antigens results in IgE crosslinking. This activation of basophils and mast cells induces them

to degranulate. This release of mediators triggers immediate hypersensitivity reactions and activates allergen-specific memory Th2 and B cells. Adapted from [115].

1.2.3. Clinical manifestations of food allergy

A break in oral tolerance leads to the elicitation of an allergic response. The effector phase of an allergic reaction produces mediators which have a systemic effect including the skin, respiratory tract, GI tract, cardiovascular, and neurological systems [154,155] **(Table 1.1)**.

Cutaneous manifestations of IgE-mediated food allergy can include erythematous rashes, pruritus, urticaria, and angioedema. Hives and angioedema are pruritic and can cause significant discomfort, while erythematous macular rash without hives may also occur.

Symptoms related to the upper and lower airway tract can manifest as occasional sniffing or nose rubbing in mild cases. However, more severe symptoms can include complete nasal blockage, wheezing, and in some cases, stridor, which can result in airway obstruction. Subjective GI symptoms include itching of the mouth or throat, nausea, or abdominal pain, while objective symptoms include vomiting and intermittent or persistent diarrhoea [156].

In severe cases, life-threatening anaphylaxis occurs. It is characterized by the involvement of more than one organ system, including cutaneous, respiratory, GI tract, cardiovascular, and neurological systems. Although rare, anaphylaxis can sometimes present with only respiratory, cardiovascular or neurological symptoms such as dizziness, weakness or unconsciousness [156].

To alleviate these deleterious responses, affected individuals usually take up avoidance diets. This may cause a reduced quality of life with nutritional deficiencies and growth impairment [156–166]

Table 1.1: IgE-Mediated Food Allergy: Clinical manifestations by organ system. Adapted from [9]

Organ	Symptoms	Mild	Moderate	Severe
Skin	Rash	Faint erythema	Erythema	marked erythema (covering more than 50% of the body surface area)
	Urticaria/ angioedema	< 3 areas of hives; mild lip edema	More than 3 areas of hives; significant lip or face edema	Swelling of the lips, eyelids and face
Upper airway	Pruritus	Scratching occasionally	Continuous scratching	Scratching continuously; excoriations
	Sneezing/ itching	Occasional sniffing	Rubbing of nose or eyes intermittently; sniffing frequently	Rubbing of nose or eyes continuously periocular swelling; persistent rhinorrhea
Lower airway	Wheezing	Expiratory wheezing	Inspiratory and expiratory wheezing	Use of accessory muscles; audible wheezing; increased work of breathing
	Laryngeal manifestation	Episodes of throat clearing or cough; persistent throat tightness	Hoarseness, frequent cough	Stridor
Gastrointestinal tract	Subjective symptoms	Nausea or mild abdominal pain	Moderate abdominal pain with normal activity	Notably distressed due to GI symptoms with decreased activity
	Objective symptoms	1 episode of emesis or diarrhoea	More than 1 but less than 3 episodes of emesis or diarrhoea	More than 3 episodes of emesis or diarrhoea
Cardiovascular and/or Neurological		Subjective weakness, dizziness; tachycardia	Drop in blood pressure; a significant change in mental status (anxiety, confusion)	Severe cardiovascular collapse; unconsciousness
Other			Loss of bladder control	Pelvic pain

1.2.4. Prognosis of food allergic reactions

Allergic reactions to food usually occur early in life. This can be attributed to low levels of mucin production [167] and an increased GI tract permeability [168] in new-born babies. Infants also have a reduced gastric acidity [169,170] which may contribute to food antigens reaching the GI tract in their intact form. This increases the chances of food antigens passing through the mucosa and causing sensitization at an early age.

The ability to outgrow some food allergies has been reported. Skripak et al. (2007) showed that by the age of 4 years, 19% of individuals become tolerant to cow's milk. This percentage increases to 42% by the age of 8 years, 64% by the age of 12 years, and 79% by the age of 16 years [171]. Savage et al. (2007) showed a different tolerance profile for egg proteins. Only 4% of individuals develop tolerance by the age of 4, while 12% of them do so by the age of 6. The percentage of those who develop tolerance increases to 37% by the age of 10 and 68% by the age of 16 [172]. However, in the case of peanut and tree nuts which cause the most prevalent and fatal allergic reactions [173,174], a lifelong phenotype with only 20% of individuals outgrowing the allergy is observed [175].

1.2.5. The nature of food allergens

According to Radauer et al. (2008) out of over 12,000 known protein families, only 0.6% of them (71 protein families) have been identified as representing approximately 400 food allergens [176]. Having such a small number of allergenic foods suggest unique characteristics which allow for their allergenicity. The most commonly implicated foods in causing allergy are cow's milk, peanut, egg, tree nuts, wheat, fish/shellfish, sesame seed, mustard, soy, and sulphites (a food additive) [177,178]. The allergenic epitopes are often 10-70kDa water-soluble proteins with high resistance to gastric acid degradation, heat and cold denaturation which allow them to remain intact after digestion, cooking or processing [6,179–182].

The resistance to processing is attributed to protein glycosylation [183] which may also aid in immunogenicity as seen by the development of antibodies against glycans in some studies [184–186]. Shreffler et al. (2006) showed the inability of the major peanut allergen and glycoprotein Ara h1 to induce Th2 cell differentiation after deglycosylation [187]. This is in line with other studies which indicate the need for protein glycosylation to play a role in DC uptake [188] and induction of Th2 differentiation [70,185,189–192]. As such carbohydrate glycosylation is viewed as an adjuvant for the sensitization and elicitation of food allergies. However, there has been a growing interest in the ability of carbohydrates to bind to IgE.

Studies by Aalberse et al. (1981) showed patient serum IgE binding to carbohydrates of pollen, vegetable food and bee venom via immunoblotting [193]. This binding was abolished after periodate treatment. Other studies show IgE from honeybee-allergic patients to cross-react with a similar carbohydrate from plant glycoproteins [194,195]. These cross-reactive carbohydrates are called cross-reactive carbohydrate determinants (CCDs). Generally, IgE towards CCDs does not result in clinical allergy [195,196] and can give false positive results in invitro assays [197–200]. However, alpha-gal syndrome (AGS) is an exception.

1.3. Alpha-gal and alpha-gal syndrome

Reactions to alpha-gal include immediate type I hypersensitivity reactions to parenterally administered drugs or a delayed hypersensitivity reaction after oral consumption of food or use of pharmaceutical products which contain galactose alpha-1,3-galactose (alpha-gal). The syndrome of delayed anaphylaxis after oral ingestion of alpha-gal in alpha-gal sensitised individuals is referred to as alpha-gal syndrome (AGS). The diagnosis of AGS relies on the presence of typical symptoms after exposure as well as the presence of alpha-gal-specific IgE. An alpha-gal IgE level of >5.5kU/L and alpha-gal sIgE: total IgE ratio of >2.12% is associated with clinical reactivity in 95% of individuals [201]. In research studies skin prick or intradermal testing with beef, pork, lamb, or bovine thyroglobulin may also be used. The gold standard for diagnosis is an oral food challenge with (non-primate mammalian) "red" meat [202].

1.3.1. Natural immunity against alpha-gal

Alpha-gal is a major constituent of membrane-bound and secreted glycoproteins and glycolipids in non-primate mammals. In contrast Old World monkeys, apes and humans do not possess the enzyme α-1,3-galactosyltransferase due to the inactivation of the α-1,3-galactosyltransferase (GGTA1) gene and thus cannot synthesize alpha-gal [203–206] **(Figure 1.6)**. The identification of alpha-gal by the human immune system results in the production of alpha-gal specific antibodies. Studies show the production of anti-alpha-gal IgG and IgM antibodies from infancy, and these contribute between 1-5% of all naturally circulating IgG antibodies [177,203]. At steady state, production of anti-alpha gal IgG and IgM antibodies is driven by commensal GI tract bacteria such as *Escherichia coli*, *Klebsiella*, and *Salmonella* strains which express alpha-gal on their surface [207–211]. Interestingly, the presence of anti-alpha-gal IgA, IgG and IgM in colostrum and breast milk suggests anti-alpha-gal antibody maternal transfer through breastfeeding [212]. Anti-alpha-gal IgA strongly binds to gram-negative bacteria from throat and stool isolates and confers protection at mucosal surfaces by inhibiting *Neisseria meningitidis* binding to buccal epithelia [212]. Anti-alpha-gal IgM and IgG antibodies have been suggested to protect against alpha-gal expressing pathogens such as *Leishmania spp.* [213,214], *Plasmodium spp*. [215], *Schistosoma mansoni* [216], *Mycobacterium marinum* [217,218], *Aspergillus fumigatus* [216], *Trypanosoma cruzi* [219], *Anaplasma phagocytophilum* and *Borrelia spp.* [220].

On an evolutionary basis, the development of anti-alpha-gal antibodies has been suggested to ensure protection against alpha-gal expressing pathogens. Anti-alpha-gal antibodies have also been implicated in xenotransplantation rejection [221–229]. Hence alpha-gal can be protective (fighting against alpha-gal expressing pathogens) or detrimental (as in the case of xenotransplants and alpha-gal syndrome).

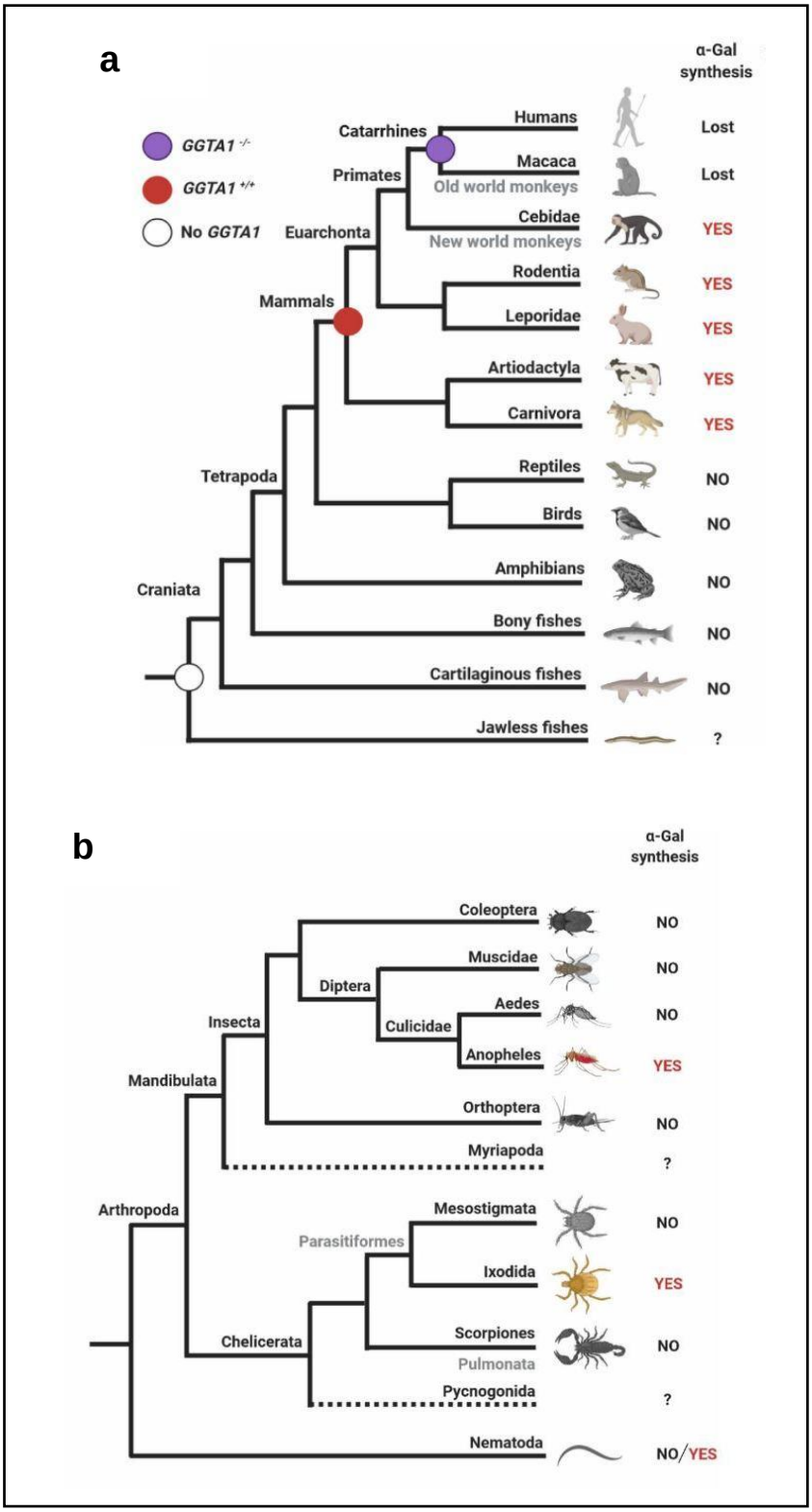


Figure 1.6: Differential GGTA1 gene expression and alpha-gal synthesis among metazoans. (a) GGTA1 gene distribution among craniates [204,217,230,231] **(b)** GGTA1 gene distribution among Arthropods [215,231–238] and Nematodes [216,232,239,240]. Taxa with missing information on the presence of alpha-gal are marked with (?). Adapted from [231].

1.3.2. Allergenicity of alpha-gal

Alpha-gal is a disaccharide sugar which retains its stability after heat treatment and pepsin digestion [241,242]. These properties increase the chances of alpha-gal being transported across the GI tract and induce Th2 cells differentiation which leads to elicitation of an allergic response. Individuals with AGS report reactions after the consumption of red meat which have been reported to contain varying quantities of alpha-gal [243–248]. Food additives such as carrageenan, a clarifying and hydrating agent from boiled red algae (*Chondrus crispus* and *Gigartina mamitiosa*), found in infant's milk, and in toothpaste can also trigger AGS [249]. Alpha-gal IgE can bind to cow milk proteins namely, γ-globulin, lactoferrin, and lactoperoxidase [250], however, clinical reactivity to milk or milk products among alpha-gal allergic patients is rare [250–253].

Pharmaceutical drugs made from mammalian cell lines have been implicated as potent inducers of alpha-gal allergic reactions. Cetuximab, a chimeric mouse/human monoclonal antibody with alpha-gal glycosylation on the Fab region may be responsible for anaphylactic reactions in alpha-gal sensitised patients getting treatment for colorectal cancer [254–256]. Straesser et al. (2021) showed the development of type I hypersensitivity reactions after the administration of snake antivenom to be due to the presence of alpha-gal on the Crotalidae-polyvalent fab region of the antivenom [257]. Recent studies demonstrate the ability of gelatine-containing vaccines (varicella vaccine, an attenuated V-zoster vaccine and the measles, mumps, and rubella live vaccine) to activate basophils invitro [258,259]. Thus, vaccine delivery in alpha-gal allergic patients can cause life-threatening anaphylaxis. The presence of alpha-gal in prosthetics has also been linked to early and peri-operative valvular deterioration after alpha-gal sensitization [260]. Wilson et al. (2018), demonstrated an increased association between alpha-gal IgE and an increase of atherosclerosis and plaque instability in individuals under the age of 65 years [261].

1.3.3. Clinical manifestations of AGS

Patients with AGS may have mild or moderate reactions and a high prevalence of reactions involving the gastro-intestinal tract, including individuals with isolated subjective abdominal symptoms (abdominal cramping only) [201,262]. This cohort may potentially be misdiagnosed as having an intolerance to mammalian meat or food poisoning. The other group of individuals may experience a combination of reactions, including severe abdominal symptoms such as abdominal pain, vomiting, and diarrhoea. They may also exhibit skin reactions such as scratching, hives, erythema, or angioedema. In severe cases, they may develop respiratory complications, experience hypoperfusion, or even anaphylaxis, which is a severe and

potentially life-threatening allergic reaction [201,263]. However, immunoassays do not reveal any differences in the level of sIgE against alpha-gal in these phenotypes [201]. This may suggest elicitation of alpha-gal allergic reactions can occur through multiple pathways. Individuals with the AB or B blood group have been shown in some studies to produce less anti-alpha-gal IgE and may be partially protected against the development of AGS [264–267]. A likely explanation is the similarity of alpha-gal to the B blood group antigen with the difference between the two being a fucose residue on the former. Hence, individuals with blood group B identify alpha-gal as a self-antigen and do not mount an immune response against it.

1.3.4. Onset of signs and symptoms in AGS

A major characteristic of alpha-gal allergic reactions is the delayed onset of symptoms after consumption of alpha-gal containing foods, particularly red meat. Pork innards elicit more severe and rapid symptoms within 2 hours after meat consumption [246,247]. Since they contain more alpha-gal epitopes quantitatively in comparison to muscle meat, severity and onset of symptoms may be associated with the bioavailability of alpha-gal determinants in red meat [246,247,268]. The occurrence of severe reactions after eating fattier meat portions while leaner cuts are tolerated suggests the role of alpha-gal glycosylated glycolipids in triggering an allergic response [247,269]. During metabolic processes, glycolipids also take more time to break down resulting in a delayed presentation of alpha-gal determinants to the immune system. Studies suggest the delay to be a consequence of the time taken to digest the meat, absorb, transport and present it to the immune system [249,270,271] **(Figure 1.7)**

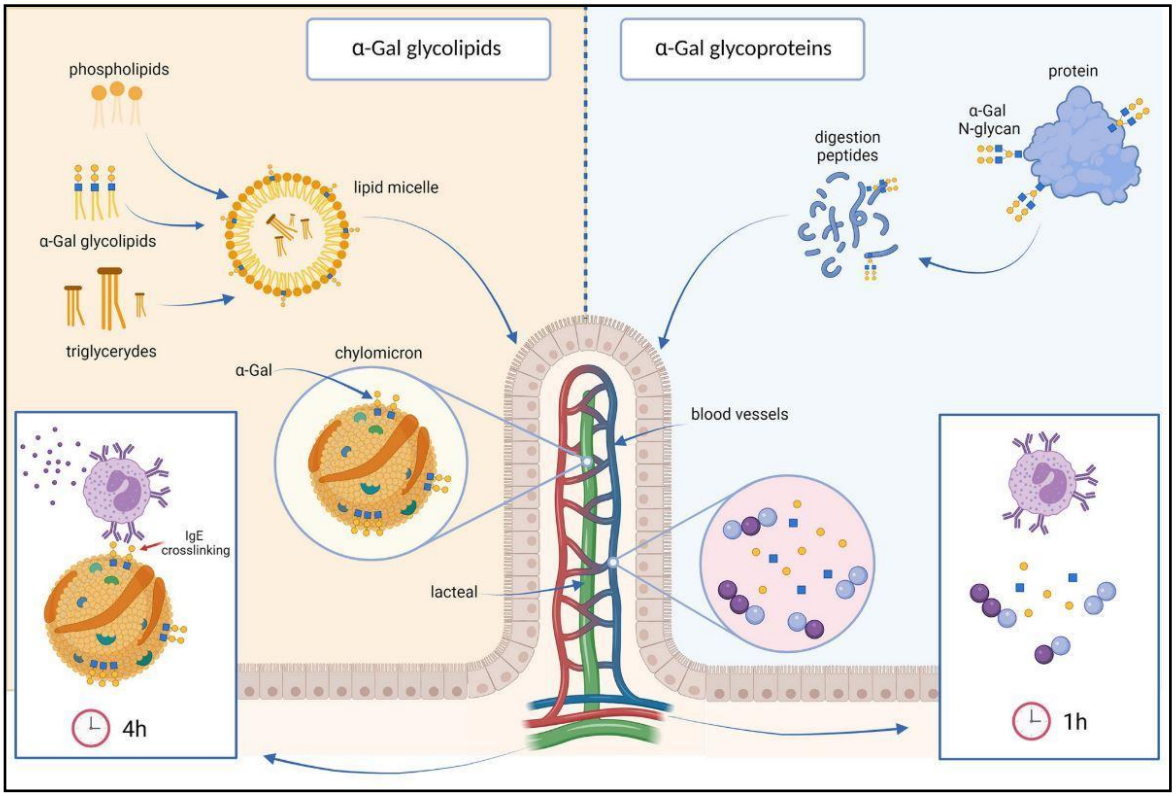


Figure 1.7: Delayed onset of reactions after the consumption of red meat. ***(on the left)*** Glycolipids carrying alpha-gal moieties are broken down into small droplets which form lipid micelles after coating with bile salts and phospholipids. These are absorbed by enterocytes where the micelles are packed to form lipoprotein particles, called chylomicrons which are transported to the lymph through the lacteal vein thus reaching the bloodstream after 3-4 hours after food consumption. ***(on the right)*** Alpha-gal carrying glycoproteins are digested into alpha-gal carrying monosaccharides which reach the bloodstream within 1-2 hours after food consumption. Hence digestion, absorption, and uptake of glycoproteins and glycolipids can explain the delay in symptoms in the development of alpha-gal allergy. Adopted from [249]

Alcohol consumption, physical exercise and use of non-steroidal anti-inflammatory drugs have been shown to cause early onset of AGS syndrome [247,272–274]. This shortening of AGS onset has been attributed to the ability of these cofactors to increase GI tract permeability allowing for increased allergen uptake, blood circulation, and histamine release [275–277].

As previously mentioned, in contrast to the consumption of red meat, intravenous injections of alpha-gal containing drugs results in classical type I hypersensitivity reactions with immediate onset of reactions since the allergen is directly introduced into the circulation where they can easily trigger basophil and mast cell degranulation. Hence, the variation in reaction times

exhibited by AGS patients is based on the time needed for processing, uptake, and presentation of the alpha-gal antigen.

1.4. Sensitization to alpha-gal

Exposure to food and environmental antigens is generally met with immune tolerance in most individuals. Intake of food or medication containing alpha-gal determinants does not cause clinical allergic reactivity in most individuals. However, a break in immune tolerance to food antigens with alpha-gal determinants induces the production of anti-alpha-gal IgE antibodies which is associated with anaphylactic responses to red meat.

1.4.1. Generation of anti-alpha-gal IgE

Two pathways have been suggested for the generation of IgE B plasma cells by the use of IgE reporter mice. Firstly, IgE-producing plasma cells and IgE memory B cells can be generated directly from IgM naïve B cells in the germinal centres [278]. Secondly, IgE plasma cells can arise from indirect isotype class switching whereby germinal centre-derived IgG1 B cells and IgG1 memory B cells undergo somatic hypermutation after antigen exposure. This leads to antibody class switching into IgE B plasma cells and IgE memory B cells [279,280]. Somatic hypermutation produces IgE antibodies with a high antigen affinity due to the positive selection of IgE memory and plasma B cells.

Human studies suggest sensitization to induce the production of allergen-specific memory B cells which triggers the proliferation of allergen-specific IgE upon antigen re-exposure [281]. Since serum IgE is short-lived. Restimulation of allergen-specific memory B cells replenishes serum IgE levels. However, these cells are found in very small quantities among healthy, atopic, and food-allergic donors [282,283] thus IgE replenishment through memory B cells of a non-IgE isotype (IgM+ or IgG+) [281] seems more viable as their levels in both healthy and allergic donors are much higher [284,285]. IgE production has also been suggested to be driven by long-lived IgE plasma cells [286] which reside in the bone marrow. Clinical studies show the transfer of peanut allergy to donors from recipients after a bone marrow transplant [287–289].

Cox et al. (2019) identified four main B cell subsets in the blood of alpha-gal allergic individuals [290]. Unlike isotype class-switched memory B cells which express IgG and are CD27 high [291,292], their studies showed B cell subsets with shared higher IgD and lower IgM expression with CXCR4, CCR6 and CD25 expression [290]. Interestingly, in-vitro stimulation of these cells resulted in isotype class switching and the production of alpha-gal specific IgE in alpha-gal allergic patients and not in healthy controls [290]. This corresponds with studies which describe

CCR6 expressing B cells to be functionally mature and capable to respond to antigen challenge [293]. Such expression is restricted to the bone marrow, umbilical cord blood, and peripheral blood-derived naive and/or memory B cells but is absent from germinal centres (GC) [293]. CD25 expression on B cells of alpha-gal allergic patients indicates a memory B cell phenotype with antigen-presenting capacity [294]. However, the low CD27 expression exhibited in this subset suggests an association with previously described **a)** memory B cells in human tissue and near epithelial surfaces [295,296] or **b)** peripheral blood memory B cells that express IgM with or without IgD, or express IgD only [291,297–300]. These differentiate into IgE-secreting cells through somatically mutated IgV genes. Therefore, anti-alpha-gal IgE antibodies have a high affinity and require a Th2 environment for their production.

1.4.2. Risk of alpha-gal sensitization

The risk of developing AGS has been variably associated with atopy (a genetic predisposition to produce exaggerated amounts of IgE). Studies by Fischer et al. (2017) show a correlation between an increase in alpha-gal IgE levels and total IgE while Gonzalez-Quintela et al. (2014) show this correlation to be true if alpha-gal IgE is driven by tick bites [272,301]. However, Commins et al. (2013) found no correlation between AGS and atopy [302]. This corresponds with other studies which show alpha-gal allergic responses in individuals with no history of atopy [251,303].

Alpha-gal allergic reactions were initially identified during a clinical trial for the treatment of advanced bowel, head, and neck cancer. The trial involved the use of a mouse-human chimeric monoclonal antibody called cetuximab [304,305]. During the trial, patients located in the South-Eastern part of the USA had a prevalence of 22% severe hypersensitive reactions characterized by anaphylaxis, while the rest of the country only showed mild hypersensitive reactions in 3% of the patients [306]. Analysis of pre-trial collected serum from the affected patients showed the presence of pre-existing IgE antibodies to alpha-gal, present on the murine portion of cetuximab [256,307,308]. Simultaneously there had been a rise in the number of cases of individuals in the same region having delayed hypersensitivity reactions after the consumption of red meat which was also associated with an increase in alpha-gal IgE [202]. Both these groups were restricted to a region with a high prevalence of the lone star tick, *Amblyomma americanum*. Development of hypersensitivity reactions in three of the AGS investigators after bites from this tick resulted in an awareness of the association between exposure to ticks and the development of AGS. van Nunen et al. (2009) also proposed a link between ticks and AGS in her Australian cohort as all affected patients reported a history of tick bites [309] while Kim et al. (2020) demonstrated a reduction of alpha-gal IgE after tick avoidance [310].

1.4.2. The role of ticks in alpha-gal sensitization

1.4.2.1 Allergic sensitization via the skin

The skin is the largest organ of the human body which acts as the first line of defence against invading pathogens. The involvement of the skin in allergic sensitization has been proposed through the dual exposure hypothesis **(Figure 1.8)**. This argues cutaneous exposure to low doses of food predisposes to allergic sensitization while ingestion of the food at high doses leads to the induction of oral tolerance [311]. Children with atopic dermatitis (AD) show high levels of sensitization to food antigens and then clinical reactivity to foods on the first oral exposure [254,312,313]. AD is a condition in which the skin of affected individuals is dry, itchy, and inflamed. Disruption of the skin barrier induces penetration of allergens and irritants and aberrant immune function, in part due to systemic IL-33 secretion which causes expansion of mast cells via the ST2 receptor [46,314]. The inflammation resulting from this disruption and allergen penetration facilitates local sensitization to food allergens and may result in symptoms of food allergy [46,315]. Martin et al. (2015) showed a 50% rate of food challenge-proven allergy to egg, peanut, or sesame seed allergy in children with severe AD by 12 months of age [316]. Infants between 4-11 months of age with a higher scoring of AD associate with an increase in dose-dependent food sensitization [254]. As such, early treatment of AD may decrease the chances of food sensitization [317].

Epicutaneous exposure to food allergens creates a pro-allergic Th2 environment, driven by a Th2 IL-4 secretion which induces high levels of allergen-specific IgE-secreting B cells [318–320]. Upon challenge, this drives systemic food allergic reactions through a mechanism of IgE-dependent intestinal mast cell expansion [321]. Using a mouse model, Dunkin et al. (2011) showed an adjuvant-dependent sensitization to cow's milk allergen (α-lactalbumin) through the skin which induces the proliferation and migration of dermal DCs to draining lymph nodes creating a pro-allergic Th2 response [322]. Application of peanuts together with milk proteins onto intact skin also results in sensitization and allergic response upon challenge to both milk and peanut allergens [323]. Therefore, these studies demonstrate the possibility of mechanical disruption and/or inflammation of the skin to result in sensitization to food antigens.

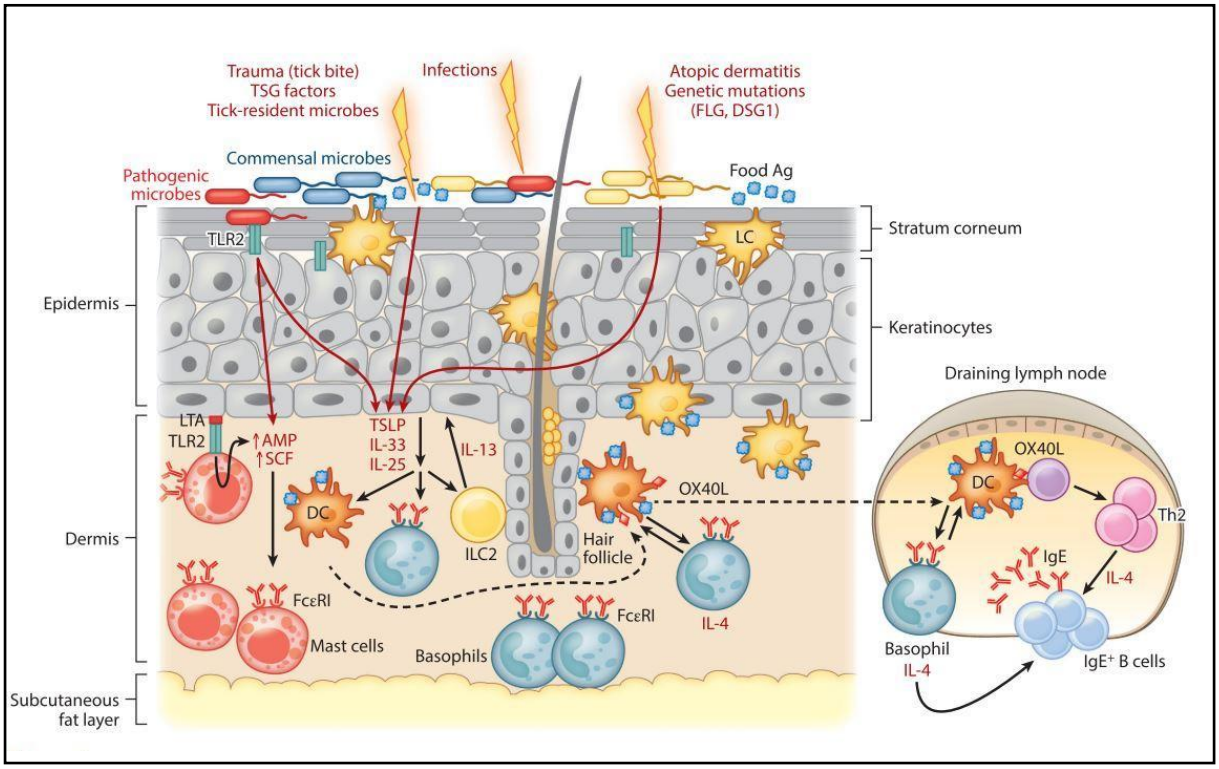


Figure 1.8: Skin barrier disruption causes allergic sensitization to food. Skin epithelium defects through trauma, commensal microbiome dysfunction, atopic dermatitis and genetic defects trigger the release of alarmins (IL-33, IL-25 and TLSP). Upon activation via pathogen recognition receptors, keratinocytes induce secretion of SCF and TLSP. The presence of pro-inflammatory cytokines activates DCs resulting in their expression of the costimulatory ligand, OX40L. This induces naïve T-cell differentiation into Th2 cells which induces B cells isotype class switching in an IL-4 mediated manner. Alarmins also promote Th2 responses through the activation of ILC2 and inducing IL-4 production by basophils. Following the binding of allergen-specific IgE antibodies to FcεRI receptors on mast cells and basophils, allergic sensitization occurs. Adapted from [324]

1.4.2.2 Immune response to tick bites

Ticks are ectoparasites which feed by disrupting the host skin in order to draw blood. Injury to the skin through intrusion by the tick's mouthparts initiates homeostatic defences by the host such as blood coagulation, platelet aggregation, and vasoconstriction [325–327]. Upon encountering tick antigens, host keratinocytes, endothelial cells, and skin resident leukocytes are activated to release antimicrobial peptides, pro-inflammatory cytokines, and chemokines such as IL-8, IL-1b and TNF-α resulting in the recruitment of neutrophils and other inflammatory cells [328]. This activates the adaptive immune system leading to the proliferation of memory T- and B-cells which produce cytokines and/or antibodies to fight against the tick

antigens during a secondary exposure [325]. Studies indicate an increase in basophil infiltration, histamine release, epidermis thickening, and hyperplasia to restrict tick attachment and feeding during second exposure [329]. This hindrance of secondary tick attachment is called acquired tick resistance (ATR) and is considered a form of allergic klendusity (generation of a hypersensitive reaction which allows the host to avoid disease or pathogen transmission through tick bites) in humans [231,330].

To repair tissue injury and promote wound healing at the site of tick attachment, a subtype of macrophages called M2 macrophages migrate to the skin [331]. These suppress inflammation by secreting IL-10 or TGF-β and dampen down a Th1 response which may be detrimental to the host. This skews the immune system towards a Th2 phenotype by upregulation of Th2 cytokines IL-10 and IL-4 together with TGF-β as seen in mice following tick bites [332]. Since tick bites promote a Th2 phenotype, this can induce the production of IgE antibodies which leads to alpha-gal sensitization.

As hematophagous parasites, ticks secrete salivary immunomodulatory molecules to ensure successful feeding. These compounds promote vasodilation, inhibit platelet aggregation and coagulation, reduce pain, and itch responses and essentially reduce host inflammation, by inducing the production of anti-inflammatory cytokines TGF-β or IL-10 [332–335] which skews the host to a Th2 phenotype. Ticks' saliva contains prostaglandin E2 (PGE2), which induces vasodilation, reduces inflammation and impairs wound healing (by restricting fibroblast migration to the site of injury) while increasing macrophage migration which further secretes anti-inflammatory cytokines at the site of injury thus in turn creating a Th2 environment [336,337]. Interestingly, Gao et al. (2016) demonstrated the ability of PGE2 to induce B cell class switching to IgE [338].

As previously mentioned, IgE responses have been described as having evolved in response to toxins and venoms (toxin hypothesis) [17]. Ticks' saliva contains toxins [339,340] which cause anaphylactic reactions [341,342]. Hence Cabezas-Cruz et al. (2017) proposed sensitization to alpha-gal to occur either by **a)** a Th2 anti-inflammatory environment cascade which induces presentation of alpha-gal expressed in tick saliva by APCs resulting in the production of anti-alpha-gal IgE by B cells **(Figure 1.9)** or **b)** a PGE2 isotype class switching of pre-existing anti-alpha-gal IgG and IgM B cell clones to anti-alpha-gal IgE secreting plasma B cells [218,338].

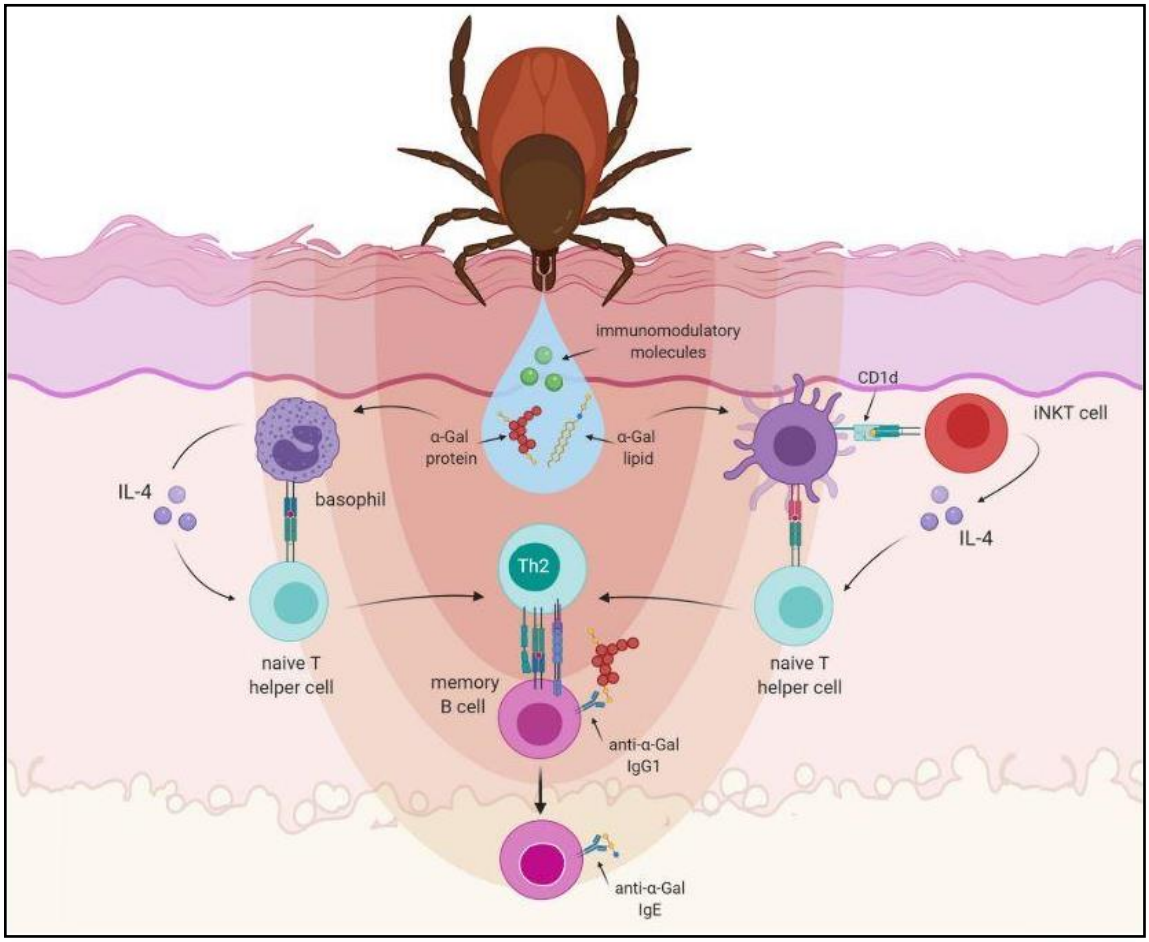


Figure 1.9: Alpha-gal sensitization through tick bites. Alpha-gal glycosylation of tick salivary glycoproteins and glycolipids triggers IL-4 production by basophils and iNKT cells (via CD1d signalling). Presentation of tick alpha-gal glycosylated proteins by DCs and basophils in an IL-4 rick environment promotes naïve T-cell differentiation into Th2 cells. These trigger antibody class switching resulting in anti-alpha-gal IgE memory B cells which release anti-alpha-gal IgE thus causing alpha-gal sensitization. Adapted from [249]

1.4.2.3 The link between tick bites and alpha-gal sensitization

Alpha-gal glycoproteins have been identified in the gut [234,235], saliva [236], salivary glands [233,234,343], ovaries [238], and cement [237] of several tick species. In adult post-fed ticks, this may be a consequence of residual alpha-gal found in a blood meal which is recycled and incorporated into the tick proteins in order to facilitate tick physiological processes and efficient feeding. In support of this are studies that show an increase in alpha-gal expression by ticks during the feeding process [234,344]. However, studies have identified tick galactosyltransferase genes b4galt7, a4galt-1 and a4galt-2 which suggest endogenous production of alpha-gal by

ticks [238]. These tick galactosyltransferase homologs increase their expression during feeding and silencing them has been shown to impair tick growth and reproduction [238].

Expression of alpha-gal on tick-borne pathogens, *Anaplasma phagocytophilum* and *Borrelia burgdorferi* [220] may act as an alpha-gal source for ticks. This can potentially drive an anti-alpha-gal IgE response when inoculated into a host, however, this is yet to be investigated. Interestingly, exposure of alpha-gal knockout mice to pathogen-free *A. americanum* proteins by subcutaneous injection demonstrated the development of systemic hypersensitive reactions upon challenge with alpha-gal-rich beef thyroglobulin [345]. This was characterized by an increase in IgE titers, basophil histamine release and inflammation [345]. Subcutaneous injection of *A. sculptum* tick saliva in alpha-gal knockout mice also induced anti-alpha-gal IgE production [236]. To mimic the human host environment which expresses alpha-gal IgM at steady state, the use of a zebrafish model has been suggested. Contreras et al. (2020) demonstrated the initiation of an allergic reaction in zebrafish only after exposure to tick saliva and these were associated with tissue-specific toll-like receptor-mediated responses and a possible role of basophils in response to tick saliva [230]. These studies implicate ticks as a driver for alpha-gal sensitization in individuals with AGS. However, exposure to helminths has not been ruled out as a possible source of alpha-gal sensitization.

1.4.3 The role of helminths in alpha-gal sensitization

1.4.3.1 Allergic sensitization via the oral-mucosal and gastrointestinal lining

Food allergic sensitization and elicitation is associated with defects in GI tract permeability [168,346–351] **(Figure 1.10)**. The GI tract is vital not only for the uptake of nutrients but also for the provision of a barrier against invading pathogens [352]. Antigen translocation may occur via microfold cell-mediated transcytosis [353,354] goblet cell antigen passages (GAPs) [35], transepithelial dendrites [82] and paracellular leak [355]. These pathways may differ in their ability to promote tolerance or result in a lack of tolerance. In particular, dysregulation of antigen translocation via GAPs has been reported to break oral tolerance and promote inflammatory T-cell responses against food antigens [356].

Stress to the GI tract triggers the activation of DCs via the release of alarmins (IL-25, IL-33 and TSLP). In their activated state DCs cause naïve T-cells to differentiate into Th2 cells which produce high levels of IL-4. Mice with elevated IL-4α signalling through a gain of function mutation (IL4rαF709 mice) promote allergen-specific Th2 and IgE responses after oral food exposure in the absence of an adjuvant [357,358]. Elevated IL-4 levels induce the reprogramming of T-regs to Th2 cells [144], enhance the proliferation of IL-13 producing ILC2s [144,359] and activates mast cells to induce an IgE-mediated response after food allergen exposure [125].

Noah et al. (2019) demonstrated IL-13 mediated formation of secretory epithelial cell antigen passages (SAPs) through a STAT6-independent and CD38-cADPR–sensitive pathway which requires IL-4α expression in the intestinal epithelium [360]. Housing WT BALB/c mice under thermal neutral conditions (26-30°C) alleviates the dampening of the intestinal immune environment by stress hormones [361,362]. This results in oral sensitization of WT BALB/c mice to egg antigen and high levels of allergen-specific IgE and IgG1 responses just as in IL4rαF709 mice which is attributed to the altered epithelial landscape from GAPs to SAPs in WT BALB/c mice at thermal neutrality [363]. Hence a Th2 environment promotes the altering of the antigen passage landscape of the GI tract from GAPs to SAPs which allows food antigens to cross the epithelial barrier and cause allergic sensitization.

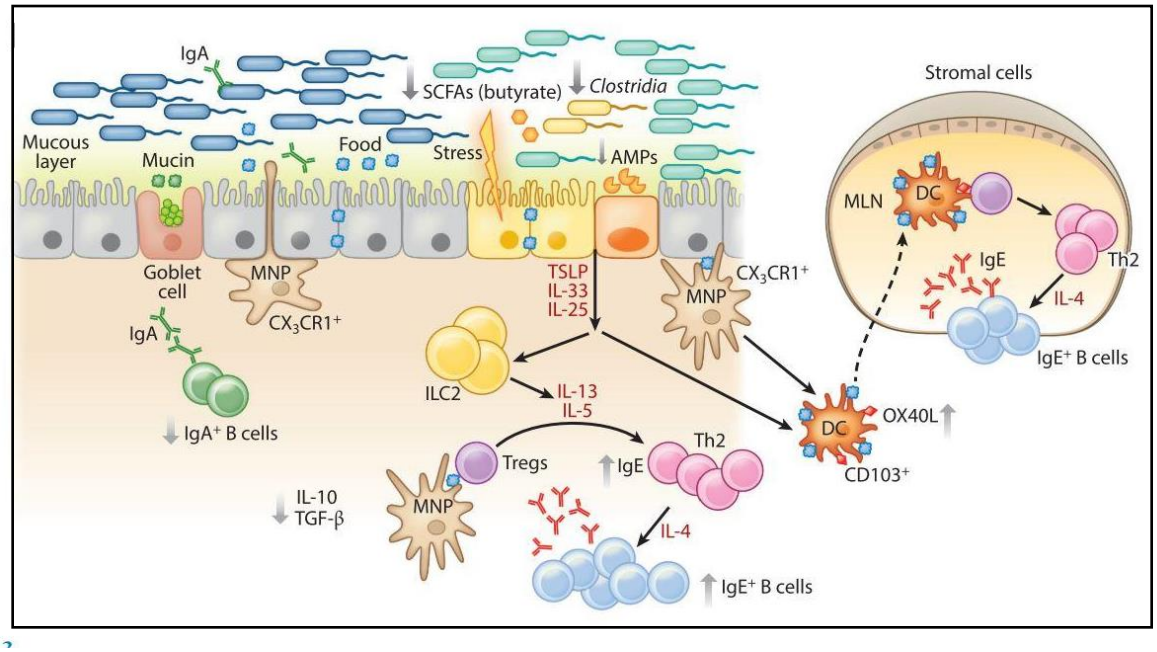


Figure 1.10: Gastrointestinal barrier disruption causes allergic sensitization to food. Microbiome dysregulation, decrease in short-chain fatty acids and trauma to the GI tract induces stress (*shown with yellow arrow*) and increased epithelial permeability of the GI tract. This triggers the release of alarmins which induce upregulation of OX40L on CD103+ DCs migrating to the mesenteric lymph nodes after uptake of food antigens. In the MLN, DCs cause naïve T cell differentiation into Th2 cells. These secrete IL-4 which triggers allergen-specific IgE production by B cells that causes allergic sensitization. Adapted from [324,364]

1.4.3.2 Immune response to helminth infections

The hallmark of immunity against helminths is the development of a robust Th2 immune response **(Figure 1.11)**. Helminth infections involve disruption of epithelial barriers (GI tract, lungs, and skin) which may result in increased epithelial permeability [365–368]. Studies show

increased jejunal permeability after *Trichenella spiralis* infection due to decreased expression of occludin in the tight junction during infection [367] while Hyoh et al. (1999) demonstrate a loss of epithelial cell adhesion as a consequence of an altered E-cadherin expression after *Nippostrongylus brasiliensis* infection [366]. *Heligmosomoides polygyrus* infection also increases epithelial permeability through altered E-cadherin expression of colonic epithelial cells via a STAT6-dependent manner [365]. Consequently, a co-infection of *H. polygyrus* and *Citrobacter rodentium* (an enteric bacteria whose infection is restricted to the colon) results in the translocation and infiltration of the bacteria into the mesenteric lymph nodes and the spleen with severe pathology indicating the ability of helminth infection to facilitate antigen translocation across the mucosa [369].

Disruption of epithelia triggers the release of alarmins IL-33, IL-25 and TLSP [370–373]. This induces the activation of ILC2s which release type 2 cytokines IL-5, IL-9 and IL-13 that play a role in the recruitment and activation of alternatively activated macrophages (AAM) [152]. IL-33 has the capacity to recruit and cause DCs to migrate to the injured epithelial barrier [374]. Presentation of helminth antigens by DCs induces naïve T-cells to differentiate into Th2 cells [375–378] due to the upregulation of the costimulatory molecule OX40 ligand on DCs by TLSP [379,380]. Activated Th2 cells release cytokines IL-4, IL-13, IL-9, and IL-5 which further aid in worm expulsion. An increase of mucus production by IL-13 activated goblet cells, traps and immobilises helminths, stopping them from causing more tissue damage [152,381]. Smooth muscle contraction and changes in GI tract permeability by IL-4 and IL-13, initiates a 'weep and sweep' response which promotes clearance of the helminths [368,382–385]. Additionally, IL-4 and IL-13 from Th2 cells activate AAMs which migrate to the source of injury where they regulate inflammation, initiate tissue repair, and provide resistance to secondary exposure [386–390]. Studies show their ability to downregulate TGF-β (a Th1 cytokine) in *Litomosoides sigmodontis* infection [386] and enhance survival after Schistosomiasis infection in a mouse model [387].

Helminths are extracellular and often too big to be phagocytosed. Parasite killing can be initiated by granulocytes. IL-5 production by Th2 cells induces activation of eosinophils [391,392] which migrate to the site of infection via chemokine receptor 3 [393] binding by eotaxin. Upon arrival, eosinophils release their granules containing major basic protein, eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxidase which are detrimental to the parasite [394,395]. If left unchecked, an exaggerated eosinophil response leads to inflammation and tissue damage [396]. Eosinophils also produce IL-4 and IL-13 which regulate Th2 responses [397,398]. Therefore, this Th2 environment with high levels of IL-4 production may result in allergic sensitization through transferring antigen translocation from GAPs to SAPs.

As previously mentioned, the development of IgE has been associated with a protective immune response towards helminths. During helminth infection B cell isotype class switching is initiated via an IL-4/STAT6 signalling pathway [382,399–403]. Parasite-specific IgE opsonises parasites and triggers degranulation of eosinophils, basophils, and mast cells after crosslinking via the IgE receptor on their surface [389,404,405]. This process is called antibody-dependent cell-mediated cytotoxicity (ADCC). Studies by Matsumoto et al. (2013) have demonstrated the need for IgG and IgE responses for the expulsion of *S. venezuelensis*, but not *N. brasiliensis* in the presence of mast cells [402]. Additionally, basophils ensure protective immunity upon secondary helminth infection [406] and are major producers of IL-4 in primary infection [407]. Therefore, the involvement of effector cells in helminth immune responses and IgE production creates a unique immune environment which allows for allergic sensitization to occur.

However, chronic helminth infections have also been shown to induce a T regulatory response in both murine [408–412] and human models [413–416]. Excretory-secretory products from helminths are capable of suppressing the host immune system through molecular mimicry and induction of IL-10 and TGF-β secretion [417–422]. *H. polygyrus* secretes a TGF-β mimic which potentially induces T-reg function and suppresses skin allograft rejection [422]. *H. polygyrus* survival is enhanced by the secretion of Alarmin Release Inhibitor (HpARI) which binds IL-33 and abrogates the activation and recruitment of ILC2s [423]. *Ascaris suum* releases an immunosuppressive protein named protein 1 (PAS-1) which suppresses LPS-induced leukocyte migration and pro-inflammatory cytokine production and stimulates IL-10 secretion [424]. Helminth immune-regulation can also occur through helminth-bacteria interactions [425–427]. Lisa et al. (2014) demonstrated the ability of experimental infection with *Lactobacillus* species to promote *H. polygyrus* infection in a T-reg environment [427].

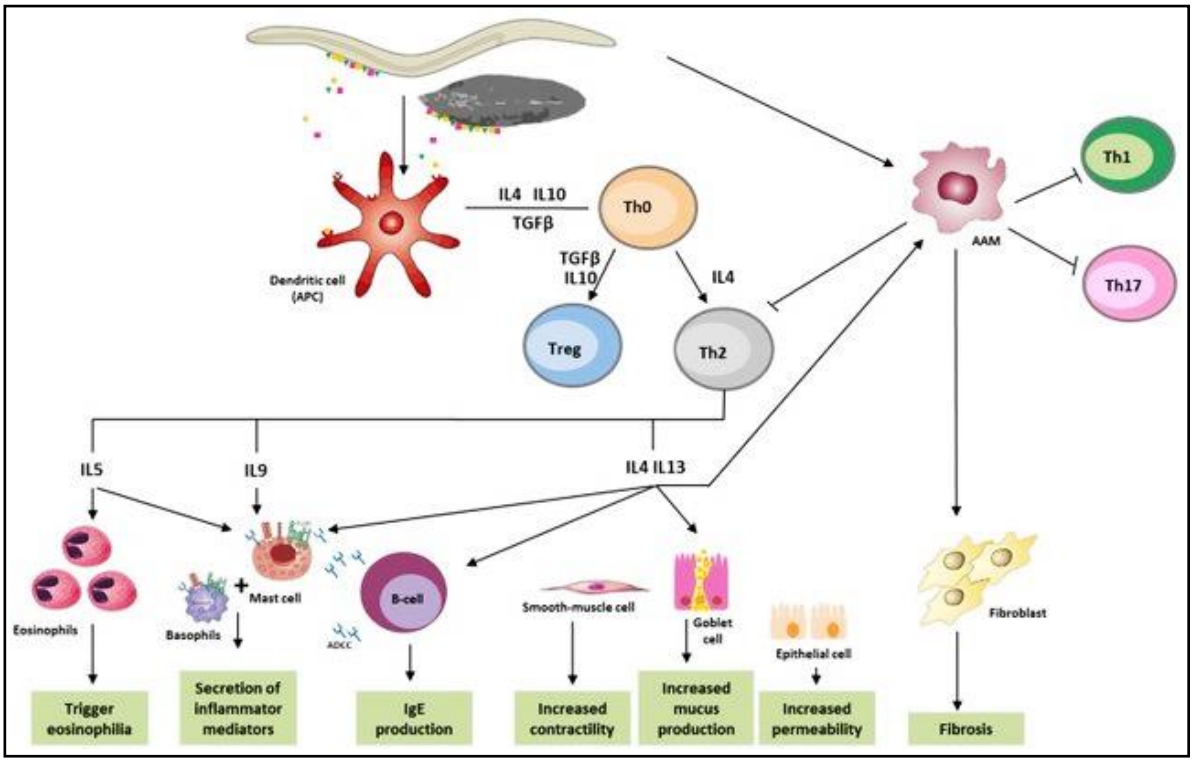


Figure 1.11: An overview of an immune response against helminths. Antigen presentation of helminth antigens by DCs induces naïve T cell differentiation into Th2 cells. These produce cytokines which trigger eosinophilia and IgE production by B cells which facilitate antibody-dependent cellular cytotoxicity by mast cells and basophils. Th2 cytokines also initiate a weep-and-sweep response for helminth expulsion via increasing intestinal permeability, upregulation of mucus production by goblet cells, and increased smooth muscle contraction. Epithelial damage during helminth invasion and migration also activates alternatively activated macrophages to facilitate tissue repair. Adapted from [428]

1.4.3.3 The relationship between helminth infection and allergy

Geohelminth infections include the roundworm *Ascaris lumbricoides*, the whipworm *Trichuris trichiura* and hookworms namely *Necator americanus* and *Ancylostoma duodenale*. According to the World Health Organisation 2023 report [429], geohelminths infect 24% of the world's population which amounts to 1.5 billion people. These infections happen early in most individuals' lives and can last a lifetime in endemic regions [430,431]. Acute infections are also prevalent among expatriates, migratory populations and through occupational exposure [432–438]. Immune responses towards a helminth infection may either be beneficial or detrimental depending on the type of helminth, intensity of infection, and duration of the infection **(Figure 1.12)** [439].

Helminth can confer protective immune regulation through mast cell saturation, and the production of IgG4 blocking antibodies. Helminth E/S antigens are highly immunogenic [440–442] and trigger the production of high levels of polyclonal IgE [430,431,443]. The saturation of non-specific polyclonal IgE on basophils and mast cells FcεRI and FcεRII inhibits binding and crosslinking of allergen-specific IgE [444–447]. Continual induction of polyclonal IgE secretion can also be exhaustive and hinder the production of allergen-specific IgE [448]. Alternatively, the triggering of Th2 responses by schistosome egg SmTAL2 proteins daily upon secretion and embedding in host tissues in small doses may have a desensitization effect that resembles allergen-specific immunotherapy (SIT) [22,449].

Chronic helminth exposure is associated with the development of IgG4 antibodies [450–453]. Studies demonstrate the ability of IgG4 to block the activity of anti-filarial IgE among children in endemic regions thus causing immunosuppression [451]. Tissue invasive helminths induce the production of immunosuppressive IL-10 and TGF-β which in turn suppress allergic sensitization [454–456]. An inverse relationship between asthma and hookworm infection has been reported, [457–459] for example a study on children in East Germany showed worm infections mainly *Ascaris,* and *Oxyuris* spp to have a negative association with eczema and allergic sensitization [460]. Additionally, moderate and severe geohelminth sensitization reduces the risk of polysensitization to HDM, *Bermuda* grass, *Blomia tropicalis*, and cockroach allergens while light infections had non-significant results [461]. Of course, there is a possibility that reverse causation may be responsible for these associations due to people with high allergic potential being able to more effectively control parasite infestation [462].

Helminth regulation of the host microbiome may affect food allergic sensitisation [463]. Microbiome dysbiosis is associated with a rise in food allergies [324,464] and can be caused by adopting a high-fat diet, low-fibre, highly processed food, and high use of antibiotics [465]. In a study conducted in mice by Harris and colleagues, it was observed that helminth infection caused a change in the bacterial microbiota and an increase in the concentration of short-chain fatty acids (SCFAs) [463]. This increase in SCFAs led to a reduction in allergic asthma through the activation of G-protein-coupled receptors (GPR41). The increase in intestinal SCFA concentrations was consistent across various host and parasite species.

However, a helminth immune response may contribute to the development and exacerbation of an allergic response. Studies by Kennedy et al. (1986) suggest the primary immune response against helminths to be directed towards the infective L2 and lung invasive L3/4 stage [440]. In these larval stages, helminths induce a strong Th2 response characterized by high eosinophilia and allergic inflammation in the lungs. An example of such is the development of a type I hypersensitivity reaction characterized by migratory pulmonary

infiltrates and peripheral eosinophilia called Loeffler's syndrome [466,467]. Studies by Jogi et al. (2022) demonstrated *A. lumbricoides* infection to be associated with a decreased lung capacity and higher odds for asthma development in males [468]. Other studies demonstrate an association between anti-Ascaris IgE [469–473] and active *A. lumbricoides* infection [474–477] with the risk of developing and the exhibition of asthma symptoms. A study in China reported a higher prevalence of allergies among children with active *A. lumbricoides* infections (determined by the presence of *A. lumbricoides* eggs in stool) in comparison to non-affected children [448]. Interestingly, anti-helminthic treatment decreased total IgE levels and abrogated allergic reactivity to *Dermatophagoides* in Venezuela and Gabon [444,478,479]. Conversely, a study involving 1632 children from Ecuador showed anthelmintic treatment for a year (albendazole administration every 2 months) to have no effect on both allergic reactions and allergen skin test reactivity [480].

Pre-conception helminth infection has been shown to influence the offspring immune phenotype [481,482]. Elevated umbilical cord blood IgE (≥0.90kUl) was described as a positive predictor for the development of atopic disease in 80.2% of 1,651 children before the age of six years [483]. Cooper et al (2018) reported maternal geohelminth infection to increase the risk of wheeze in offspring by an odds ratio of 1.41 [484]. However, geohelminth infection of children under the age of 3 years was associated with a decreased risk of wheeze and asthma [484]. Interestingly, a study in Uganda demonstrated a lower risk for the development of eczema in infants born from mothers with active helminth infection at the time of delivery. Maternal albendazole treatment was associated with an increased risk for infantile eczema development in comparison to mothers given a placebo, however, this effect lacked statistical significance [485].

Hypersensitivity reactions involving the skin and GI tract together with non-specific bronchial hyperresponsiveness have been reported after *Anisakis simplex* infection which increases total IgE, plasma histamine levels and expression of mast cell protease 1 (MMCP-1) [486–492]. Elevated IgE and positive skin prick test to *Anisakis simplex* is reported in individuals with recurrent hypersensitivity reactions to fish [488,493–496]. Oral challenge with Anisakis L3 crude extract in mice after a primary and secondary *Anisakis simplex* infection resulted in a hypersensitivity reaction [486]. However, this reaction was abolished when IL-4Rα-/- mice were used, indicating the necessity of IL-4 to drive the Anisakis-induced Th2 response [486]. Concerning food allergy, a study among Gabonese school going children showed an association between anti-helminth IgE and increased IgE levels against food antigens [497]. Therefore, helminth infection may play a vital role in allergic sensitization.

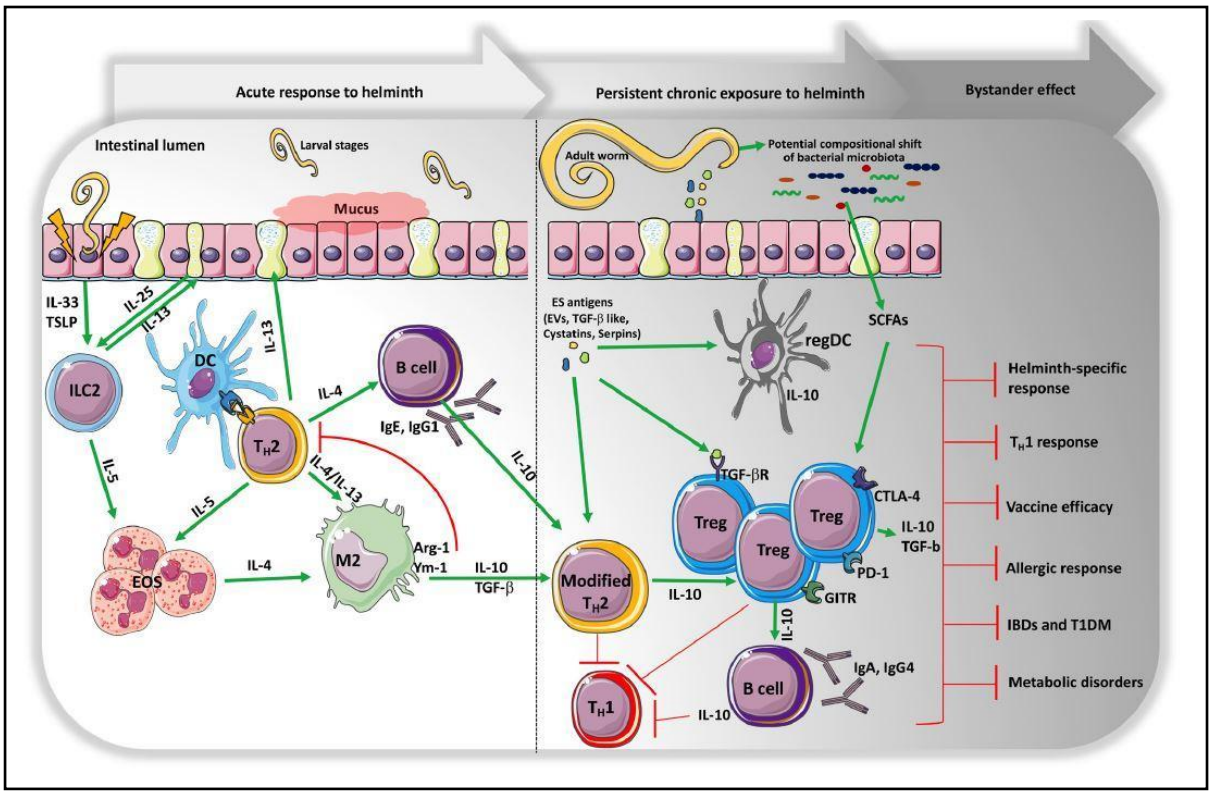


Figure 1.12: Different immune profiles shaped by the severity and duration of helminth infection. Early helminth infection is marked by an increase in epithelial cell alarmin secretion due to the trauma caused by invasion and migration through the lungs and/or intestinal mucosa. This skews the immune response to a Th2 phenotype with increased production of antigen-specific IgG1 and IgE which is capable of causing allergic sensitization. Prolonged helminth exposure leads to a modified Th2 immune response characterised by expansion of CTLA-4, and PD-1 expressing T-regs. There is also IL-10 production which induces IgG4 production by B cells. This results in the suppression of allergic responses. Adapted from [498]

1.4.3.4 The link between helminth infection and alpha-gal sensitization

Screening 5 European patients from northern Spain with alpha-gal allergy confirmed by a positive SPT test to cetuximab, beef proteins and a history of delayed anaphylaxis after the consumption of red meat showed the presence of specific-IgE for Ascaris (3/5 patients), Echinococcus (4/5 patients) and Anisakis (1/5) [499]. Commins et al. (2011) showed the prevalence of alpha-gal IgE in rural and urban Kenya to be 76% and 29% respectively [500]. In these cohorts, the Ascaris IgE was at 48% in children from rural Kenya while those from the urban regions were 17% [501]. Another cohort from Ecuador with a geohelminth prevalence of 63.4% [502] also showed the presence of alpha-gal IgE in 37% of its participants. Interestingly, Wilson et al. (2021) reported that despite sensitized and non-sensitized children having a similar frequency of *A. lumbricoides* eggs in their stool, there was a significant positive

association between Ascaris IgE, IgG, and IgG4 with alpha-gal IgE [503]. Arkestal et al (2011) also showed 85% of participants with helminth exposure to have elevated IgE to alpha-gal [504].

No studies have shown the presence of alpha-1,3 galactosyltransferases in helminths. However, immunoblotting experiments have demonstrated the presence of alpha-gal in somatic and E/S extracts from *Haemonchus contortus* [239], *Echinococcus granulosus* [505], *Schistosoma mansoni* [216], *Fasciola hepatica* [506] and *Parelaphostrongylus tenuis* [507]. Alpha-gal expression in helminths has been suggested as a form of molecular mimicry, however, this trait is selectively expressed as shown by its absence in other *Parelaphostrongylus* species namely *P. cantonensis* and *P. costaricensis* [507].

Taken together, the presence of alpha-gal in helminths and the association between helminth IgE and alpha-gal IgE suggests a role of helminth exposure in causing sensitization to alpha-gal.

1.5. STUDY RATIONALE

The causative parasitic vectors of alpha-gal sensitization in Africa are largely unknown. Studies in the USA [234,500,508], Europe [235,266,267], Asia [343] and Australia [309] have reported an association between ticks and sensitization to alpha-gal. However, not every tick species has been shown to express alpha-gal [234]. Interestingly, studies in Ecuador, Kenya and Zimbabwe demonstrate an association between helminth exposure and alpha-gal IgE [503,504]. Here we present a cohort with oral food challenge-proven alpha-gal allergy in an environment with both tick and helminth exposure. It is therefore important to determine the role of either tick or helminth exposure or both in causing sensitization to alpha-gal.

1.6. AIMS AND OBJECTIVES

The aim of this thesis is to identify the associations between parasite exposure and the development of allergic responses to alpha-gal. Firstly, the detection of alpha-gal in candidate parasitic vectors was assessed to ascertain antigen source. Co-sensitization and cross-reactivity to parasites was analysed using serum collected from individuals with and without alpha-gal allergy. The ability of different parasites to induce allergic reactions was investigated by using a luminescent IgE reporter cell line. Lastly, we probed into the identity of parasite alpha-gal glycosylated proteins with possible adjuvant capabilities resulting in sensitization to alpha-gal.

Objective 1: Demonstration of parasite and/or parasite-derived proteins as the source of alpha-gal epitopes.

Rationale: For parasitic exposure to drive a specific anti-alpha-gal IgE response, there is a need for alpha-gal to be present in parasite homogenates. We hypothesize antigen presentation of parasite antigens with alpha-gal glycosylation to induce a Th2 response characterized by the production of high-affinity anti-alpha-gal IgE.

Objective 2: Investigating the cross-reactivity between serum-from alpha-gal allergic individuals and antigens from ecto- and endoparasites.

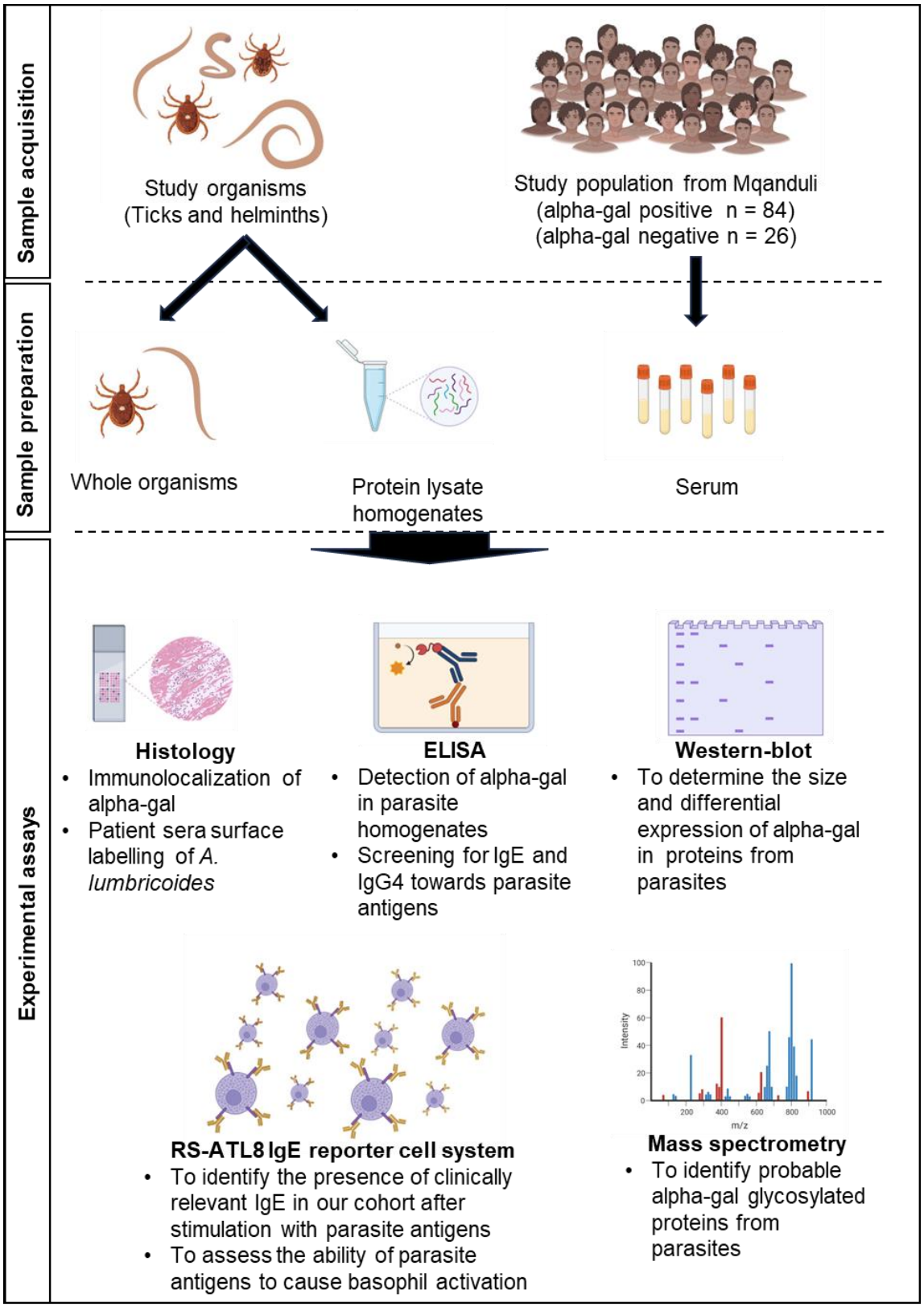
Rationale: The development of AGS is associated with exposure to either ticks or helminths. Therefore, we hypothesize patients with AGS to have antibodies directed towards parasites responsible for sensitization to alpha-gal.

Objective 3: Identification of possible alpha-gal glycosylated proteins in parasites.

Rationale: General exposure to alpha-gal glycosylated products does not elicit a type I hypersensitivity reaction. To date, AGS primary sensitization occurs after human exposure to parasites. Therefore, we hypothesize that proteins from parasites which are glycosylated with alpha-gal have adjuvant properties which induce the production of allergen-specific IgE against alpha-gal.

CHAPTER 2: METHODS AND MATERIALS

Graphical abstract



2.1. Participants

The study made use of serum samples from 114 patients between 5 years and 60 years, who underwent an oral food challenge in the Adverse reactions to mammalian meats and correlations with alpha-gal sensitisation in rural Eastern Cape South Africa clinical study. Subjects with alpha-gal allergy proven by oral food challenge were identified in Mqanduli district, Eastern Cape province, South Africa as previously described [509]. The study enrolled participants who had a history of symptoms following the consumption of red meat, as well as participants who had no adverse reactions to red meat and regularly consumed it at Zithulele Hospital in the Mqanduli district of the rural Eastern Cape. Researchers collected demographic information, assessed clinical symptoms, and recorded the participants' history of exposure to tick bites, scabies, or local parasites using questionnaires. Blood samples were taken from all participants to measure total IgE and specific IgE antibodies using ImmunoCAP testing Phadia. The antibodies were tested for alpha-gal antigen, *A lumbricoides* spp, mammalian meat extracts, cat dander, and cat serum albumin, with results reported in kilounits per liter. Sera were analysed using an Immunocap100 device (ThermoFisher Scientific), which has a lower level of detection (0.35 kU/L). Allergens used have been previously mentioned to either contain alpha-gal [243–248] and/or people with alpha-gal allergy to have IgE reactivity to them [499,503,504]. Cat serum albumin was used as a control. Upon invitation to participants with a history of adverse reactions to meat, an oral food challenge was conducted using cooked beef sausage as previously described [509]. Participants who tested positive for alpha-gal exhibited either subjective or objective symptoms. Subjective symptoms were specifically characterized as severe and persistent abdominal cramping. Objective symptoms, on the other hand, included symptoms such as vomiting, diarrhoea, scratching, hives, erythema, and angioedema, as well as more severe reactions like respiratory problems and hypoperfusion **(Figure 2.1)**. Alpha-gal negative participants were described as individuals with a negative ImmunoCap and no symptoms and/or individuals with a positive ImmunoCap and no reaction after an oral food challenge. A control group of 40 participants of similar characteristics (age and gender) with the study participants with oral food challenge confirmed alpha-gal allergy was included. These where conveniently sampled from the medically stable participants in the outpatient department of Zithulele hospital. The study was approved by the Human Research Ethics Committee of the University of Cape Town (174/2017) and informed consent, parental consent, and assent were obtained from all participants. Serum samples from participants were kept at -80^{o}C till further use.

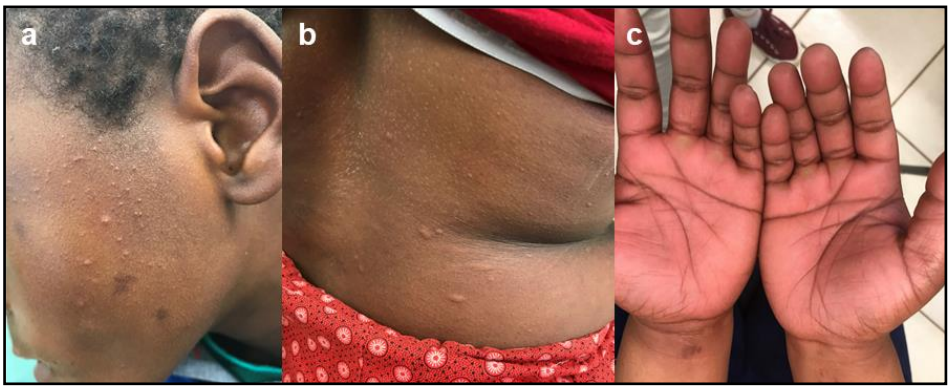


Figure 2.1: Objective symptoms after a red meat challenge. Presentation of hives on participants' **a**) face and **b)** back. **c)** reddening of the palms

2.2. Antigen acquisition and preparation

2.2.1. Ectoparasites

Ticks were collected from livestock (predominantly cows, sheep, pigs, donkeys, and mules) in Mqanduli district, Eastern Cape. Tick samples from animals were kept in different tubes for each animal respectively. Tick taxonomy was carried out by visual inspection under a stereomicroscope (Leica) and using keys appropriate for the classification of South African species [510]. Ticks were acquired from 11 indigenous cattle, 1 donkey, 3 goats, 6 sheep, 7 pigs, 10 dogs, 7 horses, and 4 mules. 6 species of hard ticks (Ixodidae) from 3 genera of *Amblyomma* (1 species: *A. hebraeum*), *Rhipicephalus* (4 species: *R. appendiculatus, R. decoloratus, R. evertsi* and *R. microplus*) and Haemaphysalis (1 species: *H. elliptica*) were identified. The largest numbers found were of *A. hebraeum* **(Figure 2.2)** and *R. evertsi*. Collected ticks were frozen down at -80^{o}C till needed.

To investigate the presence of alpha-gal at different feeding stages, the Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR) *R. evertsi* and *A. hebraeum* colony was expanded as previously described [511]. For this study, female and male ticks were grouped into three categories, that is, unfed, partially fed and fully fed (engorged). These were fed on the back of a pathogen-free South African meat merino sheep weighing 92 kg at a ratio of 1.7 ticks/kg bodyweight with defined protocols [511]. Detached fully engorged ticks were removed with a spoon from the inside of the calico bags attached to the sheep's back. To collect partially fed ticks, fine curved forceps were used to grab the ticks' mouthparts and detach them from the host. After collection, ticks were separated from excreta and other debris by using a fine sieve. Running tap water was used to wash away blood or other animal fluid exuded from feeding wounds. The ticks were left to dry thoroughly on filter paper. Ticks at different feeding and developmental stages were dissected for the collection of salivary glands and guts.

Protein extraction was done in Tris-HCl, pH 8.0 by sonification and pelleting of cell membranes and debris by centrifugation at 16 000g, prior to protein quantification and storage of the protein lysates at -80^{0}C till needed. Whole ticks at different feeding and developmental stages were also frozen down at -80^{0}C till further use.

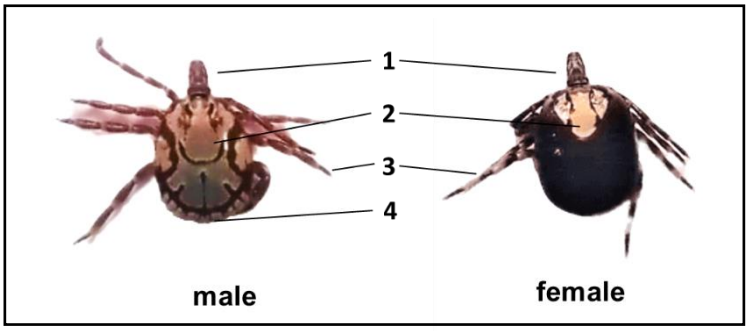


Figure 2.2: Identification of *Amblyomma hebraeum* ticks using taxonomic keys. *A hebraeum* male and female ticks were identified based on their; 1- long mouth parts, 2- banded legs, 3- ornate scutum with discrete lateral patches of colour, and 4- the uniform yellow colour of the festoons (males).

2.2.2. Endoparasites

A. lumbricoides adult worms were collected from human subjects at the Red Cross War Memorial Children's Hospital (Cape Town, South Africa) with parental consent and ethical approval (HREC 174/2017) from the University of Cape Town. Upon collection of live adult *A. lumbricoides spp*, worms were incubated with tap water for an hour **(Figure 2.3a)** to allow the expulsion of host-derived excretions. The worms were transferred to a 5-litre bucket containing 1L of DMEM with 10% Pen-Strep overnight in an incubator at 37^{0}C, this was marked as day 1. In the morning the worms were extracted from the media and washed with PBS once. Some of the worms were frozen down at -80^{o}C for use in somatic antigen preparation. The remaining worms were transferred into T175 culture flasks **(Figure 2.3b)** with DMEN containing 1% Pen-strep (ThermoFisher Scientific), 1% L-glutamine (Merck), 1% Gentamycin (ThermoFisher Scientific) and 2,22% of 45% Glucose. The day 1 media was spun at 400g for 5 minutes, and the egg pellet was transferred to a 50ml falcon tube with 0,1% formaldehyde **(Figure 2.3c)** and stored at 4^{0}C until time for embryonation. The supernatant containing the worm's E/S was transferred to 50ml falcon tubes and frozen down at -80^{o}C until *A. lumbcrioides* E/S preparation. The worm culture flasks were kept at 37^{o}C in an incubator with removal and changing of media every day for three days.

Adult *A. lumbricoides* E/S products in the extracted media were defrosted and concentrated using an Amicon (Merck) ultra-concentrator and the concentrated proteins were re-suspended

in 5mL of PBS. Another batch was differentially centrifuged at 400g, 2000g and 4000g for 30 minutes respectively before ultracentrifugation at 240 000g for 3 hours at 4ºC. The pellet formed was washed with PBS and spun down at 240 000g for 2 hours at 4ºC. The resultant pellet was resuspended in 500ul of PBS and stored at -80^{0}C until needed.

Figure 2.3: (a) Adult *A. lumbricoides* worms left in tap water for an hour to release host contaminants **(b)** *A. lumbricoides* in T-175 culture flasks with 50ml of cell culture media and left overnight in an incubator at 37ºC **(c)** *A. lumbricoides* eggs in 0.1% formaldehyde.

A. lumbricoides larval worms were obtained by embryonating eggs from adult *A. lumbricoides* previously stored at 4ºC. Embryonation was initiated by washing eggs with 0,5M of NaOH 5 times to remove the outer sticky layer, followed by another 5 washes with PBS (pH 7.0). In between the washes, the eggs were spun for 5 mins at 400g. The eggs were transferred to an Erlenmeyer flask with either PBS (pH 2.0) or 0.1% formaldehyde and left to incubate in an incubator at 30^{0}C with shaking at 100 rpm. The flask was covered with perforated parafilm to allow oxygenation of the eggs. After every seven days, the eggs were analysed to assess larval development **(Figure 2.4)**. After 32 days eggs were spun down and washed with saline 3 times followed by a 30-minute incubation with 5-6% sodium hypochlorite (NaOCl) with glass beads at 37^{0}C, 250 rpm. The disrupted eggs were spun down and washed with HBBS media before plating in a 24-well plate with KW-2 media overnight to allow all larvae to break out of their eggshells. After overnight incubation, the larvae were separated from the egg debris via a Bearman's technique. Isolated larvae were plated out onto a 24-well plate and cultured at 37^{0}C for three days until they reached the L3/4 stage upon which they were either fixed in 4% formalin or spun down and frozen at -80^{0}C until needed. Culture media with larval E/S was also spun down and concentrated using an Amicon (Merck) ultra-concentrator and the concentrated proteins were re-suspended in 5 mL of PBS.

E. granulosus cyst wall and cyst fluid were obtained from human subjects at the Red Cross War Memorial Children's Hospital (Cape Town, South Africa). All organisms were stored in sterile conditions at -80°C until used. Taenia crassiceps (L3) homogenates and excretory-

secretory products were provided as a gift from the Joseph Raimondo lab (University of Cape Town). Pork kidney procured from a commercial butchery was used as a positive control, and a boiled whole hen's egg as a negative control.

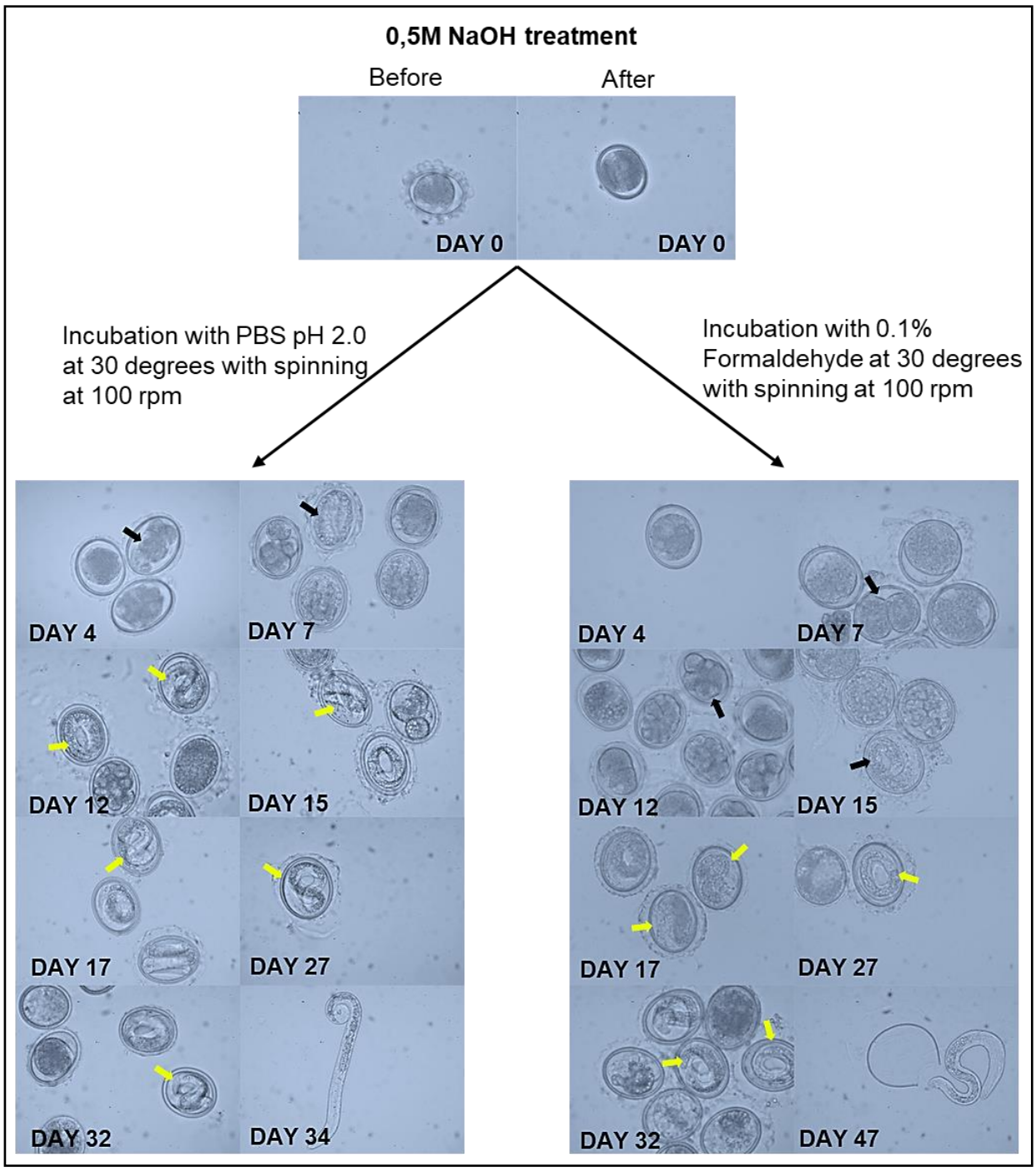


Figure 2.4: Incubation of *Ascaris lumbricoides* eggs with PBS pH 2 increases the rate of embryonation. Eggs cultured in PBS pH 2.0 showed the development of larvae from day 12 and were mature enough for hatching by day 32. However, eggs cultured with 0.1% formaldehyde showed larval formation on day 17 and could only be hatched 15 days later in comparison to the PBS pH 2.0 culture. (*black arrows show subsequent cell divisions for*

embryo formation; yellow arrows show the embryo morphing into a worm). Images were taken at X400 magnification.

2.2.3. Protein Extraction

A. lumbricoides (adult and larvae worms), *Nippostrongylus brasiliensis* (third larval stage - L3), *E granulosus* cyst wall, *A. hebraeum*, *R. evertsi*, pork kidney, and boiled egg were washed with phosphate-buffered saline (PBS), cut into small pieces, then suspended into RIPA buffer (Sigma-Aldrich) on ice for 30 minutes. The tissue was homogenized with a Wiggens handheld homogenizer (D-130), left to stand for an hour on ice and then centrifuged at 2000g for 30 mins at 4^{o}C followed by removal of the supernatant for protein quantification.

BCA protein estimation kit (Pierce, Rockford) was used to determine protein concentration of the resultant lysates and antigens which were normalised to 500ug/mL and stored at -80^{o}C before use.

2.3. Detection of alpha-gal glycoproteins

2.3.1. ELISA (Enzyme-Linked Immunosorbent Assay)

Alpha-gal presence in glycoproteins was determined by ELISA. NUNC-coated maxisorb ELISA plates were coated overnight at 4^{o}C with 50µg of glycoproteins from pork kidney, whole egg, ectoparasites, and endoparasites diluted in carbonate buffer. After blocking with 5% BSA (Bovine Albumin Fraction V mol. biol. grade, ≥98% purity, Carl Roth, Karlsruhe, Germany) for an hour at 37 degrees celsius, the plates were incubated with anti-alpha-gal chicken single chain variable fragment (scFv) antibody (1:5000 for 2 hours) which is highly specific for terminal Galα1,3Gal-R binding [512]. Biotin anti-6-his epitope tag antibody (BioLegend) in 1% BSA diluted in PBS-T (1:5000) was added to the plates followed by Streptavidin Horseradish Peroxidase (Strep-HRP) (BioLegend) incubation; with one wash step in between the antibody incubations and five washes after incubation with Strep-HRP. As validation, biotinylated Griffonia simplicifolia isolectin B4 (GSI- B4 lectin) (Merck) at a dilution of 1:5000 was added to another set of antigen-coated plates, respectively. TMB microwell peroxidase substrate system (Thermo Fisher Scientific) was used to develop the plates and the reaction stopped with 2M sulfuric acid. The plates were read at an absorbance of 450nm (Lm1) and 570nm (Lm2; background) using a VersaMax microplate reader (Molecular Devices Corporation, CA, U.S.A).

2.3.2. Western-Blot

Immunoblotting was performed using 5µg of each antigen preparation. The samples were separated on a 10% SDS-PAGE gel at a voltage of 120-150V and transferred to a 0.4µm nitrocellulose membrane (Bio-Rad) at 80V for 2 hours. Ponceau S staining was carried out to validate wet transfer. The nitrocellulose membrane blocking with 3% BSA (Bovine Albumin Fraction V mol. biol. grade, ≥98% purity, Carl Roth, Karlsruhe, Germany) in PBS at room temperature on the laboratory bench, preceded overnight incubation with anti-alpha-gal chicken scFv antibody (1:5000). Detection of binding to alpha-gal was achieved by the addition of Biotin anti-6His tag as a secondary antibody (1:5000) and Strep-HRP conjugate (1:5000) with 3-5 washes for 5 minutes of the blot with Tris-buffered saline-tween (TBS-T) in-between antibody blot incubations. GSI-B4 lectin was also added to another set of blots overnight before the detection of binding by the addition of Strep-HRP.

2.3.3. Immunohistochemistry

A. hebraeum, R evertsi, and *A lumbricoides* were preserved in 4% formaldehyde before embedding in paraffin wax blocks for staining. Cut sections 4µm were hydrated in varying concentrations of alcohol and blocked with 3% H_2O_2 for 15 minutes. 0,1M citrate buffer (pH 6.0) was used for antigen retrieval for 2 minutes in a pressure cooker. Tick sections were blocked with 1% BSA (Bovine Albumin Fraction V mol. biol. grade, ≥98% purity, Carl Roth, Karlsruhe, Germany) for optimal staining conditions. Staining for alpha-gal was done by adding anti-alpha-gal chicken scFv antibody (1:200) on whole cut sections in an overnight incubation at 4°C. Detection of binding was performed by sequentially adding Biotin anti-6-His tag as a secondary antibody (1:1000), Strep-HRP (1:400). For *A. hebraeum* and *R. evertsi* VIP substrate (Vector laboratories) was used for visible colour development to avoid false positives as in the case of using DAB substrate **(Figure 2.5).** Slides were counterstained with methylene green, dehydrated and cover slipped. *A. lumbricoides* slides were blocked with 5% BSA which was followed by a biotin block step for the removal of background staining **(Figure 2.6)**. The rest of the treatments were the same as for ticks except for treatment with DAB then counterstaining with haematoxylin to avoid false positives **(Figure 2.7)**. Pork kidney and Chicken breast protein were used as a positive and negative control respectively **(Figure 2.8)**.

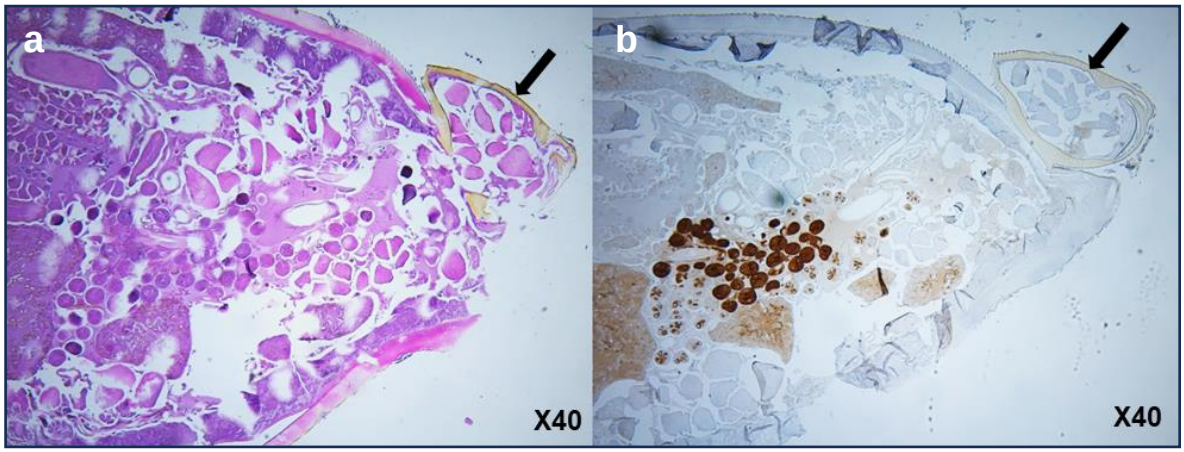


Figure 2.5: DAB staining of *Am hebraeum* whole sections may cause false positives. DAB-positive staining is brown. Tick surface structures and mouth parts maintain a brown colour regardless of **a)** H&E or **b)** alpha-gal staining with scFv chicken antibody. False positive staining is indicated by a black arrow.

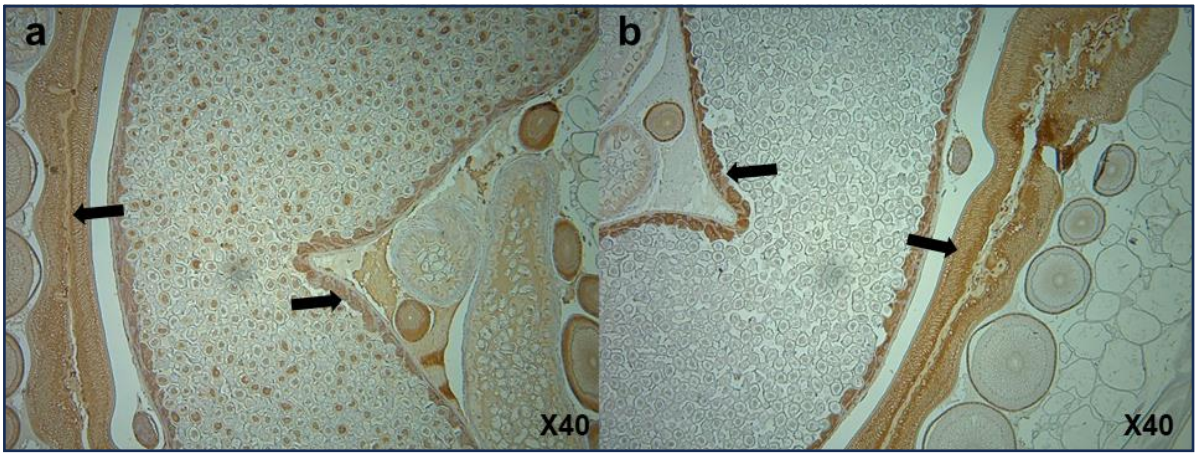


Figure 2.6: *Ascaris lumbricoides* sections contain endogenous biotin which causes high background and false positives. Anti-alpha gal staining with **(a)** anti-alpha-gal chicken scFv antibody and **(b)** secondary staining only results in high background staining in both the stained **(a)** and unstained control **(b)** as indicated with the black arrows if biotin blocking is not done.

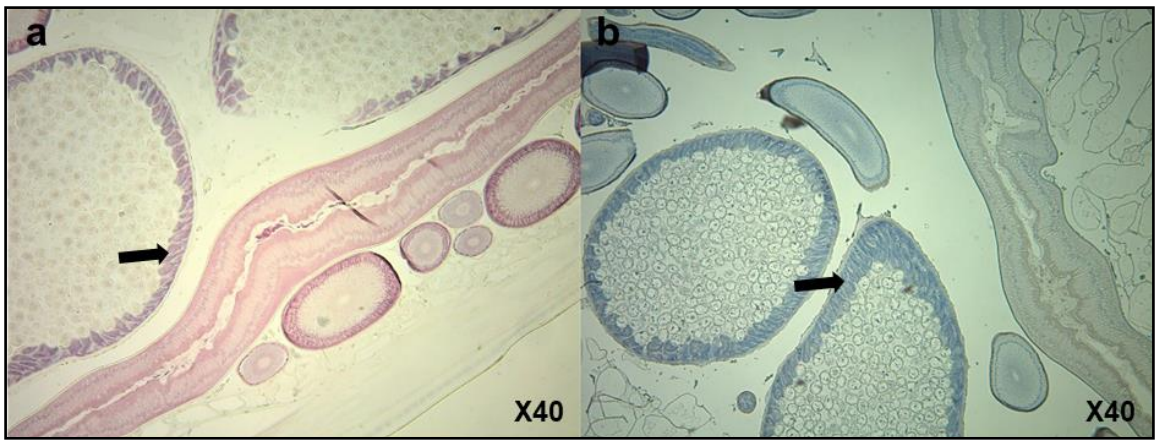


Figure 2.7: Developing *Ascaris lumbricoides* sections with VIP substrate may cause false positives. Immunolocalization of alpha-gal using **(a)** anti-alpha-gal chicken scFv antibody results in some structures of the worm staining purple. However **(b)** secondary staining only also results in a purple staining. Black arrows show endogenous purple structures in *A lumbricoides*.

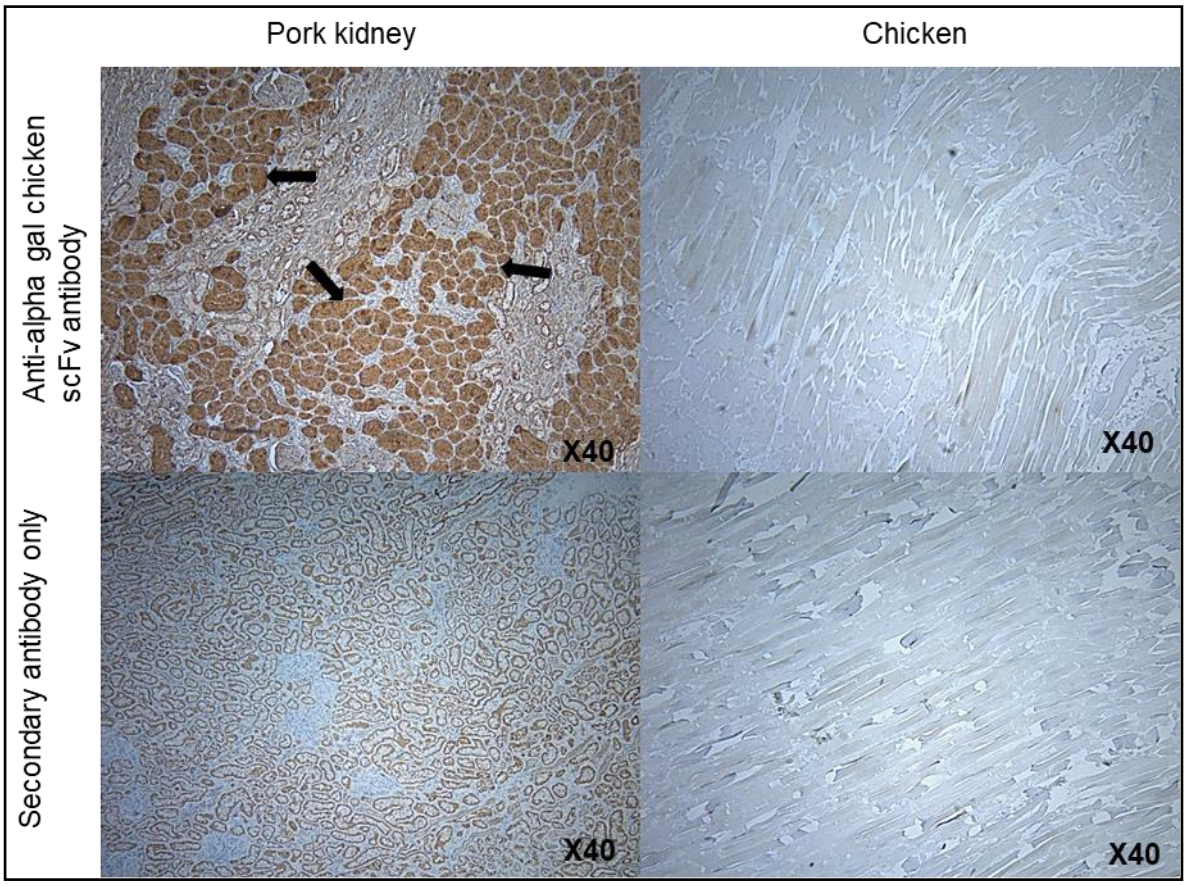


Figure 2.8: Validation of pork kidney and chicken tissue as controls for anti-alpha gal staining. Paraffin wax embedded 4µm tissue sections were incubated with anti-alpha-gal chicken scFv antibody. Binding of antibody was confirmed by staining the tissues with DAB

substrate. Brown spots on tissue showed the presence of alpha-gal in pork kidney as indicated by the black arrows.

2.4. Detection of human IgG4 and IgE antibodies

2.4.1. ELISA

IgG4 and IgE antibodies against *A. lumbricoides*, recombinant ABA-1, and *A. hebraeum* were measured using an indirect ELISA. Ninety-six-well Nunc Maxisorb plates (Thermo Scientific) were coated overnight at 4°C with 20µg/mL of either Ascaris somatic Ag, *A. hebraeum* Ag or 2ug/ml of recombinant ABA-1 antigen diluted in 50mM carbonate buffer at pH 9.6. Plates were washed three times with 200µl PBS/0.05% Tween-20. After blocking with 200µl of 5% BSA (Bovine Albumin fraction V with a purity ≥ 98%, Carl Roth, Karlsruhe, Germany) for 2 hours at 37°C plates were washed another three times and incubated with sera from participants in triplicates at a dilution of 1:10 in PBS for another 90 min at 37°C. After another three washes bound antibodies were incubated with alkaline phosphatase-conjugated mouse anti-human IgG4/IgE antibodies (SouthernBiotech Sigma-Aldrich) at a dilution of 1:1000 in PBS-T in 5% BSA at 4°C overnight. Plates were washed 3 times, followed by one final wash with PBS without Tween-20. p-Nitrophenyl Phosphate (PNPP) was used for visualization (incubated for 30 minutes at 37ºC), the reaction was stopped with 50µl of 2N NaOH and plates were read (ClarioStar Plus, BMG, Labtech, Germany) at 405 nm to determine the optical density (OD).

Inhibition of alpha-gal IgE binding to ecto- and endoparasites extracts by 2mg/mL bovine thyroglobulin (BTG; Merck) was assessed by ELISA. Briefly, F96 Maxisorp ELISA plates (ThermoFisher Scientific/Nunc Roskilde, Denmark) were coated overnight at 4°C with 50µL of 50µg/mL *A. lumbricoides, A. hebraeum,* and *R. evertsi* extract in 50 mM carbonate buffer pH 9.6. Plates were washed three times with 200µL PBS/0.05% Tween-20 (PBS-T; wash buffer) followed by blocking with 200µL of 5% BSA (Bovine Albumin Fraction V mol. biol. grade, ≥98% purity, Carl Roth, Karlsruhe, Germany) in PBS for 2 hours at 37°C. After three more washes with 200µL of wash buffer, the plates were incubated with 50µL 1:10 sera diluted in PBS in the presence or absence of 2mg/mL BTG and incubated at 37°C for a further 90 min in triplicates. After another 3 washes with 200µL wash buffer, 50µL of anti-human-IgE-HRP (mouse monoclonal [B3102E8] anti-human IgE Fc (HRP), (Abcam), diluted 1:500 in PBS-T with 5% BSA (antibody buffer) were added and incubated at 4°C until the next morning. Plates were washed 3x with 200µL wash buffer, followed by one final wash with 200µL PBS without Tween-20. Following the final washing step, 50µL of 1-Step Ultra TMB-ELISA (ThermoFisher Scientific) were added and the plates incubated for 90 min at 37°C. Reactions were stopped

by the addition of 50µL 2M sulfuric acid and absorbance was measured at 450 nm in a spectrophotometer (CLARIOstar Plus, BMG Labtech, Ortenberg, Germany).

2.4.2. Human sera labelling of endoparasites

Formalin-fixed L3-4 larvae obtained from embryonated eggs were subjected to centrifugation (Thermo Scientific Cytospin 4 Centrifuge) at 800 rpm for 5 minutes. The larvae were then deposited onto positively charged glass slides and allowed to dry for 72 hours. Next, the slides were incubated overnight at 4°C with 25µl of undiluted sera obtained from both alpha-gal positive and alpha-gal negative patients. Following this, the slides were washed three times with PBS-T and incubated for 1 hour at room temperature with 100µl of Hoechst 33342 (Invitrogen, USA) that was diluted 1:1000 in PBS. After additional washes, the slides were incubated with 50µl of Alexa-Fluor 647-conjugated anti-human IgG4 (Invitrogen, USA) diluted 1:200 in 1% BSA-PBS for 1 hour at room temperature. For visualizing IgE binding, Alexa-Fluor 647-conjugated anti-human IgE (Invitrogen, USA) was used, following the same steps. To visualize IgG, DayLight 547 (Invitrogen, USA) was utilized. The slides were fixed using moviol (Merck, Darmstadt, Germany) and anti-fade (Thermo Fisher Scientific GmbH, Germany) which was diluted 1/50. Finally, the slides were imaged using an Axioscope 7 microscope with a Colibri 5 fluorescent LED light source.

2.5. Identification of clinically relevant IgE via RS-ATL8 assay

2.5.1. Recombinant expression and purification of Ascaris allergens

Two *A. lumbricoides* antigens Asc l 1 [513] (UniProt O46207) and Asc l 3 [514] (UniProt. C0L3K2) were chosen for recombinant expression in HEK293-6E suspension cell culture system. Both coding sequences were synthetically produced by GeneArt (ThermoFisher Scientific) with NheI/BamHI restriction sites flanking the CDS for direct cloning into pTT28 vector (National Research Council - NRC Canada, NRC patent file 11266) for extracellular recombinant expression of C-terminal His-tagged proteins. Codons were optimized for expression in human cells. All final constructs were confirmed by DNA sequencing.

The transfection of the cells was performed according to the protocol described by Durocher et al. (2002) [515]. Briefly, 2µg of purified vector (pTT28-Asc l antigens) per 106 cells were used for transfection of suspension HEK293-6E cells using 25 kDa branched PEI from Polysciences (Warrington, PA) in 3:1 (PEI:DNA) ratio. 24 hours after transfection, the cells were stimulated with a final concentration of 0.5% (w/v) Tryptone N1 of the total volume of the culture and incubated at 37°C in a humidified incubator under constant shaking for three more days for higher protein yield production. The cell culture was harvested and after centrifugation the

medium supernatant was collected and filtered before purification by affinity chromatography using HisTRAP-HP column in ÄKTA Start (Cytiva), as directed by the manufacturer. Purified proteins were quantified by BCA assay (Pierce BCA Protein Assay Kit) before using in further analysis.

2.5.2. Cell culture

RS-ATL8 cells **(Figure 2.8)** [516] were cultured in Eagle's Minimum Essential Medium (Merck) supplemented with 10% heat-inactivated Foetal Bovine Serum (Merck), 2 mM L-glutamine (Merck) and Penicillin/Streptomycin (100U/mL and 100µg/mL, respectively; both ThermoFisher Scientific) in a T-75 flask (Nunc Easy flask, ThermoFisher Scientific). Details of passaging can be found in Wan et al. (2014) [517].

2.5.3. Serum samples

Individual serum samples from alpha-gal positive and alpha-gal negative participants were heat inactivated at 56°C for 5 minutes to avoid cytotoxicity and added to the cell suspension at 1:100 dilution factor respectively. This heat treatment inactivates the complement without affecting the ability of IgE to bind to the FcεRI receptor.

2.5.4. Cell sensitization

RS-ATL8 cells were resuspended in medium to a concentration of 1x10^6 cells/mL. The cells were sensitized using heat-inactivated serum at a 1:100 dilution. 50µL of the sera and the cell suspension were added to NUNC™ white-96 well plate (Thermo Scientific) and incubated in a humidified at 37ºC and 5% CO_2 for 18-20 h.

2.5.5. Controls and Allergens Stimulation

The next day, the medium was removed, and the cells were washed once with PBS. 50µL of each of the following conditions using phenol red-free MEM medium (Thermo Scientific) were added to the appropriate wells: negative control (cells sensitized with serum, but unstimulated); the positive controls 10µg/mL Concanavalin A (Merck) and 1µg/mL polyclonal goat anti-human IgE antibody (Merck); the test samples contained 10µg/mL Ascaris E/S protein or the recombinant allergens of Asc l 1 and Asc l 3 both at 1µg/mL and incubated for 3.5 hours in a humidified incubator at 37ºC and 5% CO_2. Using the ONE-Glo Luciferase Assay System (Promega), 50 µL of ONE-Glo Reagent was added to each well. The luminescence was then measured using a CLARIOstar plus multimode microplate reader.

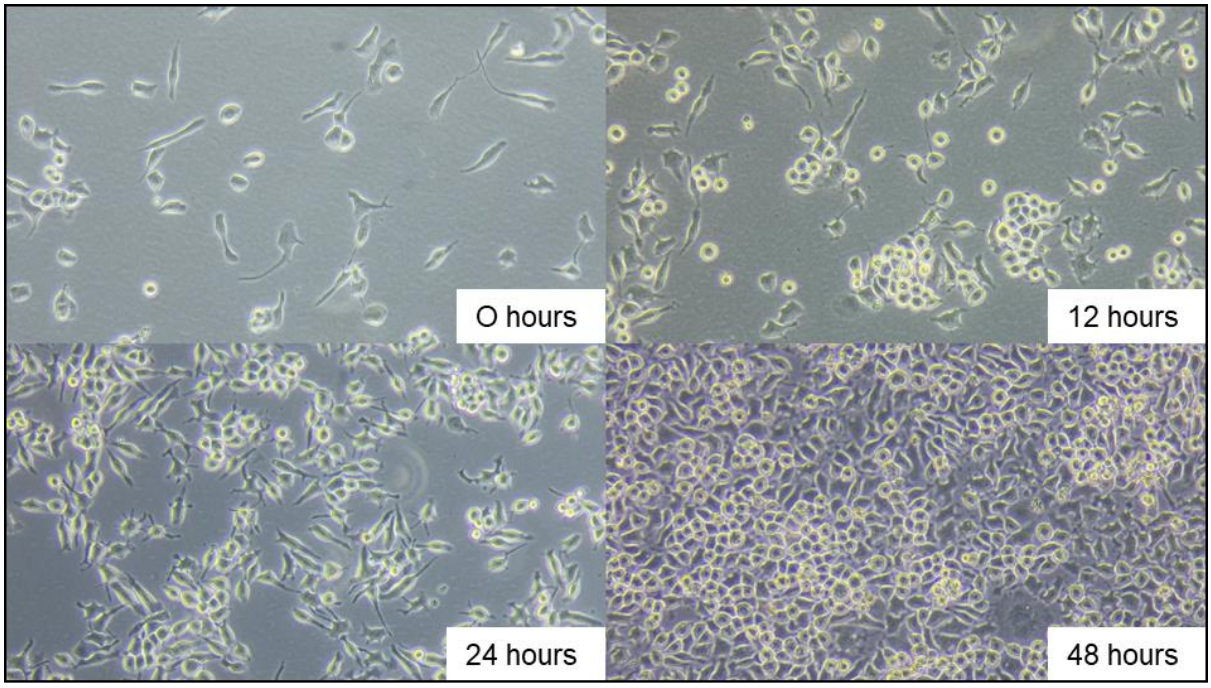


Figure 2.9: RS-ATL8 cells. Culturing of RS-ATL8 cells in basal media carried out in T-25 flask before sensitization with patient sera.

2.6 Proteomic analysis of endo-and ectoparasites

2.6.1 Mass spectrometry

Selected protein bands were cut out from the gel using a scalpel. Gel pieces were washed in 20 mM ammonium bicarbonate (ABC) followed by acetonitrile (ACN). Reduction of cysteine bonds was done using 10mM of dithiothreitol at 56^{o}C for 45 mins with agitation followed by alkenylation with 20mM iodoacetamide at room temperature for 45 mins in the dark. After washing the gels with ABC, the gel pieces were dehydrated with ACN for 15 mins. Samples were incubated with 12.5ng/µL of trypsin (Promega, Madison, WI, USA) overnight at 37^{o}C. The protein digest step was quenched using extraction buffer A (50% v/v ACN; 0.1% formic acid) and extraction buffer B (80% v/v ACN; 0.1% formic acid) for 30 mins under agitation. A C18 resin was used to capture extracted proteins. The eluted protein was subjected to analysis using LC-MS/MS (Liquid Chromatography-Mass Spectrometry/Mass Spectrometry) utilizing an HPLC (High-Performance Liquid Chromatography) system connected to a ThermoFisher Q Exactive mass spectrometer. Peptides were loaded onto a trapping column and then separated on a 75µm analytical column packed with Luna C18 resin (Phenomenex, Torrance, CA, USA) at a flow rate of 350 nL/min. The mass spectrometer operated in data-dependent mode, with the Orbitrap operating at a resolution of 60,000 FWHM (Full Width at Half Maximum) for MS analysis and 17,500 FWHM for MS/MS analysis. The 15 most abundant

ions were selected for MS/MS analysis. The obtained data were searched against the NCBI *A. lumbricoides, R. microplus*, and *A. americanum* databases using a local copy of Mascot [518] with monoisotopic mass values, 10 ppm peptide mass tolerance, 0.002 Da fragment mass tolerance, and maxed missed cleavages of two. The Mascot DAT files were parsed into Scaffold (Proteome software, Portland, OR, USA) for validation, filtering, and creating a non-redundant list per sample. Data were filtered using a 1% protein and peptide false discovery rate (FDR), which requires at least two unique peptides per protein.

2.6.2 Protein characterisation

The gene ontogeny annotations of identified proteins were established by searching Uniprot database [519]. N glycosylation prediction was achieved through NetNGlyc1.0 [520] while signal peptide prediction was deduced via SignalP6.0 [521]. Domain analysis through Motif scan [522] showed the presence of galactose binding domains and the relationship of key identified proteins with other organisms was achieved through Phylogeny.fr [523–530].

2.7 Statistical analysis

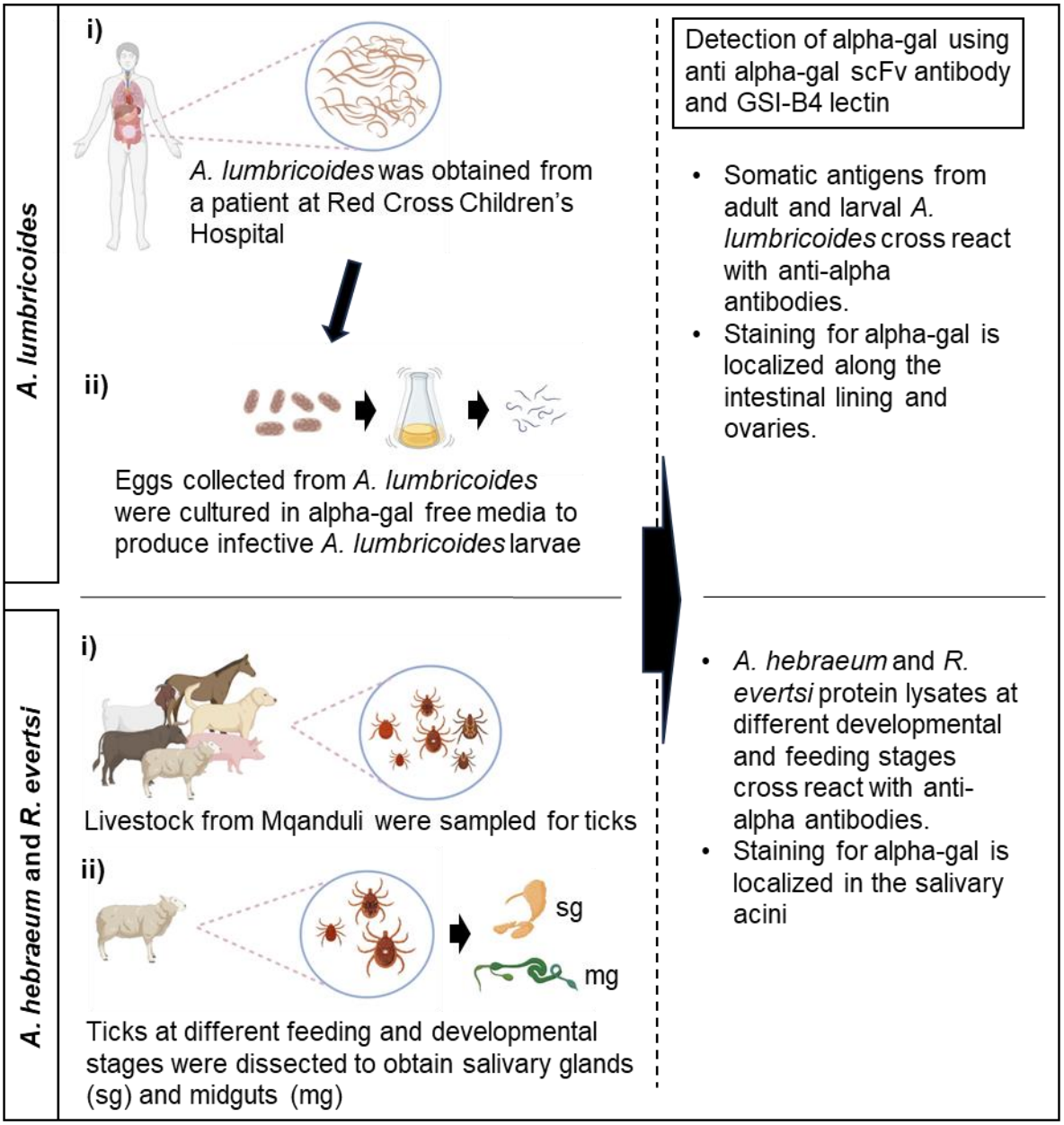
Analysis of antibody levels and a history of exposure to common parasites between alpha-gal allergy positive and negative participants was done in GraphPad Prism v8 (GraphPad Prism software; La Jolla, CA). To test the difference in numerical exposure variables, Mann-Whitney tests were employed. This choice was made because the continuous data was skewed and did not meet the assumption of a large sample size. For categorical versions of the data, Fisher's exact test was utilized. To assess the linear correlation between serum IgE levels, a correlation analysis was conducted using the Pearson correlation coefficient (r). Three-dimensional data scatter plots were curated using SPPS v28 (IBM SPSS Inc)

Luminescence (counts/s) was measured using CLARIOstar plus multimode microplate reader Data is presented as mean ± SD obtained from N=111 sera samples, analysed in triplicates. P values and significances were determined using either the two-tailed Mann-Whitney T-test or non-parametric one-way ANOVA (GraphPad Prism software; La Jolla, CA). Groups were judged to be significantly different if the P value was less than 0.05 (*p≤0.05, **p≤0.01, ***p≤0.001, **** p≤0.0001).

OBJECTIVE 1: Demonstration of parasite and/or parasite-derived proteins as the source of alpha-gal epitopes.

Rationale: For parasitic exposure to drive a specific anti-alpha-gal IgE response, there is a need for alpha-gal to be present in parasite homogenates. We hypothesize antigen presentation of parasite antigens with alpha-gal glycosylation to induce a Th2 response characterized by the production of high-affinity anti-alpha-gal IgE.

Graphical abstract



CHAPTER 3: Detection and immunolocalization of alpha-gal in endoparasites

Some of the data presented in this chapter contributed to a larger publication **(Figure 3.1, 3.2 and 3.3)**:

Murangi T, Prakash P, Moreira BP, Basera W, Botha M, Cunningham S, Facey-Thomas H, Halajian A, Joshi L, Ramjith J, Falcone FH, Horsnell W, Levin ME. Ascaris lumbricoides and ticks associated with sensitization to galactose α1,3-galactose and elicitation of the alpha-gal syndrome. Journal of Allergy and Clinical Immunology. 2022 Feb;149(2):698-707.e3. doi: 10.1016/j.jaci.2021.07.018. Epub 2021 Jul 29. PMID: 34333031.

Key findings

- Somatic protein homogenates from adult and larval *A. lumbricoides* cross-reacts with anti-alpha-gal antibodies.
- This cross-reactivity is localised along the lining of the GI tract and ovaries.

3.1. Introduction

Endoparasites establish their parasitism by living within the host GI tract, lungs, heart, blood vessels and tissue [531]. These parasites predominantly belong to the protozoan or helminth families. They contain complex glycoconjugates on their cell surfaces and/or release excretory-secretory (E/S) products which are essential for virulence and viability [532].

Glycosylation of protozoan lipids and proteins with alpha-gal has been demonstrated in arthropod-borne pathogens, such as *Plasmodium* [215,533], *Trypanosoma* [534] and *Leishmania* [533,535]. In these endoparasites, the alpha-gal epitope is recognized as a driver of protective immunity in humans [213,215,536].

The presence of alpha-gal in helminths is of major interest. Helminths have complex life cycles which allow them to mutually evolve with their host. In part, this co-evolution has been attributed to the presence of carbohydrate moieties linked to their lipids and proteins. For example, the antennae of somatic and/or excretory-secretory (E/S) antigens from *Haemonchus contortus* [239], *Echinococcus granulosus* [505], *Schistosoma mansoni* [216], *Fasciola hepatica* [506] and *Parelaphostrongylus tenuis* [507] have been shown to have alpha-gal glycosylation. This may serve as a form of molecular mimicry by helminths in non-primate mammalian hosts which recognize alpha-gal as a self-antigen. However, according to Duffy et al. (2006) use of alpha-gal in this fashion is not a trait shown by all nematodes as their studies could not detect alpha-gal residues in other *Parelaphostrongylus* species namely *P. cantonensis* and *P. costaricensis* [507].

Individuals from helminth endemic regions have been shown to have elevated alpha-gal IgE. A Zimbabwean cohort with high titers of anti-helminth antibodies demonstrated impaired diagnosis of cat allergy when using an alpha-gal glycosylated cat antigen, Fel d 5, due to the prevalence of elevated IgE to alpha-gal in 85% of the helminth-infected individuals [504]. Children from rural and urban Kenya with elevated Ascaris IgE [501] also showed the presence of elevated alpha-gal IgE in 76% and 29% of the helminth-infected groups respectively [500]. A study in Ecuador established an association between Ascaris IgE, IgG, and IgG4 with alpha-gal IgE in children with active helminth infection although the number of eggs between sensitized and non-sensitized children was not significantly different [503].

The presence of alpha-gal has not been reported in *A lumbricoides*, hence we sought to investigate the presence of alpha-gal in this nematode.

3.2. Results

3.2.1. Galactose alpha 1,3 galactose is present in adult *A. lumbricoides* somatic antigen

To determine the presence of alpha-gal in *A. lumbricoides* we probed glycoproteins from different helminths with anti-alpha-gal chicken scFv antibody and anti-alpha-gal GSI-B4 lectin via western blotting and ELISA. Pork kidney was used as a positive control and whole egg as a negative control. Immunoblotting with anti-alpha-gal chicken scFv antibody showed bands for *A. lumbricoides* antigens at 130kDa and 70-100 kDa (smear) **(Figure 3.1a)**. Pork kidney glycoproteins formed a band at 80kDa while there was no binding to whole egg, *N. brasiliensis,* and *T. crassiceps* proteins. There was faint binding to *E. granulosus* at 120kDa, 60kDa and 30kDa. Immunoblotting with GSI-B4 lectin showed binding to *A. lumbricoides* at 70-100kDa and for pork kidney at 80kDa **(Figure 3.1c)**. *E. granulosus* antigens showed faint bands at the same position as with the anti-alpha-gal chicken scFv antibody at 120kDa, 60kDa and 35kDa while no binding was evident to whole egg*, N. brasiliensis*, and *T. crassiceps.* With ELISA both anti-alpha-gal chicken scFv antibody and GSI-B4 lectin significantly bound to *A. lumbricoides* and pork kidney proteins **(Figure 3.1b and d)** while binding to the other helminth antigens was not different to the negative control. However, there was no significant difference in the binding of anti-alpha-gal chicken scFv antibody and GSI-B4 lectin to *A. lumbricoides* and pork kidney although there is a trend for an increase in binding to *A. lumbricoides*.

To investigate if the presence of alpha-gal in adult *A. lumbricoides* was present in both somatic and E/S antigens, we carried out immunoblotting on both adult *A. lumbricoides* antigens. Immunoblotting with scFv Ab showed cross-reactivity in somatic antigens only at 130kDa and 100kDa but not E/S **(Figure 3.2a)**.

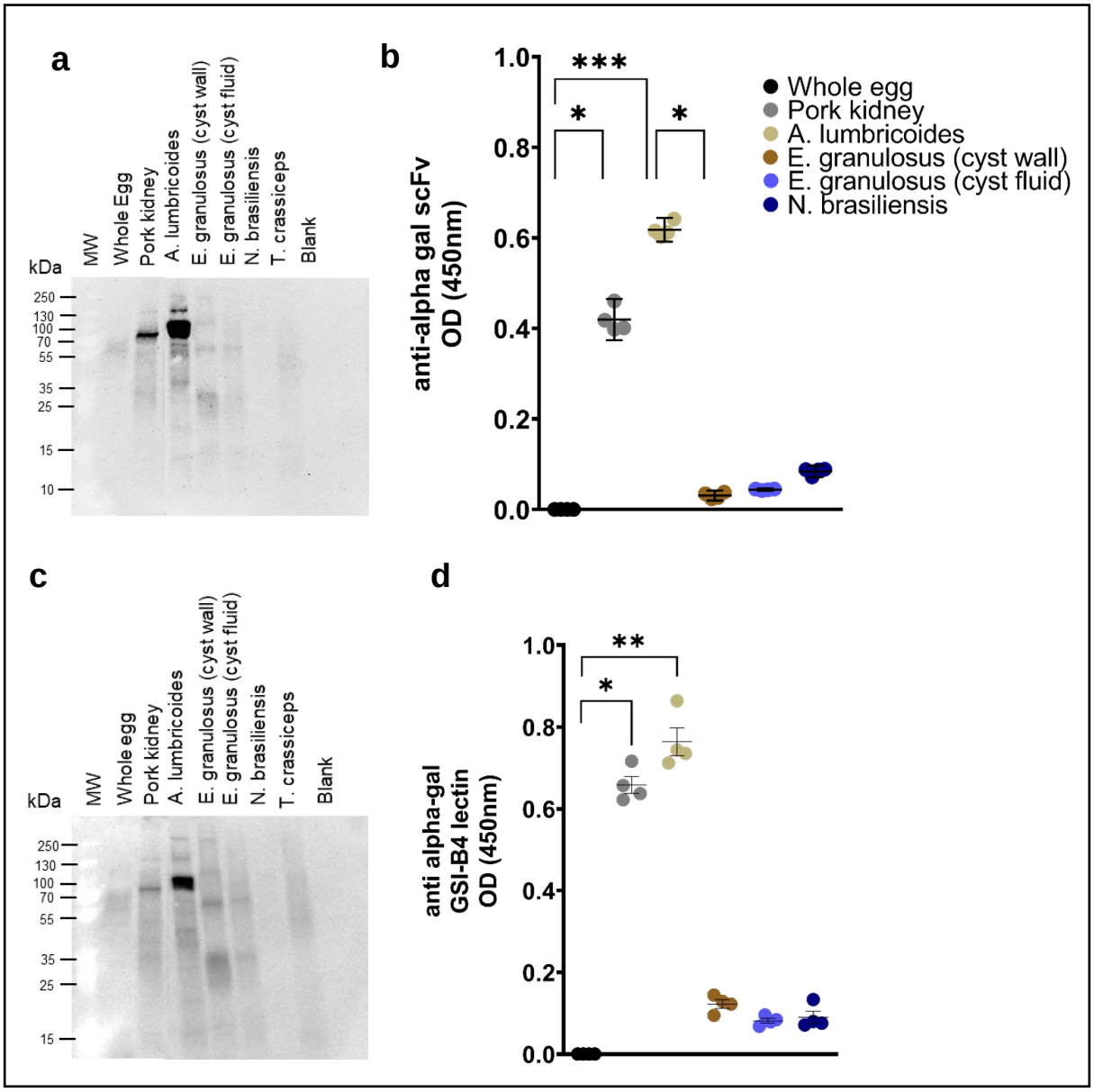


Figure 3.1: Galactose alpha-1,3 galactose is present in adult *Ascaris lumbricoides*. Immunoblotting on 5µg of helminth protein using **(a)** anti-alpha gal scFv chicken antibody and **(c)** anti-alpha gal GSI-B4 lectin. ELISA plates were coated with 50µg of helminth protein prior to alpha-gal detection with **(b)** anti-alpha gal chicken scFv antibody and **(d)** anti-alpha gal GSI-B4 lectin. Data shown is representative of two experiments with four replicates (mean±sem). Statistical significance was calculated by one-way ANOVA and Friedman multiple comparison tests. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$

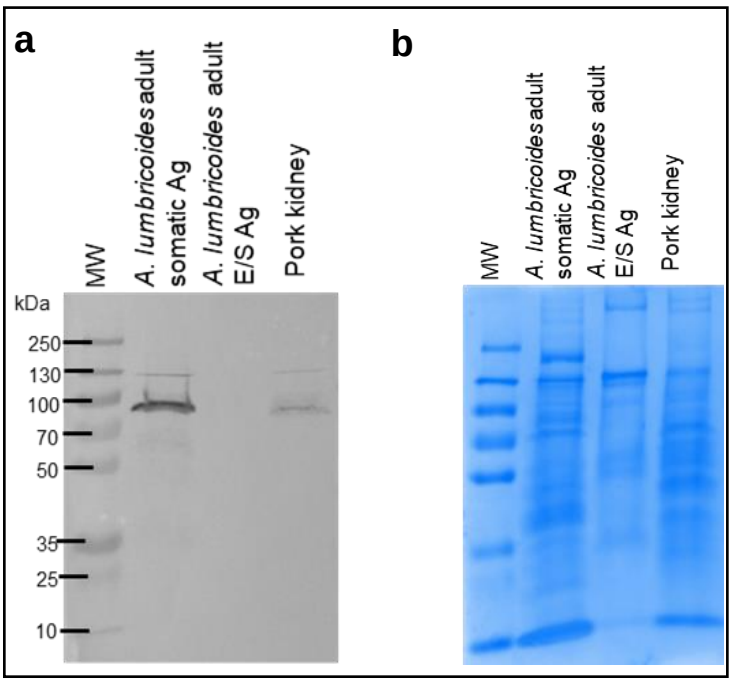


Figure 3.2: Presence of Galactose alpha-1,3 galactose in adult *Ascaris lumbricoides* is dependent on antigen type. (a) Immunoblotting of 5µg *Ascaris lumbricoides* somatic and E/S antigen for alpha-gal using anti-alpha-gal scFv chicken antibody **(b)** 10% SDS-PAGE. Data shown is representative of two independent experiments.

3.2.2. Immunolocalization of alpha-gal in adult *A. lumbricoides*

Next, we sought to localize alpha-gal in *A. lumbricoides* via immunohistochemistry. To check the integrity of the tissue we carried out a hematoxylin and eosin stain. Periodic acid Schiff (PAS) staining was done to identify glycoproteins. To detect alpha-gal in the *A. lumbricoides* tissue, anti-alpha-gal chicken scFv antibody and GSI-B4 lectin were used. Anti-alpha gal negative staining was done by using Biotin anti-His antibody and Strep-HRP.

Positive PAS staining was seen along the GI tract, ovaries and slightly on some parts of the uterine wall **(Figure 3b)**. Staining for alpha-gal was positive along the GI tract, and the lining of ovaries when anti-alpha-gal chicken scFv antibody was used **(Figure 3c)**. GSI-B4 lectin additionally stained the eggs **(Figure 3e)**. There was no staining noted when Biotin anti-His antibody and Strep-HRP were used **(Figure 3d and f)**.

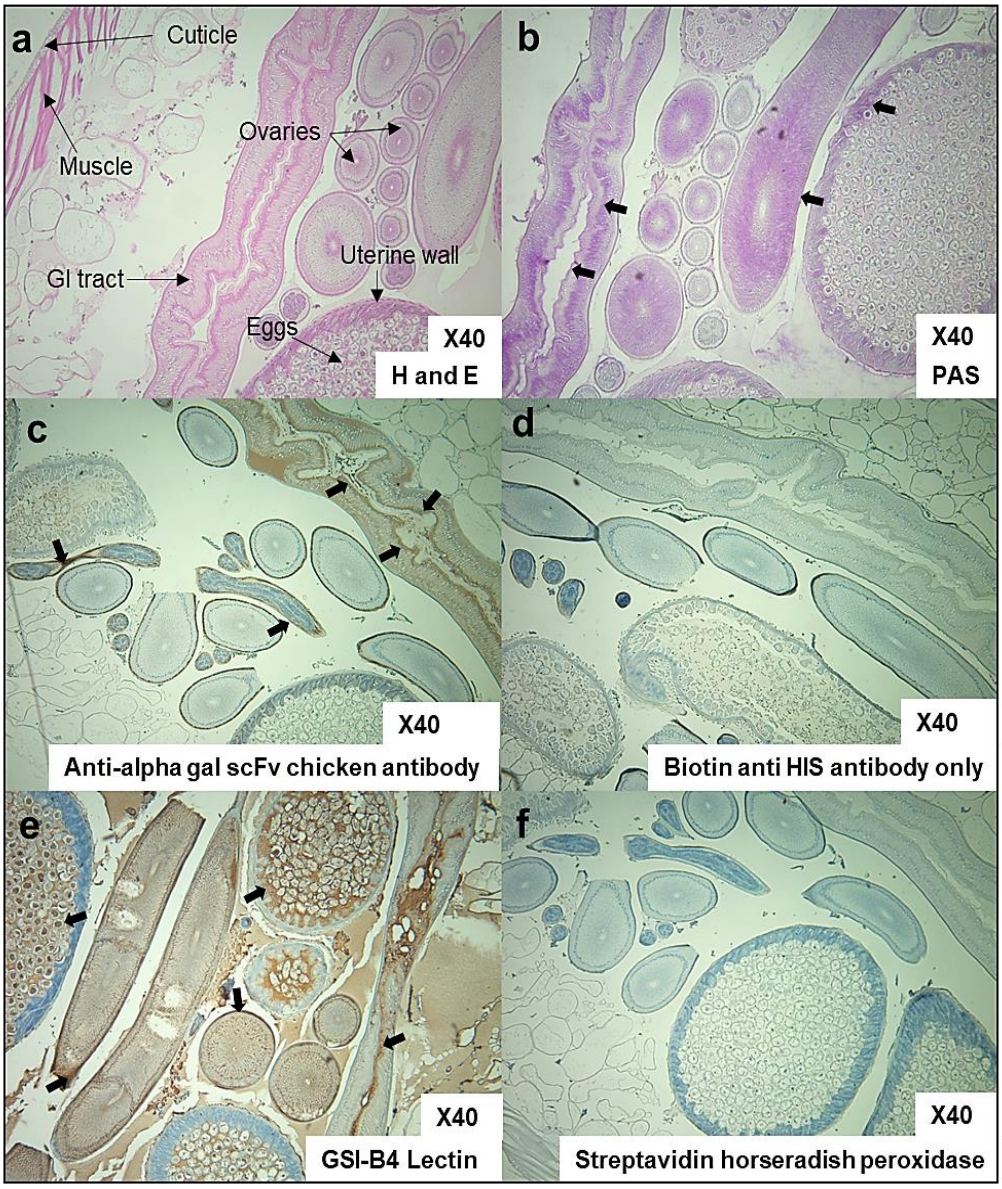


Figure 3.3: Immunolocalization of Galactose alpha 1,3 galactose in adult *Ascaris lumbricoides*. (a) Hematoxylin and Eosin staining **(b)** Periodic acid Schiff staining **(c)** anti-alpha gal scFv chicken antibody staining **(d)** biotin anti-HIS antibody staining **(e)** anti-alpha gal GSI-B4 lectin staining and **(f)** streptavidin horseradish peroxidase staining of *Ascaris lumbricoides* longitudinal sections imaged at X40 magnification. Data is representative of two independent experiments. Positive staining is indicated by a thick black arrow.

3.2.3. Presence of alpha-gal is not dependent on *A. lumbricoides* developmental stage

To determine whether alpha-gal is expressed at various stages of development of *A. lumbricoides*, we carried out immunoblotting on glycoproteins from *A. lumbricoides* in the adult and larval stages. Consistent with previous findings, adult somatic antigens cross-reacted with anti-alpha-gal scFv antibody at 130kDa and 100-70kDa. Larval somatic antigen showed binding at 90kDa slightly above pork kidney at 70kDa (**Figure 4a**). There was no binding of the anti-alpha-gal antibody to larval E/S Ag or KW-2 media **(Figure 4b)**.

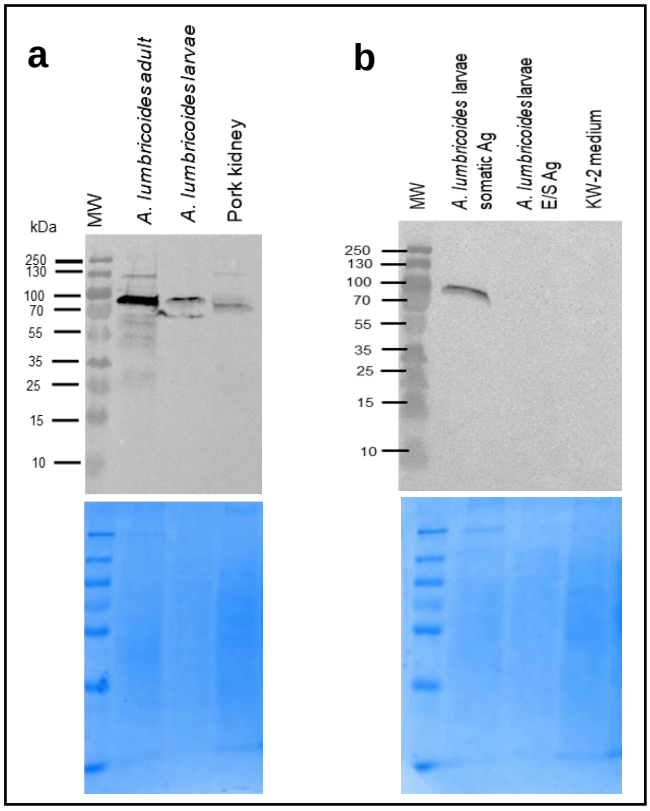


Figure 3.4: Galactose alpha-1,3 galactose is present in the developmental stages of *Ascaris lumbricoides*. Immunoblotting on 5µg of **(a)** *Ascaris lumbricoides* adult and larval somatic antigen **(b)** *Ascaris lumbricoides* larval somatic, E/S and culturing media for alpha-gal using anti-alpha-gal scFv chicken antibody. Data shown is representative of two independent experiments.

3.3. Discussion

A. lumbricoides is known for establishing acute and chronic infections and may only be detected when the worm burden within the host has greatly increased to the point where there is intestinal blockage [177,537,538]. This is evidence of the ability of this helminth to successfully evade the host's immune system during an infection. Glycosylation of helminth antigens has been attributed as a factor allowing host immune evasion [184,532].

In this chapter, we show for the first time the presence of alpha-gal in glycoproteins from the nematode *A. lumbricoides*. Previously, Duffy et al. (2006) showed the presence of alpha-gal in another nematode *P. tenuis* [507]. In line with our findings, they found binding to alpha-gal homogenates at 50-91kDa. However, unlike in our study, the presence of alpha-gal in *P tenuis* was restricted to the adult stage. We found alpha-gal in larval somatic extracts to be only at 90kDa (in the same range as binding in adult worms) while binding in adult worms was also at 130kDa. This indicates differential expression of proteins across the helminth developmental stages [532,539,540]. We found alpha-gal containing antigens to be restricted to somatic antigens and absent in E/S products. This is in contrast to studies showing alpha-gal in *P. tenuis* and *H. contortus* E/S products [239,507]. Interestingly, van Stijn et al. (2010) shows no production of anti-alpha antibodies in sheep vaccinated with *H. contortus* alpha-gal containing E/S [239].

In the case of a human host, alpha-gal is recognized as a foreign antigen which triggers the immune system [255,256,307,541]. The presence of alpha-gal in the larvae may be immunogenic. During the development of *A. lumbricoides*, there is a phase of larval migration through the lung parenchyma [542,543]. This results in the activation of the pulmonary and systemic immune response leading to transient diffuse lung infiltrates [544] and occasional eosinophilic pneumonia [545,546]. This is characteristic of a strong Th2 immune response making *A. lumbricoides* larvae one of the major causes of allergic parenchymal lung disease [537,547]. Hence, the presence of alpha-gal in *A. lumbricoides* larvae can lead to allergic sensitization during larval migration.

Immunolocalization of alpha-gal with the anti-alpha-gal chicken scFv antibody showed the presence of this carbohydrate moiety along the GI tract and lining of the ovaries. This follows the same trend as in the model nematode *P. tenuis* [507]. The presence of alpha-gal in the GI tract may suggest the source of alpha-gal is host-derived. *A. lumbricoides* adult worms gain their nourishment from the host luminal content. Despite no endogenous alpha-gal production by the human host, alpha-gal may be present in the gut from food products with high alpha-gal concentrations such as red meat [270]. Alternatively, alpha-1,3 galactosyltransferases from human commensal bacteria may also contribute to the development of alpha-gal in adult *A. lumbricoides*. Studies show the ability of alpha-1,3 galactosyltransferase translocation from

Klebsiella spp to add alpha-gal moieties onto the surface of senescent red blood cells in order to facilitate their clearance [548]. Therefore, the addition of alpha-gal epitopes onto *A. lumbricoides* (both adult and larval stages) by bacteria derived alpha-1,3 galactosyltransferases may be a form of immune response for the clearance of helminth infection. However, this does not account for the presence of alpha-gal in *A. lumbricoides larvae* which we cultured under laboratory conditions in an alpha-gal-free environment, suggesting endogenous production by the helminths. Hence, further studies are required to investigate if *A. lumbricoides* has active alpha-gal galactosyltransferase genes.

In our study, the lining of ovaries was stained positively for the presence of alpha-gal. This may indicate the use of this carbohydrate in *A. lumbricoides* reproduction. The glycan may coat eggs prior to release by the female. This may facilitate host immune evasion by the eggs as they mimic alpha-gal expression on the surface of gut microbes [549]. Hence, the shedding of eggs by worms may act as a source of sensitization to alpha-gal in the human host. However, alpha-gal staining of eggs in the uterus was only established by GSI-B4 lectin and not anti-alpha-gal scFv antibody. GSI-B4 lectin is used as a confirmatory marker for alpha-gal moieties although it has been known to bind to other alpha-gal moieties without an alpha 1,3 linkage [550–552]. To avoid the promiscuous binding nature of GSI-B4 lectin other studies use the anti-alpha gal M86 antibody, however, being an IgM antibody it has low affinity [553,554]. In our study, we used an anti-alpha-gal chicken scFv antibody which is specific only to our glycan of interest ie alpha-gal with the 1,3 linkage [512]. Hence, it can be postulated that the loss of staining of eggs in the uterus is due either to the presence of an alpha-gal moiety with a different linkage to alpha-gal or the anti-alpha-gal chicken scFv antibody not being able to access the egg antigens after coating with a thick protein layer before release. However, this warrants further study.

Unlike other studies which demonstrate the absence of alpha-gal in *A. suum* [216,555], our detection of alpha-gal in *A. lumbricoides* may present a difference between the two closely related organisms whose classification as either one [556–558] or two [559,560] different species has been a major point of debate. This selective expression has been reported among *Parelaphostrongylus* species with *P. cantonensis* and *P. costaricensis* showing an absence of alpha-gal expression which is present in *P. tenuis* [507].

In conclusion, in this chapter, we show the presence of alpha-gal in adult and larval somatic *A. lumbricoides* glycoproteins and their localization in the GI tract and ovaries. The localization of alpha-gal in the ovaries and not only in the GI tract as well as its detection in larvae (cultured in an alpha-gal-free environment) suggests the alpha-gal source may not be derived from the

host. The presence of alpha-gal in *A. lumbricoides* larvae may result in sensitization during larval migration in the human host.

CHAPTER 4: Detection and immunolocalization of alpha-gal in ectoparasites

Some of the data presented in this chapter contributed to a larger publication **(Figure 4.2 and 4.3)**:

Murangi T, Prakash P, Moreira BP, Basera W, Botha M, Cunningham S, Facey-Thomas H, Halajian A, Joshi L, Ramjith J, Falcone FH, Horsnell W, Levin ME. *Ascaris lumbricoides* and ticks associated with sensitization to galactose α1,3-galactose and elicitation of the alpha-gal syndrome. Journal of Allergy Clinical Immunology. 2022 Feb;149(2):698-707.e3. doi: 10.1016/j.jaci.2021.07.018. Epub 2021 Jul 29. PMID: 34333031.

Key findings

- Alpha-gal is differentially expressed during the development and feeding stages of the ticks *A. hebraeum* and *R. evertsi*.
- Staining for alpha-gal is localised in the salivary acini.

4.1. Introduction

Ticks are hematophagous ectoparasites of vertebrates [561]. This is facilitated by the presence of salivary proteins which when introduced into the host can inhibit hemostasis and modulate the host immune system through salivary components such as PGE2 which reduces inflammation and inhibits tissue repair by restricting fibroblast migration to the site of tick attachment [333,336,337,562–564]. During feeding, ticks also introduce a wide range of pathogens to their host [565] making them efficient vectors for viral, bacterial, and protozoan diseases such as Lyme disease, Rocky Mountain spotted fever, Babesiosis, Encephalitis and Theileriosis [566–570]. There has been a global rise in tick-borne pathogens [561] and associated conditions such as alpha-gal allergy [270].

Alpha-gal sensitization may be induced by ticks in three possible ways; **a)** sensitization of human hosts by mammalian blood which contains alpha-gal in the tick midgut after a blood meal [235], **b)** the presence of endogenous tick alpha-gal antigens (including those found in non-fed larval ticks) [302] or **c)** through alpha-gal producing tick-borne microbial pathogens [508].

Studies show evidence of alpha-gal epitopes in salivary glands of partially fed and fed *A. americanum* and *I. scapularis* [234], *I. ricinus* [571], *A. sculptum* [236] and *H. longicornis* [343]. Alpha-gal epitopes have also been shown in the midguts of ticks belonging to the family Ixodes particularly *I. ricinus* [235] and *I. scapularis* [234]. However, Crispell et al. (2019) noted that once fed, alpha-gal epitopes became undetectable [234].

Not all tick species have been reported to have alpha-gal. Crispell et al. (2019) showed the absence of cross-reactivity of anti-alpha-gal antibodies with unfed and partially fed salivary

glands from *A. maculatum* and *Dermacentor variabilis* as well as salivary antigens from the former [234]. Therefore, in this chapter we investigate the presence of alpha-gal in local South African tick species.

4.2. Results

4.2.1. Galactose alpha-1,3 galactose is present in adult *A. hebraeum* and *R. evertsi*

To investigate the role of ectoparasites in causing sensitization to alpha-gal we collected ticks from domestic animals in the proximity of our alpha-gal allergic cohort. From the nine villages sampled, the most common ticks collected were *Amblyomma spp, Rhipicephalus* spp and *Boophilus spp* **(Figure 4.1)** with the former two being more prevalent. These ticks were most commonly found on cows and goats (**Figure 1a and c**) mostly on the surface of the body, tail, interdigit, and anal opening **(Table 4.1).**

To determine the presence of alpha-gal in these ticks, we carried out immunoblotting and ELISA using anti-alpha-gal chicken scFv antibody and GSI-B4 lectin as previously described in Chapter 3. Immunoblotting with anti-alpha-gal chicken scFv antibody **(Figure 4.2a)** cross-reacted with *R. evertsi* female homogenates at 100-130kDa and at 150kDa. *R. evertsi* male ticks had three bands at 90kDa, 120kDa and 150kDa. *A. hebraeum* female ticks had one prominent band at 90kDa and a faint one at 150kDa while the male ticks had two bands at 80-120kDa and 150kDa. Pork kidney showed cross-reactivity with anti-alpha gal chicken scFv antibody at 80kDa while there was no staining in the lane with whole chicken's egg (negative control). Immunoblotting with GSI-B4 lectin **(Figure 4.2c)** produced faint bands although the same trend as seen with anti-alpha-gal chicken scFv antibody probing was observed. ELISA showed the binding of both the anti-alpha gal chicken scFv antibody and GSI-B4 lectin to *A. hebraeum* and *R. evertsi* antigens **(Figure 4.2b and d)**. Binding to pork kidney homogenates was higher in comparison to tick homogenates. There was no significant difference in binding between male and female ticks, although male ticks appeared to show a trend of increased binding.

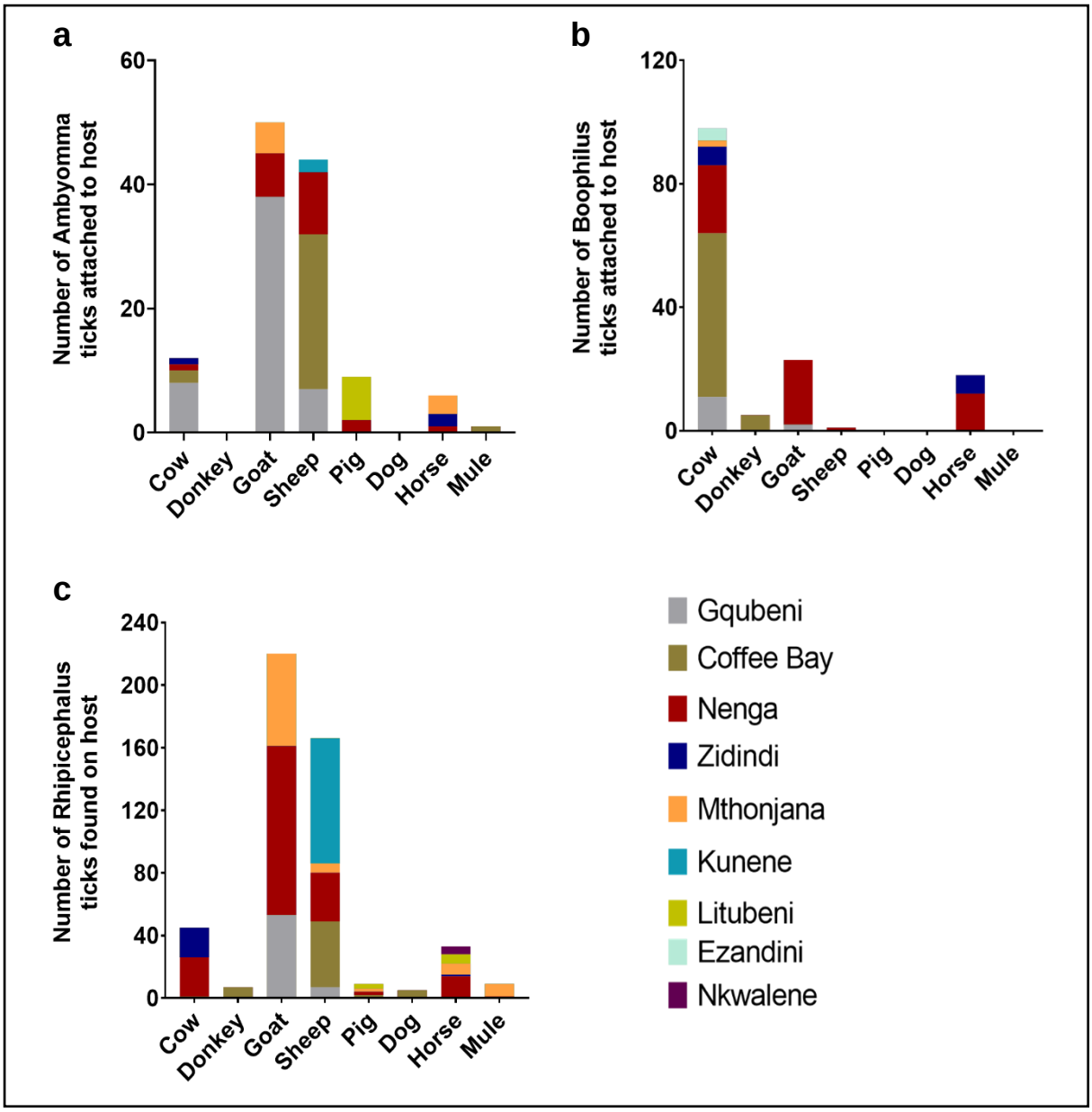


Figure 4.1: Tick distribution in Mqanduli district, Eastern Cape. Ticks belonging to the species **(a)** *Amblyomma* **(b)** *Boophilus* and **(c)** *Rhipicephalus* were randomly collected from domestic animals in 9 villages and tallied cumulatively.

Table 4.1: Site of attachment on a host by ticks collected in Mqanduli District, Eastern Cape

Tick Genus	Host	Site of attachment
Amblyomma spp	Cow	Tail, Udder, Groin, Body, Anal opening, Inside Ear
	Goat	Interdigit, Body, Anal opening, Inside and Outside Ear, Tail
	Sheep	Eyelids, Interdigit, Body, Anal opening, Inside and Outside Ear, Tail
	Pig	Body
	Dog	Inside Ear, Head
	Horse	Neck, Scrotum, Anal opening, Groin, In and outside Ear, Body,
	Mule	Tail, Body, Anal opening
Boophilus spp	Cow	Inside Ear, Body, Head, Anal Opening
	Donkey	Body
	Goat	Ear In, Interdigit, Bd, Anal
	Sheep	Interdigit, Inside Ear
	Horse	Body
Rhipicephalus spp	Cow	Body, Inside Ear
	Donkey	Anal opening
	Goat	In and outside Ear, Interdigit, Anal opening
	Sheep	Anal and vaginal area, Inside Ear, Interdigit, Body, Tail
	Pig	Groin
	Dog	Inside Ear, Body
	Horse	Anal opening, Inside Ear, Head, Body,
	Mule	Inside Ear, Body

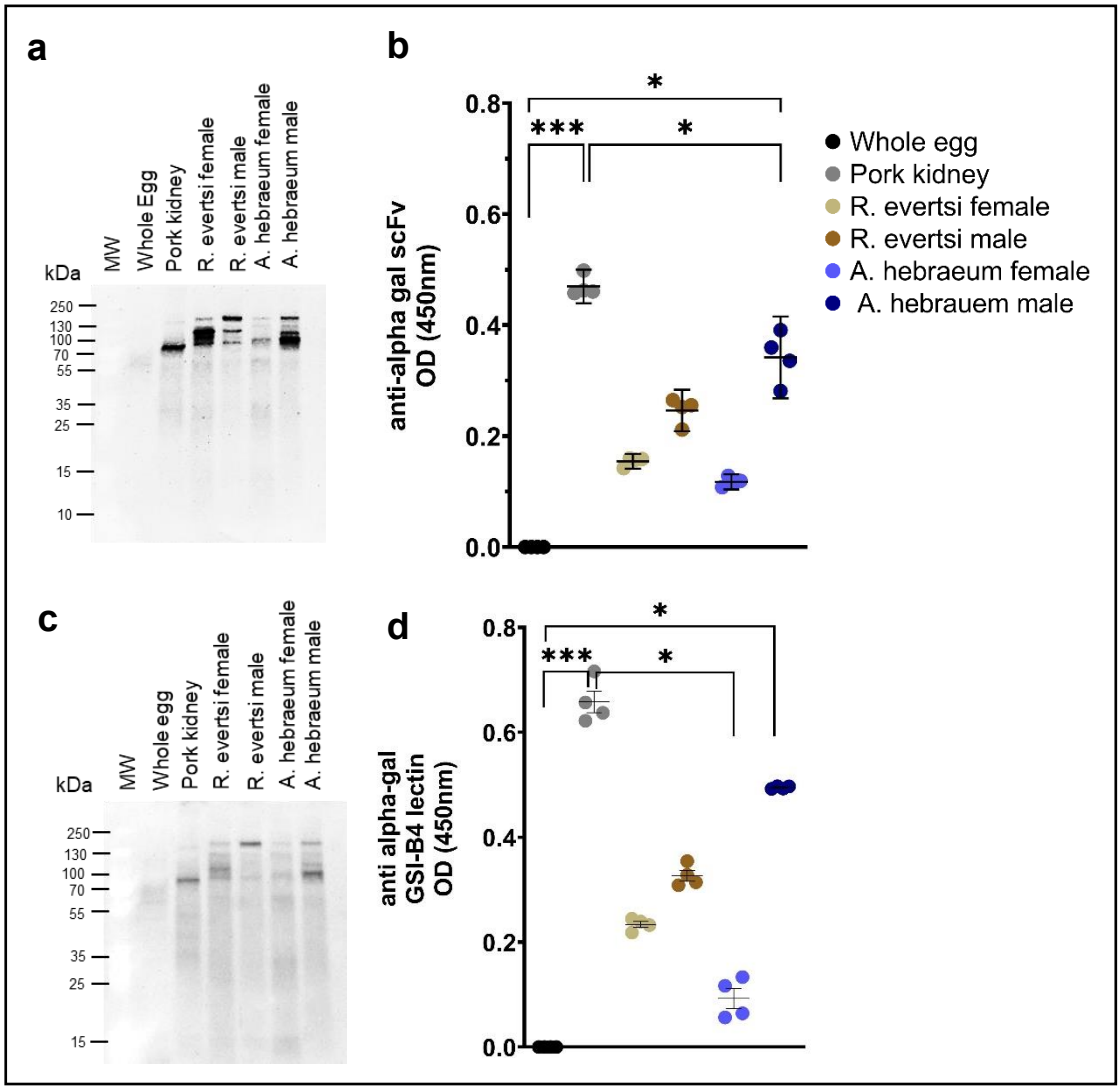
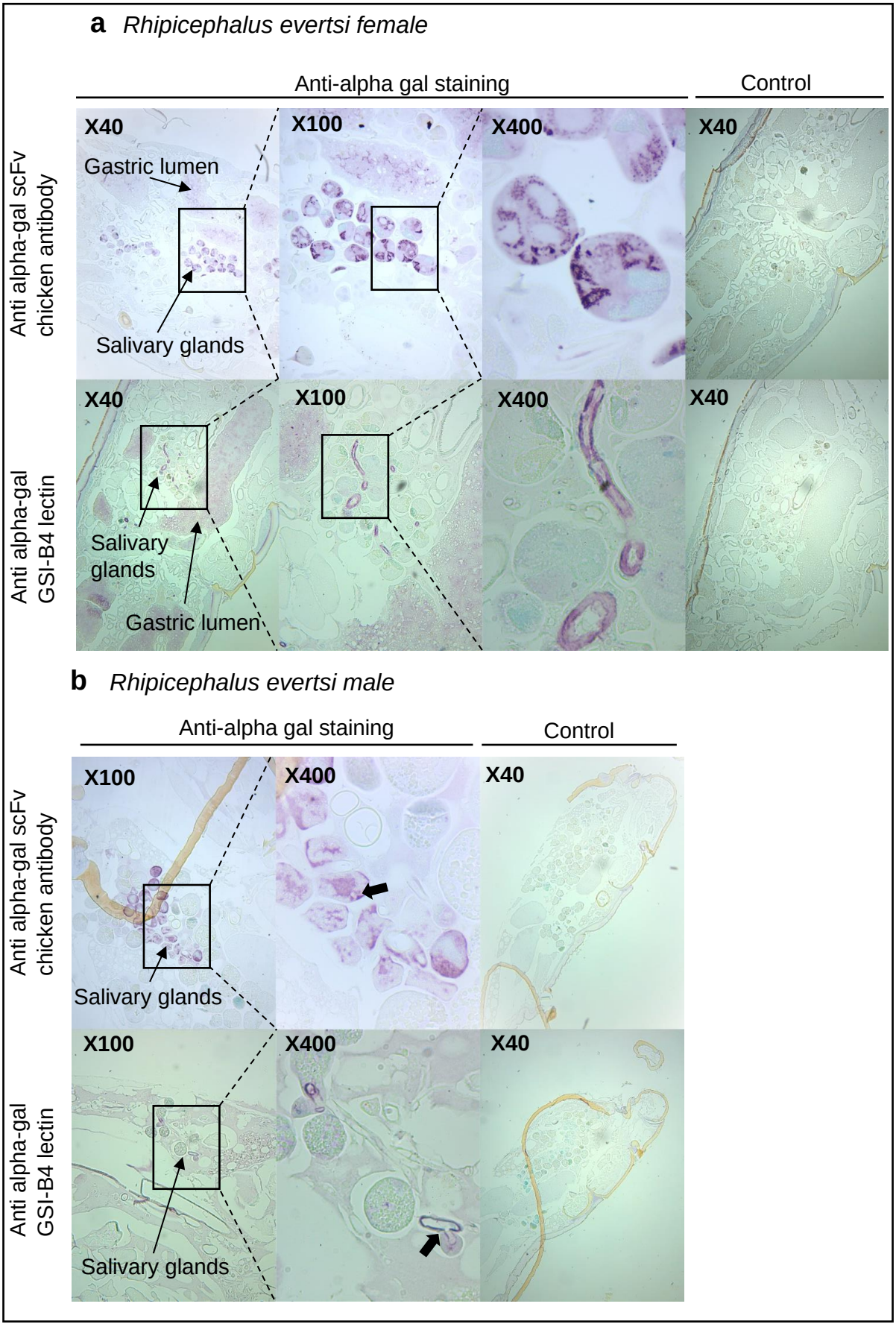


Figure 4.2: Galactose alpha-1,3 galactose is present in adult *Amblyomma hebraeum* and *Rhipicephalus evertsi* homogenates. Immunoblotting analysis on 5µg of tick protein using **(a)** anti-alpha gal chicken scFv antibody and **(c)** anti-alpha gal GSI-B4 lectin. ELISA plates were coated with 50µg of tick protein prior to alpha-gal detection with **(b)** anti-alpha gal chicken scFv antibody and **(d)** anti-alpha gal GSI-B4 lectin. Data shown is representative of two experiments with four replicates (mean±sem). Statistical significance was calculated by one-way ANOVA and Friedman multiple comparison test. * $p<0.05$, *** $p<0.0001$

4.2.2. Immunolocalization of alpha-gal in adult ticks

Using the anti-alpha gal chicken scFv antibody and a lectin as previously described in Chapter 3, we stained longitudinal sections to localise alpha-gal in *A. hebraeum* and *R. evertsi*. Firstly, ticks collected from our study cohort's proximity in Mqanduli were assessed. Both tick species namely *A. hebraeum* and *R. evertsi* showed alpha-gal staining in the ticks' salivary acini **(Figure 4.3)**. There was no difference in the staining of male or female ticks. Probing for alpha-gal using the secondary antibody only showed no staining.

To investigate the effect of feeding on the localisation of alpha-gal, we made sections from unfed and partially fed ticks fed on pathogen-free sheep. These ticks were obtained from the Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR). Unfed and partially fed female ticks **(Figure 4.4a and b)** stained with anti-alpha gal chicken scFv antibody showed staining for alpha-gal in the salivary acini. In *A. hebraeum* female ticks, a red-brownish mass was present, but it was not positive for alpha-gal staining **(Figure 4.4a)**. Staining with a secondary antibody only showed negative staining. Unfed *A. hebraeum* male ticks showed alpha-gal staining in their salivary acini and testis **(Figure 4.4c)** while in unfed *R. evertsi* alpha-gal staining was in the undefined cell and salivary glands **(Figure 4.4d)**.


a Rhipicephalus evertsi female
Anti-alpha gal staining
Control
Anti alpha-gal scFv chicken antibody
Anti alpha-gal GSI-B4 lectin
X40
X100
X400
X40
Gastric lumen
Salivary glands
Salivary glands
Gastric lumen
b Rhipicephalus evertsi male
Anti-alpha gal staining
Control
Anti alpha-gal scFv chicken antibody
Anti alpha-gal GSI-B4 lectin
X100
X400
X40
Salivary glands
Salivary glands

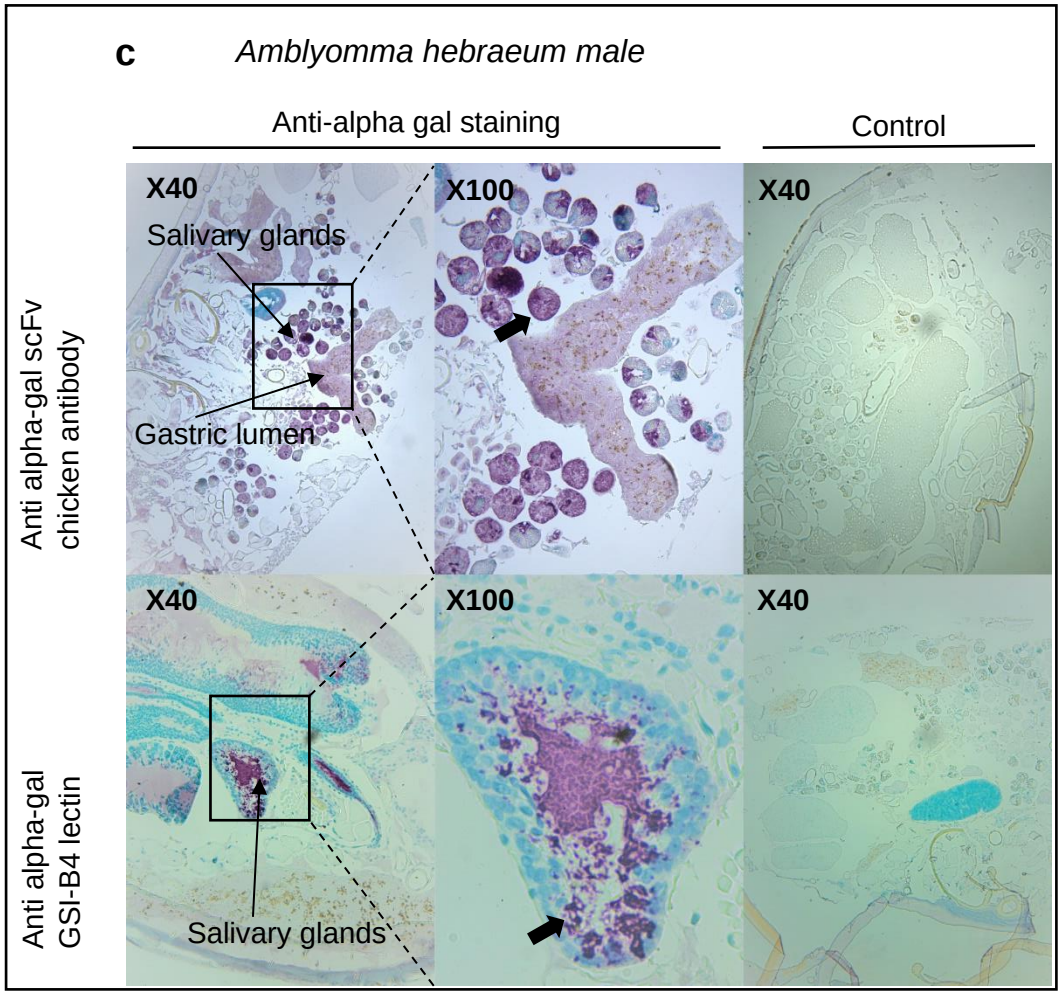
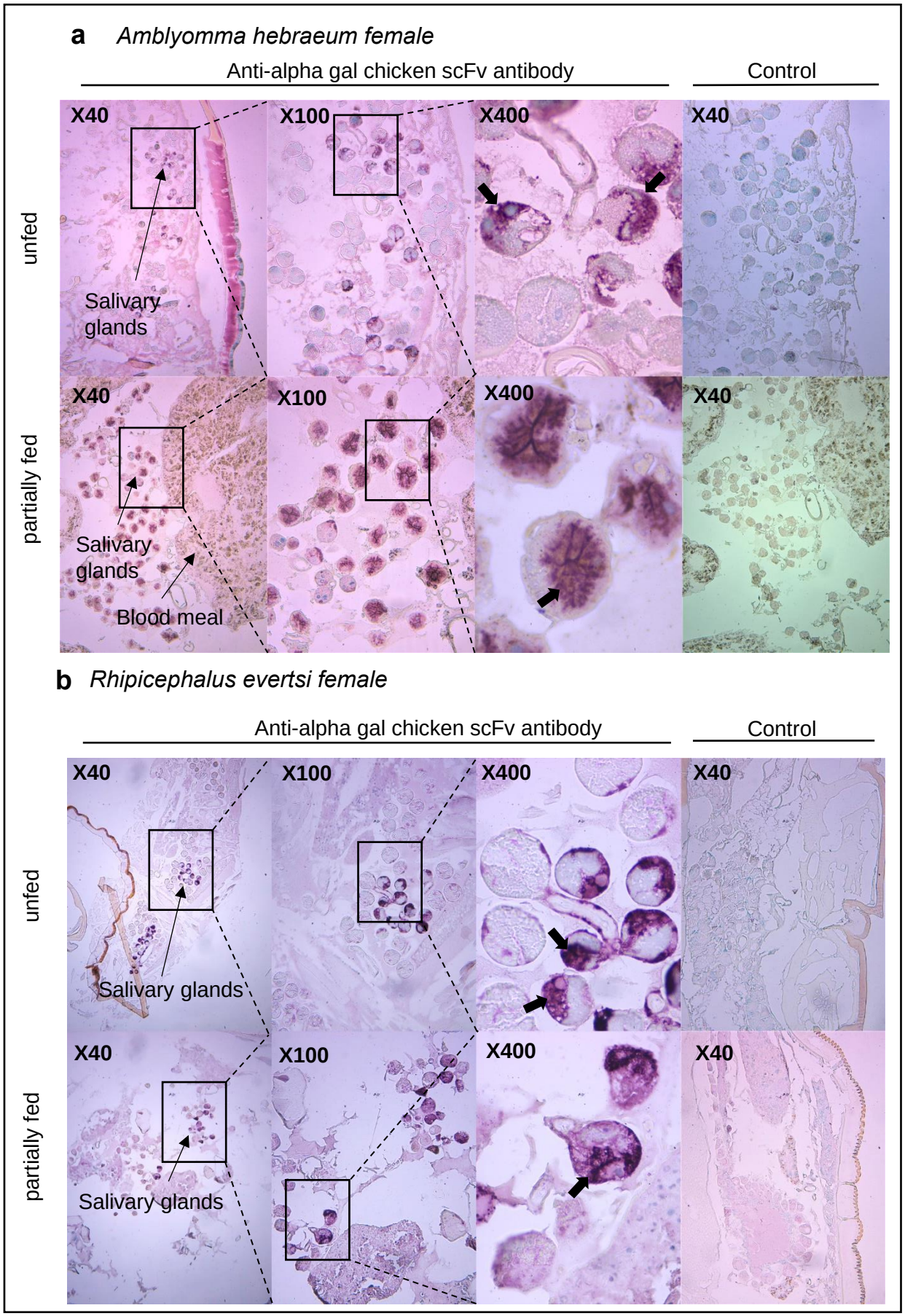


Figure 4.3: Immunolocalization of Galactose alpha 1,3 galactose in adult ticks. 4µm sections of **(a)** *Rhipicephalus evertsi* female **(b)** *Rhipicephalus evertsi* male and **(c)** *Amblyomma hebraeum* male ticks were stained for alpha-gal using anti-alpha gal scFv chicken antibody and anti-alpha gal GSI-B4 lectin. These were imaged at X40, X100 and X400 magnification. Data is representative of two independent experiments with two section replicates per slide. Positive staining is indicated by a thick black arrow.


a Amblyomma hebraeum female
Anti-alpha gal chicken scFv antibody
Control
unfed
partially fed
X40
X100
X400
X40
Salivary glands
X40
X100
X400
X40
Salivary glands
Blood meal
b Rhipicephalus evertsi female
Anti-alpha gal chicken scFv antibody
Control
unfed
partially fed
X40
X100
X400
X40
Salivary glands
X40
X100
X400
X40
Salivary glands

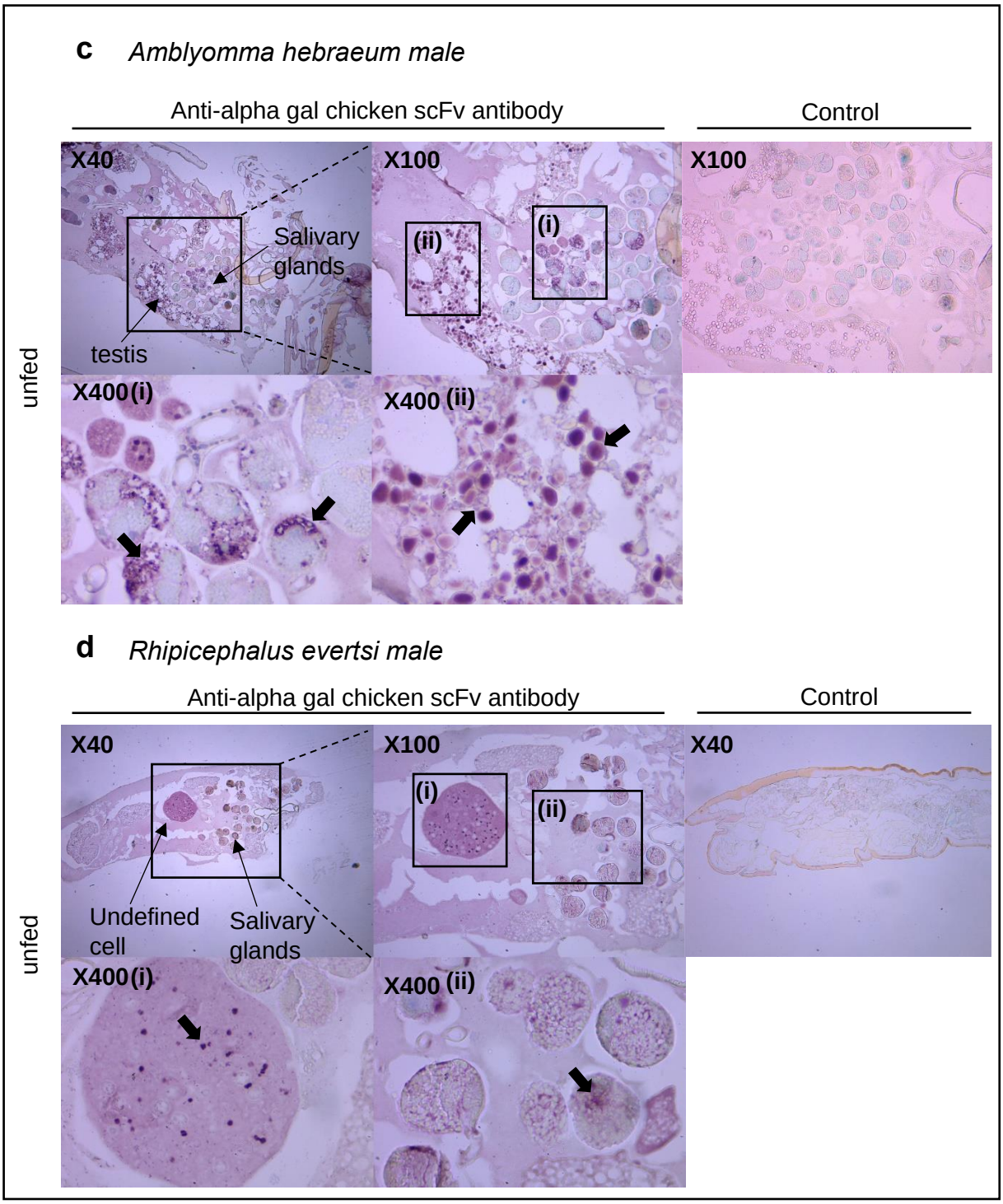


Figure 4.4: Immunolocalization of Galactose alpha 1,3 galactose in adult *A. hebraeum* and *R. evertsi* at different feeding stages. 4µm sections of **(a)** *Amblyomma hebraeum* female **(b)** *Rhipicephalus evertsi* female **(c)** *Amblyomma hebraeum* male and **(d)** *Rhipicephalus evertsi* male ticks at unfed and partially fed feeding stages were stained for alpha-gal using anti-alpha gal chicken scFv antibody. These were imaged at X40, X100 and X400 magnification. Data is representative of one experiment with two section replicates per slide. Positive staining is indicated by a thick black arrow.

4.2.3. Galactose alpha 1,3 galactose is differentially expressed during feeding

To investigate the effect of feeding on alpha-gal expression, immunoblotting was carried out on salivary glands and guts from ticks extracted at different feeding stages. In salivary glands from *A. hebraeum* two protein bands showed the presence of alpha-gal. The first band was at 130kDa and the other one at 70kDa **(Figure 4.5a)**. However, despite these bands being present in all the feeding stages, the intensity of the bands increased as feeding time increased with the band at 130kDa almost unnoticeable in the unfed state. Salivary glands from *R. evertsi* showed the same trend. Guts from *A. hebraeum* showed a single band in the unfed state at 130kDa, two bands at 130kDa and 70kDa in the partially fed state and no alpha-gal containing proteins in the fully fed state **(Figure 4.5b)**. In *R. evertsi* alpha-gal containing proteins in the partially fed state produced bands at 130kDa and 70kDa while only one protein band at 130kDa was detected for guts in the fully fed state (**Figure 4.5b**). Pork kidney showed staining at 130kDa and 70kDa. The Coomassie-stained gels showed the absence of *R. evertsi* unfed homogenates from both the salivary glands and guts. No cross-reactivity was detected in these lanes.

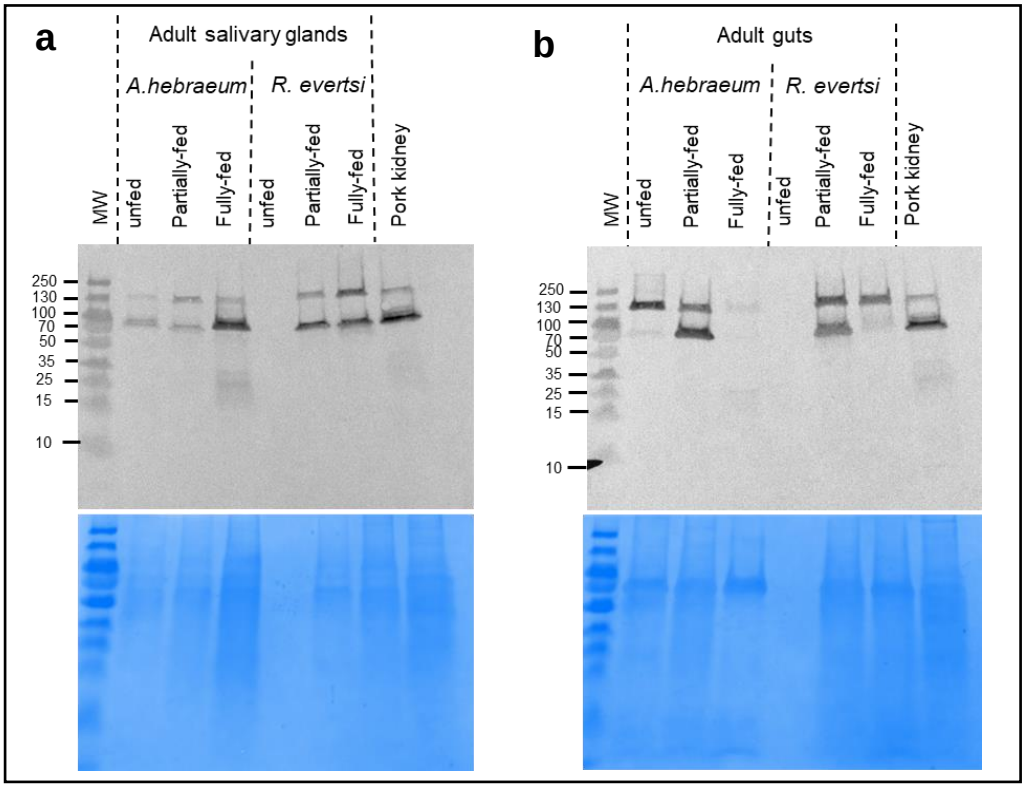


Figure 4.5: Differential expression of galactose alpha 1,3 galactose proteins in adult *A hebraeum* and *R evertsi* during feeding. Immunoblotting of 5µg **(a)** salivary gland and **(b)** gut proteins were probed with anti-alpha gal scFv chicken antibody. Pork kidney was used as a positive control. Data shown is representative of three independent experiments.

4.2.4. Galactose alpha 1,3 galactose detection is not dependent on the developmental stage

Next, we investigated the effect of *A. hebraeum* and *R. evertsi* developmental stage on the presence of alpha-gal in these homogenates. Using the anti-alpha gal chicken scFv antibody we carried out immunoblotting on whole tick lysates from unfed adult and larval ticks. Protein bands in the adult tick homogenates maintained the same trend as previously described **(Figure 4.2a and c)**, although slightly fainter. Homogenates from larval ticks (both species) only had one protein band at 130kDa which corresponded to one of the bands seen in the adult ticks' homogenates **(Figure 4.6)**.

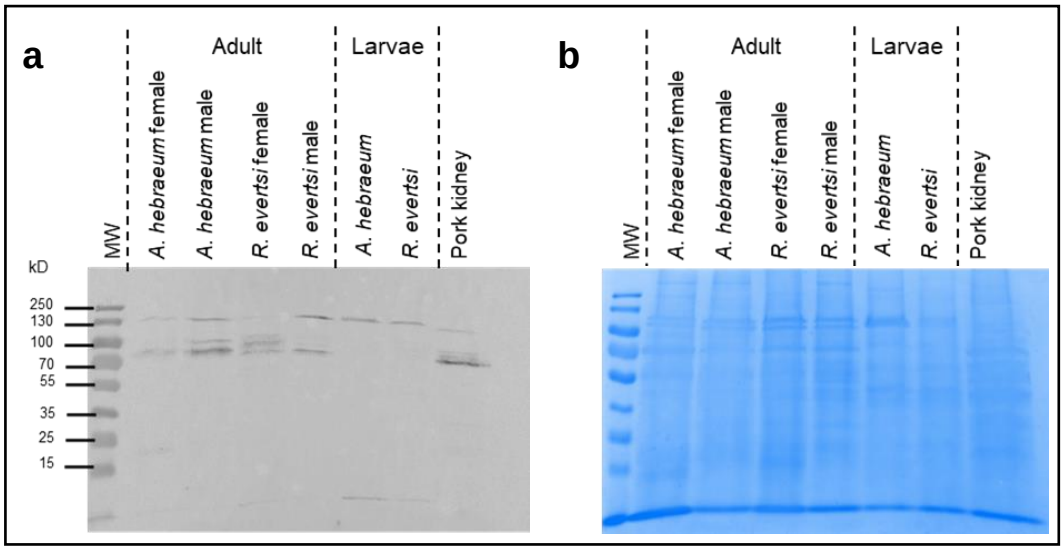


Figure 4.6: Galactose alpha 1,3 galactose is present in the adult and larval developmental stages of unfed *A. hebraeum* and *R. evertsi*. (a) Immunoblotting on 5µg tick protein probed with anti-alpha gal scFv chicken antibody. Pork kidney was used as a positive control. **(b)** 10% SDS-PAGE. Data shown is representative of two independent experiments.

4.3. Discussion

In this chapter, we report for the first time the presence of alpha-gal in two local South African ticks i.e. *A. hebraeum* and *R. evertsi*. Firstly, sampling of ticks from areas proximal to our alpha-gal allergic cohort identified ticks belonging to the genus *Amblyomma hebraeum* and *Rhipicephalus evertsi* as the most prevalent ticks in Mqanduli district. *A. hebraeum* (The South African bont tick), is a 3-host tick which feeds on cattle, goats, sheep, and large wild ruminants. *R. evertsi* (the red-legged tick) prefers horses, zebra, eland, cattle, and sheep and is a two-host tick. Engorged females from these species lay up to 20 000 (*A. hebraeum*) and 5 000 - 7 000 (*R. evertsi*) eggs and after hatching the larvae climb up onto vegetation to wait for a host [510]. The positioning of the larvae and our observation of these ticks on livestock (mostly cows and goats) as well as body parts easily in contact with humans makes it most likely for these ticks to attach to human hosts with *A. hebraeum* larvae being noted as most responsible for human bites [572].

Ticks from Mqanduli showed cross-reactive alpha-gal bands in the range of 80-150kDa in agreement with other studies on *I. ricinus* and *A. americanum* [234,235,571]. This suggests alpha-gal glycosylated proteins to be conserved among these species. We observed an increase in the intensity of bands from salivary proteins which crosslinked with anti-alpha gal antibodies as feeding time increased. Interestingly, gut proteins also showed a similar result with the development of another protein band, particularly in the partially fed guts at 70-100kDa. This 70-100kDa band disappears in *R. evertsi* guts. In *A. hebraeum* guts both the 130kDa and 70-100kDa bands disappear when fully fed. This demonstrates a system of differential protein expression of alpha-gal glycosylated proteins during feeding [508,573]. Studies by Park et al. (2020) show that during feeding, the repertoire of salivary gland N-glycans with alpha-gal increases by 16% in female and 8% in male ticks [344]. Hence alpha-gal glycosylation of proteins in ticks may be responsible for the regulation of feeding.

When feeding on a non-primate mammalian host, this increase in alpha-gal in salivary compounds enables host immune suppression, however, in humans, this has deleterious effects. Since humans generate an immune response against alpha-gal [205,225,549], exposure to tick alpha-gal glycosylated antigens during feeding is most likely to result in alpha-gal sensitization [231]. Our inability to detect alpha-gal in *R. evertsi* unfed guts and salivary glands may be due to the amount of protein loaded being below the level of detection, as Coomassie staining also showed no protein in the lane. We also noted differential expression of alpha-gal in adult vs larval stages of *A. hebraeum* and *R. evertsi* with the larvae only having bands at 130kDa. Despite having detected alpha-gal in unfed larvae, this may have been due to alpha-gal-carrying vitellogenins allowing for transovarial alpha-gal transfer from the adult stage [574].

Hence sensitization can be a consequence of bites from a tick at any developmental stage [302,571].

Immunolocalization of alpha-gal in ticks collected in the vicinity of our alpha-gal allergic patient cohort as well as those reared in the laboratory at different feeding stages showed alpha-gal localisation in salivary glands. This agrees with observations from other studies [574]. The increase in the stained proportion of salivary acini in partially fed ticks can be explained by a sialome switch which increases the production of alpha-gal glycosylated proteins in the salivary glands during feeding [344,575]. Interestingly, unfed male *R. evertsi* stained positive for alpha-gal in undefined cell structures reported to contain polysaccharide granules during the feeding secretory cycle of unfed *R. sanguineus* male [576]. Since the ticks used in our study were fed on non-primate mammalian hosts, it is expected to see staining of the blood meal in the partially fed ticks. However, this was not the case. The red-brownish mass observed is likely to be hematin particles from a digested blood meal. This lack of staining agrees with studies showing the capacity of ticks to break down and incorporate host proteins into their own [577,578]. This can be through the use of heme lipoproteins and/or enzymes such as alpha-D-galactosidase [234,508,579].

Immunofluorescence experiments by Fischer et al. (2020) show colocalization of alpha-gal and clathrin staining indicating the adsorption of blood meal during hematophagy by endocytosis in clathrin-coated pits [574]. However, the restricted localization of alpha-gal in salivary glands organized around a lumen into which the saliva is secreted suggests ticks endogenously produce alpha-gal. The expression of the alpha-gal transferase genes b4galt7, a4galt-1, and a4galt-2 by some tick species and the upregulation in the transcription of these genes during feeding [238] suggest elevated levels of the corresponding proteins during feeding to result in a higher level of alpha-gal bearing antigens. We saw an increase in the surface area of stained salivary acini in the partially fed state suggesting increased alpha-gal production during feeding. Interestingly, unfed male ticks (reared in the laboratory) stained for alpha-gal in their testis while this staining was not present in fed male ticks collected from Mqanduli. This can be explained by the likelihood of ticks in the fed state having undergone mating. Studies show that *A. hebraeum* male ticks attach to a host and feed for about six days or until maturity at which point they release pheromones which attract female ticks to attach to the host and start feeding [510,511,572]. *A. hebraeum* female ticks do not attach to a host without these signals from the male. Hence, as females feed, mating occurs, thus fed male ticks release the contents of their testes during the reproductive act. Apart from feeding this implicates alpha-gal as essential for mating in ticks.

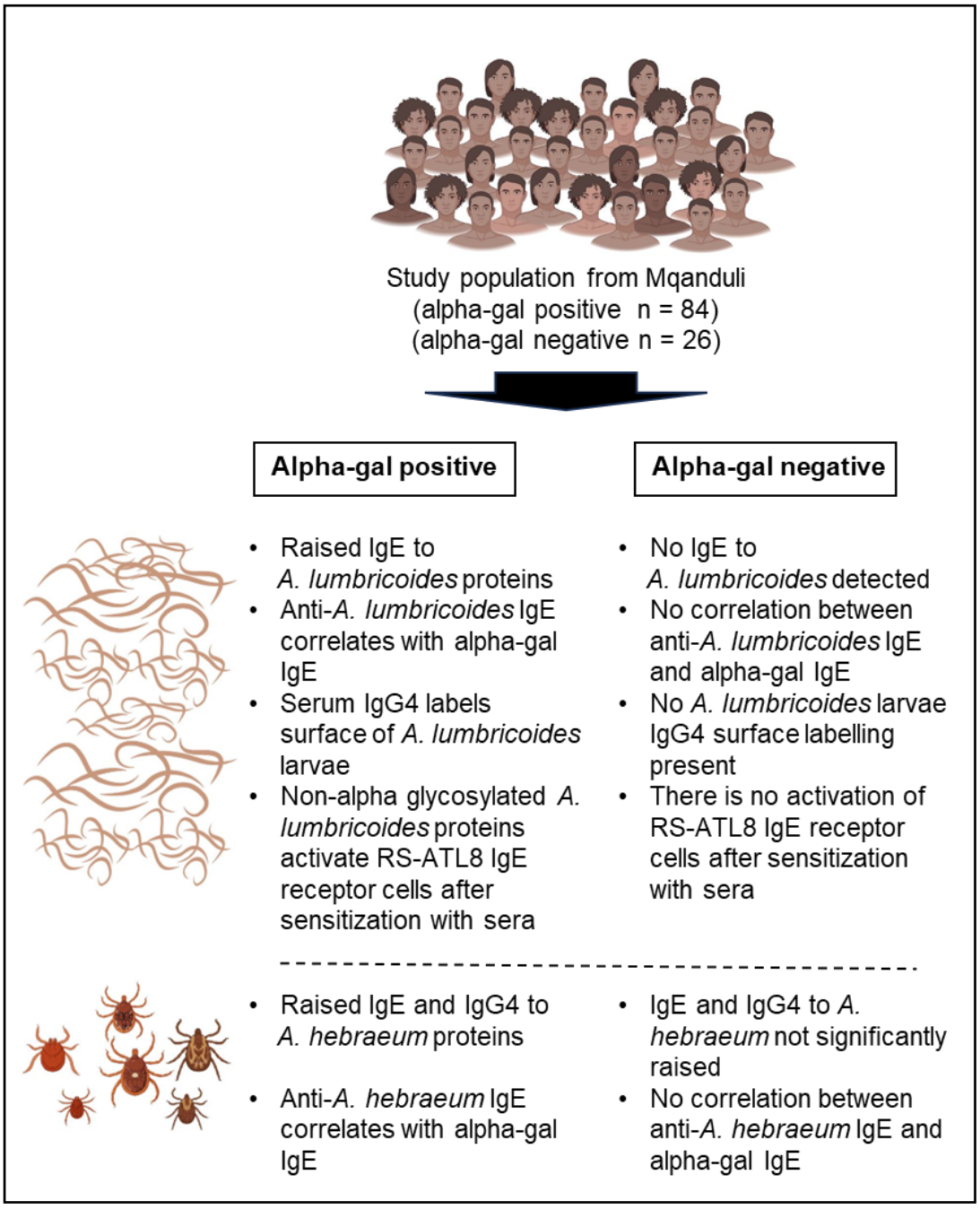
Despite our findings suggesting endogenous alpha-gal production in *A. hebraeum* and *R. evertsi*, other studies point to a possible role of the tick microbiome as an alpha-gal source [324]. Galactose is essential for microbes, serving not only as an energy source but also as a crucial molecule for producing glycosylated exopolysaccharides and lipopolysaccharides, which can act as alpha-gal antigens [580]. Silencing of alpha-D-galactosidase in partially fed ticks resulted in a sixfold decrease in the total bacterial load of partially fed tick tissues [508]. Interestingly, the presence of certain microbes, for example, *Borrelia burgdorferi* and *Borrelia mayonni* [581] in ticks led to a reduction in the relative abundance of galactose while *Anaplasma phagocytophilum* led to an increase in alpha-gal in *I. scapularis* tick cells [238]. Microbes from *Rizobiaceae* and *Caulobacteriaceae* families which infect *A. americanum* also possess a novel lipid A alpha-(1-1)-GalA transferase gene (rgtF) [582,583] which could be necessary for alpha-gal antigen development [582,584]. Although the tick microbiome could also be involved in alpha-gal sensitization during tick feeding, the presence of alpha-gal in ticks obtained not in the wild, but reared on pathogen-free sheep implies that they are unlikely to be the (sole) source of alpha-gal.

In conclusion, this chapter demonstrates the presence of alpha-gal glycosylated proteins in two local South African ticks namely *A. hebraeum* and *R. evertsi*. Alpha-gal staining localises alpha-gal in the salivary glands suggesting alpha-gal sensitization to occur during tick feeding. Detection of alpha-gal in male testes also suggests alpha-gal to be involved in tick reproduction.

OBJECTIVE 2: Investigating the cross-reactivity between serum-from alpha-gal allergic individuals and antigens from ecto- and endoparasites.

Rationale: The development of AGS is associated with exposure to either ticks or helminths. Therefore, we hypothesize patients with AGS have antibodies directed towards parasites responsible for sensitization to alpha-gal.

Graphical abstract



CHAPTER 5: Evaluating the cross-reactivity between serum from alpha-gal allergic individuals and endoparasite antigens

Some of the data presented in this chapter contributed to a larger publication **(Table 5.1 and 5.2, Figure 5a, Figure 5.10)**:

Murangi T, Prakash P, Moreira BP, Basera W, Botha M, Cunningham S, Facey-Thomas H, Halajian A, Joshi L, Ramjith J, Falcone FH, Horsnell W, Levin ME. *Ascaris lumbricoides* and ticks associated with sensitization to galactose α1,3-galactose and elicitation of the alpha-gal syndrome. Journal of Allergy Clinical Immunology. 2022 Feb;149(2):698-707.e3. doi: 10.1016/j.jaci.2021.07.018. Epub 2021 Jul 29. PMID: 34333031

Key findings

- Alpha-gal allergic individuals have raised IgE to *A. lumbricoides* which correlates with alpha-gal IgE.
- IgG4 from alpha-gal allergic patients labels the surface of *A. lumbricoides* larvae.
- Individuals with raised IgE to both *A. lumbricoides* and *A. hebraeum* have significantly higher levels of alpha-gal IgE.
- Non-alpha-gal glycosylated *A. lumbricoides* antigens can stimulate an RS-ATL8 IgE reporter cell system after sensitization with sera from alpha-gal allergic individuals.

5.1. Introduction

Helminths can modulate allergic disease and atopy. Infections may be associated with a lower risk of developing allergic disorders [457,479,585–588]. This has been attributed to the induction of high levels of polyclonal IgE after helminth infection which saturates FcεRI and FcεRII receptors on basophils and mast cells [444,447,589], thus inhibiting allergen-specific IgE crosslinking on these receptors. Other studies report helminth infection to drive an increase in the production of regulatory cytokines such as IL-10 resulting in an immunosuppressive T regulatory response characterized by unresponsiveness to allergen stimulation [454,590,591]. Alternatively, other studies have proposed helminth allergic response suppression to be a bystander consequence of a helminth-induced modified Th2 response which upregulates allergen-specific IgG4 production [592]. This competes with IgE for binding and thus inhibits allergen-specific IgE crosslinking with effector cells.

However, this protection is also likely to depend on the chronicity of infection and parasite burdens. Acute helminth infection may stimulate allergic activity [593–595] due to a strong Th2 response and an absence of a sufficiently stimulated regulatory network [503,596]. Helminth invasion and migration also disrupt epithelial surfaces (skin, GI tract, lungs) which trigger the

release of alarmins (IL-33, IL-25 and TLSP) [370–373]. In the initial stages of the immune response to helminths, these cytokines initiate the development of a strong Th2 response which has been implicated in causing wheeze [478,595,597], asthma [587,598] and atopic dermatitis [599] in the infected individuals.

Jogi et al. (2022) reported an increased odds ratio for the development of asthma and decreased lung capacity in men with prior *A. lumbricoides* infection [468]. This corresponds to other studies which implicate high Ascaris IgE [469–473] or active *A. lumbricoides* infection [474–477] as a risk factor for asthma development. Interestingly, antihelminthic treatment in some cases results in the resolution of allergy [444,478,479,599] although other studies show no difference [480]. Therefore, the disruption of a tolerogenic phenotype after *A. lumbricoides* infection [600,601] leading to a hindrance of oral tolerance induction is likely to result in allergic sensitization.

In Chapter 3, we showed the presence of alpha-gal in adult and larval *A. lumbricoides* somatic antigens. We hypothesize antigen presentation of *A. lumbricoides* alpha-gal glycosylated antigens to drive the production of anti-alpha gal IgE. However, studies by Hodžić et al. (2020) show that despite the presence of alpha-gal in *Schistosoma mansoni*, it did not induce the production of anti-alpha-gal antibodies [216]. On the other hand, alpha-gal-containing endoparasites such as *Plasmodium falciparum*, *Trypanosomes*, and *Leishmania* induce the production of high levels of IgE [602,603], anti-alpha-gal IgG [604] and anti-alpha-gal IgM [605]. Wilson et al. (2021) reported a positive association between Ascaris IgE, IgG, and IgG4 with alpha-gal IgE among children from Ecuador and Kenya [503].

In this chapter, we seek to investigate the role of *A. lumbricoides* in causing sensitization to alpha-gal in our cohort.

5.2. Results

5.2.1. Sera from alpha-gal positive individuals bind to mammalian meat and cat dander

Alpha-gal allergy is characterized by raised IgE levels to mammalian meat. We assessed this by carrying out an ImmunoCap test on patient sera to alpha-gal, beef, pork, lamb, cat dander and cat serum albumin. Alpha-gal IgE and alpha-gal IgE: total IgE ratio was significantly ($p < 0.01$) raised in the oral food challenge-proven alpha-gal allergic group **(Table 5.1)**. IgE to beef, pork, lamb, and cat dander in sera from the alpha-gal positive group was significantly elevated in comparison to the non-allergic group **(Figure 5.1)**. There was no raised IgE in both groups against cat serum albumin.

Table 5.1 Description of control vs alpha-gal allergy participants

Category		Control n=26	Alpha-gal allergy n=84	P-value
Self-reported exposure, n (%)	Tick bite	1 (3.9)	9 (10.7)	0.29*
	Scabies	14 (53.9)	39 (46.4)	0.65*
	Worms	8 (30.8)	22 (26.2)	0.63*
	Schistosomiasis	2 (7.7)	8 (9.5)	1.00*
Alpha-gal IgE kU/L, median (IQR)		0.5 (0–1.2)	12.0 (4.2–33.4)	<0.01**
Alpha-gal IgE: total IgE ratio, median (IQR)		0.1 (0-0.4)	4.2 (1.9–11.0)	<0.01**

IQR, Interquartile range
*P value calculated using Fischer's exact
**P- value calculated using the Mann-Whitney U test

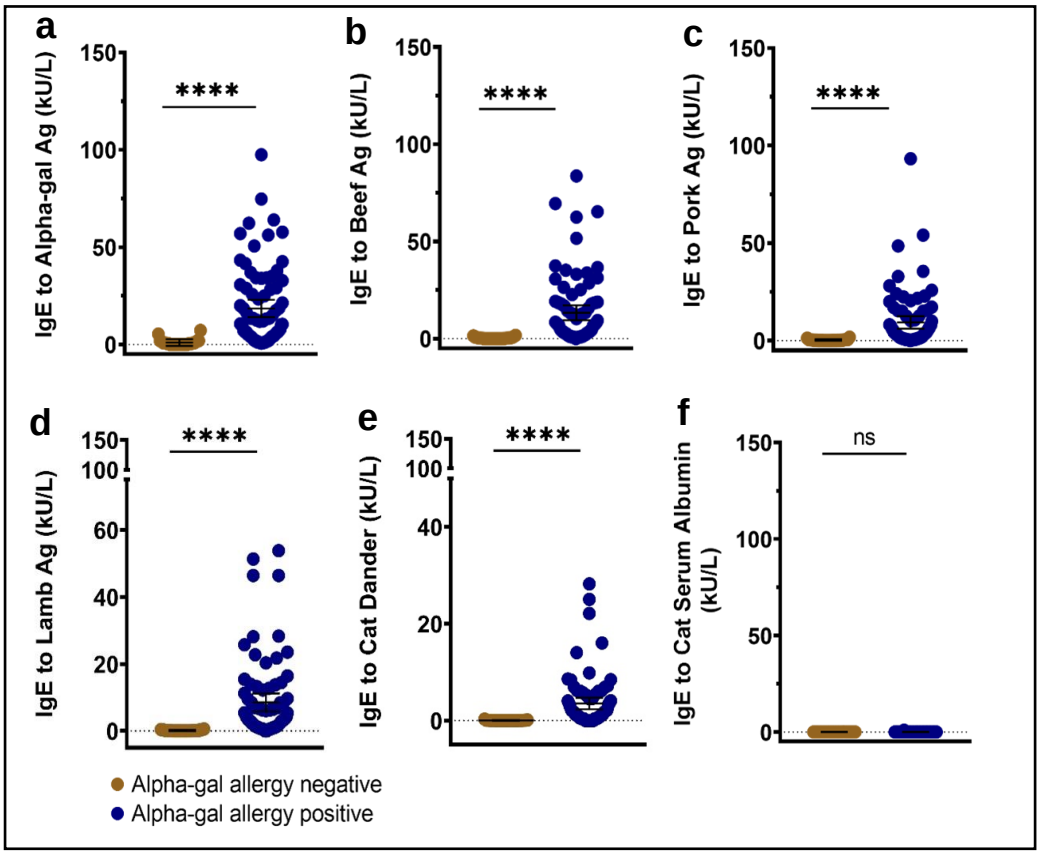


Figure 5.1: Alpha-gal positive patient serum IgE binds to mammalian meat and cat dander extracts. Patient sera from alpha-gal allergic (n = 81) and non-allergic patients (n = 26) was assessed for IgE to **(a)** alpha-gal **(b)** beef **(c)** pork **(d)** lamb **(e)** cat dander and **(f)** cat serum albumin using ImmunoCap 100 instrument. A value of ≥0.35kU/L (dotted line on the

graphs) was the lower detectable level of sensitization. Statistical significance was calculated by the Mann-Whitney U t-test. **** $p \leq 0.0001$

5.2.2. Anti *A. lumbricoides* IgE is elevated in the alpha-gal allergic group

To assess the source of alpha-gal sensitization in our cohort we asked participants if they recalled any exposure to ticks, scabies, worms and schistosomes. From the questionnaires self-reported exposure to endo- and ectoparasites was not significantly different between the alpha-gal allergic and non-allergic group **(Table 5.1)**. Secondly, we carried out an ImmunoCAP using *A. lumbricoides* adult antigen. There was significantly raised levels of anti-*A. lumbricoides* IgE ($p = 0.01$) and a high Ascaris IgE: total IgE ratio ($p<0.001$) in sera from the alpha-gal allergic group in comparison to the non-allergic control group **(Table 5.2)**.

Previously (chapter 3), we hypothesized that sensitization to alpha-gal may occur during *A. lumbricoides* larval migration through the host. Using an ELISA, we assessed for IgE and IgG4 to *A. lumbricoides* larval proteins and recombinant ABA-1. Sera from alpha-gal allergic individuals had elevated IgE to both *A. lumbricoides* larval proteins and rABA-1 **(Figure 5.2a and c)**. There was no raised anti-*A. lumbricoides* larvae IgE in the alpha-gal negative group. IgG4 was elevated in both the alpha-gal allergic and non-allergic groups **(Figure 5.2b and d)**. IgG4/IgE ratio to *A. lumbricoides* larval and adult antigens was significantly elevated in the alpha-gal negative group in comparison to the alpha-gal allergic group **(Figure 5.2e and f)**.

To assess the relevance of anti-*A. lumbricoides* IgE we assessed its correlation with anti-alpha-gal IgE and total IgE in both the allergic and non-allergic groups. There was a positive correlation between alpha-gal IgE and total IgE in the alpha-gal allergic group. In the alpha-gal allergic group, a positive correlation existed between anti-*A. lumbricoides* larval IgE and total IgE **(Figure 5.3a and b)**. A positive correlation was also found between anti-*A. lumbricoides* (both larval and adult) IgE and alpha-gal IgE **(Figure 5.3c and d)** in the alpha-gal allergic group. Correlations between IgE from the alpha-gal negative group with either alpha-gal IgE or total IgE were not significant.

Interestingly, three-dimensional analysis of the relationship between *A. lumbricoides* IgE and IgG4 with anti-alpha-gal IgE showed a trend for reduced *A. lumbricoides* IgG4 to coincide with elevated *A. lumbricoides* IgE and anti-alpha-gal IgE **(Figure 5.4b)**.

Table 5.2: Ascaris IgE (kU/L) in control vs alpha-gal allergy participants

Category	Control n=26	Alpha-gal allergy n=84	P-value
Ascaris IgE kU/L, median (IQR)	0.25 (0.03-1.13)	1.12 (0.47-2.59)	0.01
Ascaris IgE: total IgE ratio, median (IQR)	0.08 (0.05-0.10)	0.17 (0.09-0.31)	<0.001

IQR, Interquartile range

P- value calculated using Mann Whitney U test

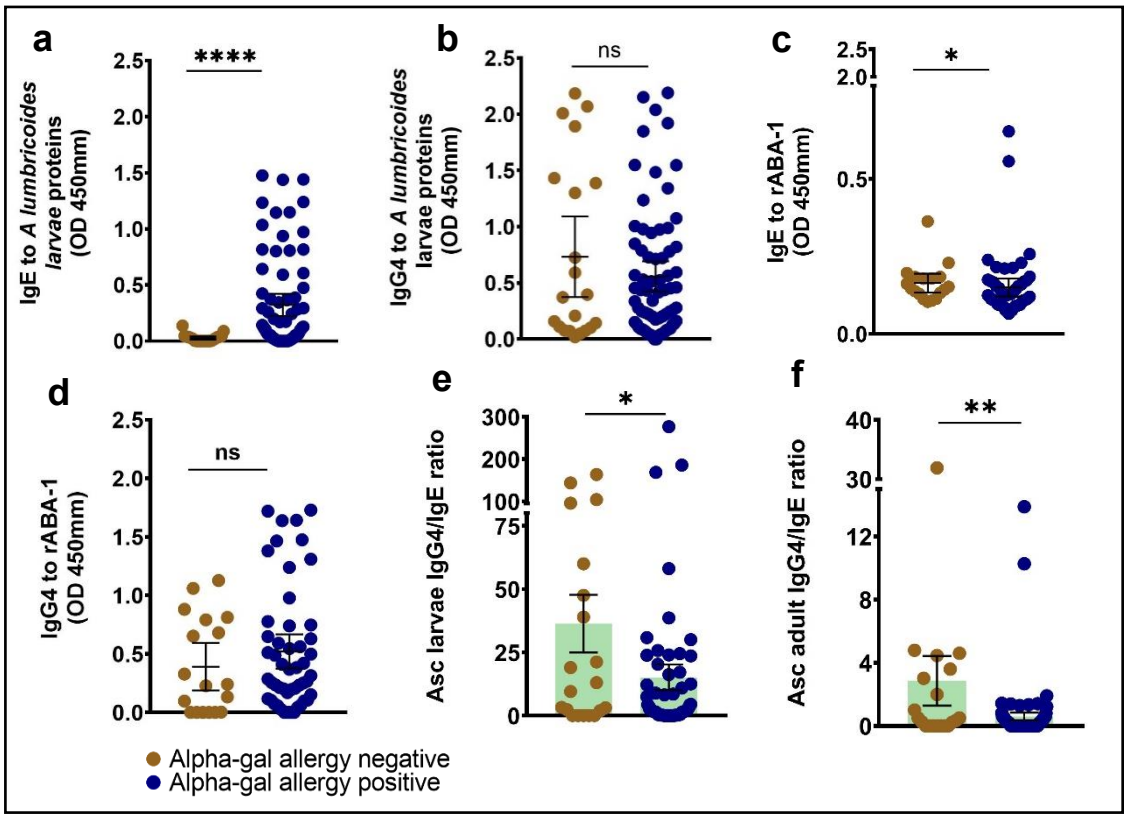


Figure 5.2: Serum IgE from alpha-gal allergic individuals binds to *A. lumbricoides* proteins. Sera from alpha-gal allergic (n = 72) and non-allergic patients (n = 21) were analyzed for *A lumbricoides* **(a)** larval IgE **(b)** larval IgG4 **(c)** rABA-1 IgE and **(d)** rABA-1 IgG4 by ELISA. IgG4/IgE ratio was also assessed *for A lumbricoides* **(e)** larvae and **(f)** adult proteins. Experiments were carried out in triplicates (mean±sem). Statistical significance was calculated by the Mann-Whitney U test. * $p \leq 0.05$, **** $p \leq 0.0001$

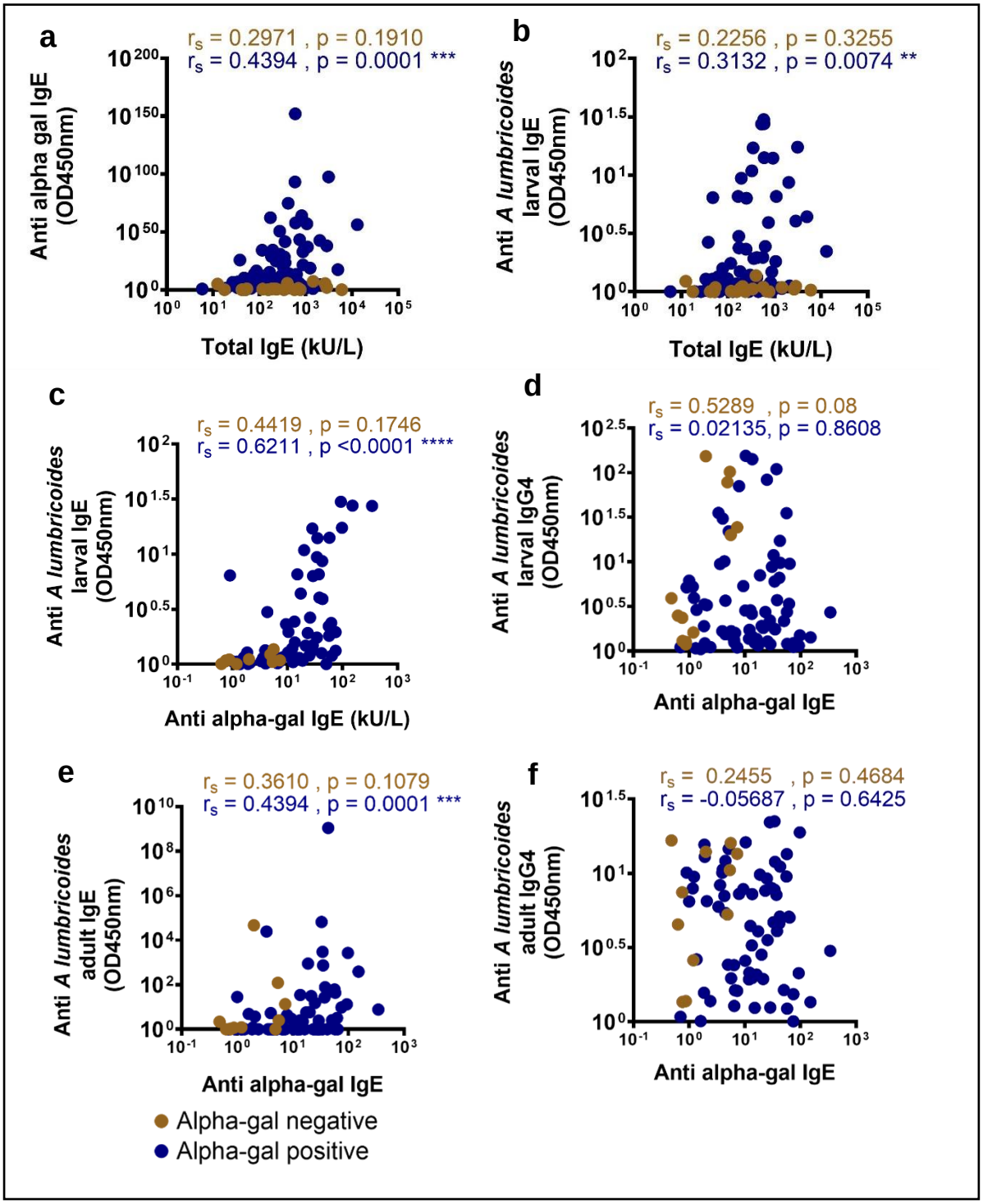


Figure 5.3: There is a correlation between anti-*A lumbricoides* IgE with anti-alpha-gal IgE and total IgE in alpha-gal allergic individuals. Spearman correlation analysis of total IgE against **(a)** anti-alpha-gal IgE and **(b)** anti-*A lumbricoides* larval IgE as well as anti-*Ascaris lumbricoides* **(c)** larval IgE **(d)** larval IgG4 **(e)** adult IgE and **(f)** adult IgG4 against anti-alpha-gal IgE in sera from alpha-gal allergic (n = 72) and non-allergic (n =21) patients. Experiment was carried out in triplicates (mean±sem). Statistical significance was calculated by Spearman rank correlation (rs). ** p = 0.001, *** p=0.0001, **** p ≤ 0.0001

5.2.3. Serum IgG and IgG4 bind to the surface of *A. lumbricoides* larvae

To further assess the possibility of alpha-gal sensitization through larval migration, we investigated the ability of sera immunoglobulin from allergic and non-allergic participants to bind to *A. lumbricoides* larvae by immunofluorescent imaging. IgG in sera from both allergic and non-allergic participants bound to the surface of *A. lumbricoides* larvae **(Figure 5.4)**. In the absence of sera, no IgG binding was observed. Serum IgG4 in the alpha-gal allergic group bound to *A. lumbricoides* larvae surface **(Figure 5.5)**. When the larvae were incubated with sera from non-allergic individuals no IgG4 surface labelling was detected. When we assessed for IgE binding to the larval surface, no binding was detected in all the groups due to high levels of background staining and non-specific binding (data not shown). This may be attributed to the capacity of IgG antibodies to compete with IgE for binding sites on allergens and cell surface receptors [606–608]. One mechanism by which IgG antibodies outcompete IgE is via steric hinderance. IgG antibodies have the capability to attach to allergens and effectively outcompete IgE, thus preventing its attachment to cell surface receptors like FcεRI [606]. Also both IgG and IgE antibodies can contest for the same epitopes on allergens, resulting in a direct competition for binding sites [608]. Ideally, depletion of IgG antibodies in sera prior to *A. lumbricoides* staining for IgE surface labelling may yield a better result.

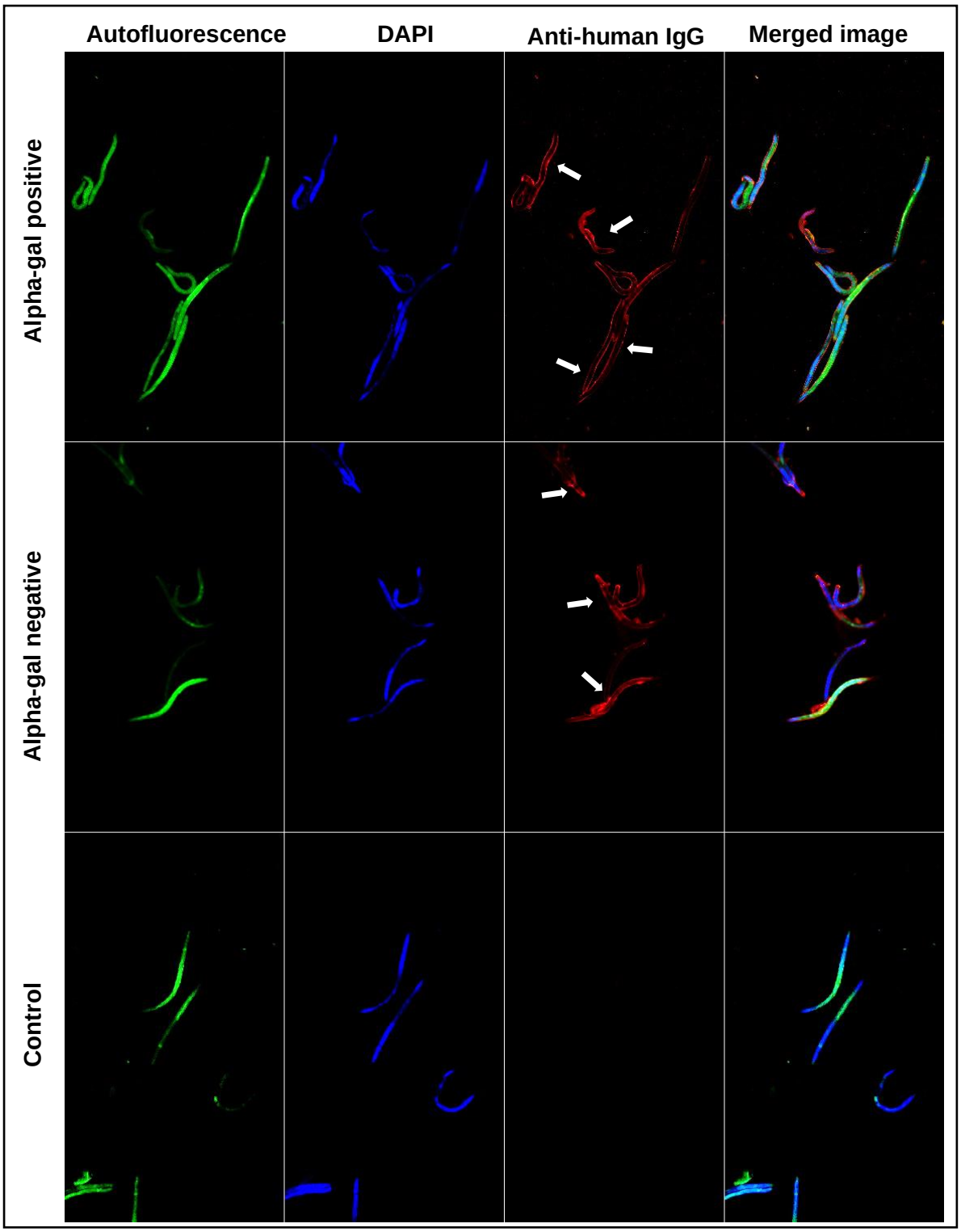


Figure 5.4: Serum IgG from alpha-gal allergic and non-allergic individuals binds to the surface of *A lumbricoides* larvae. Formalin-fixed larvae were incubated with patient sera overnight and stained with Alexa floura 647 anti-human IgG. Sera binding was imaged in the red channel. White arrows show the presence of sera surface labelling.

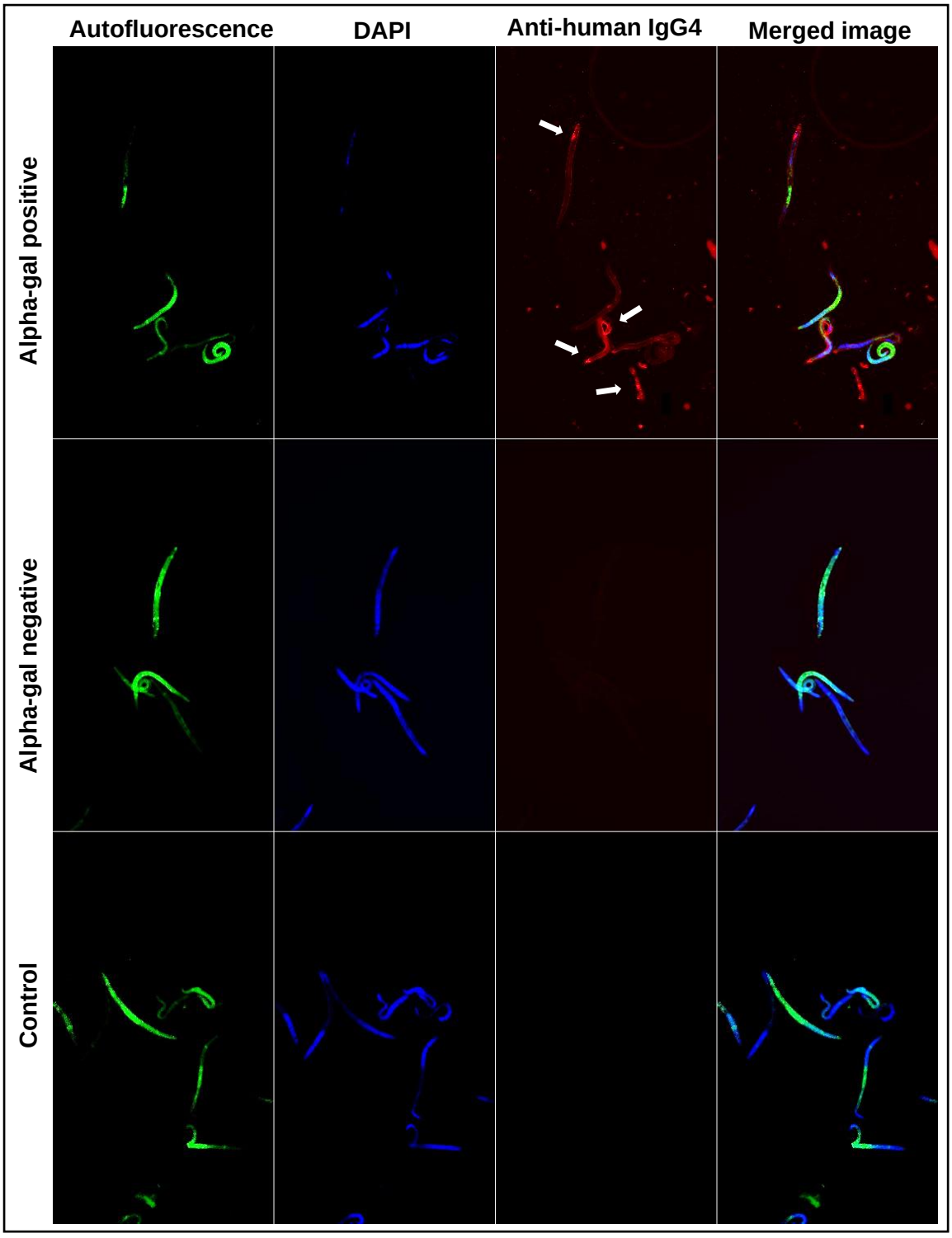


Figure 5.5: Serum IgG4 from alpha-gal allergic individuals binds to the surface of *A lumbricoides* larvae. Formalin-fixed larvae were incubated with patient sera overnight and stained with Alexa floura 647 anti-human IgG4. Sera binding was imaged in the red channel. White arrows show the presence of sera surface labelling.

5.2.4. Co-sensitized patients show significantly higher levels of alpha-gal IgE

To assess if binding to *A. lumbricoides* proteins was due to cross-reactive alpha-gal epitopes, we competitively inhibited anti-alpha-gal IgE from serum by incubating sera with bovine thyroglobulin (an alpha-gal-rich protein). This is an established method for impairing anti-alpha-gal IgE in patient serum from binding to the alpha-gal antigen [242,255,272,609]. Comparison of the ability of BTG-treated sera and untreated sera to bind *A. lumbricoides* antigen revealed a clear reduction in the binding to *A. lumbricoides* antigens in the BTG treated sera. However, IgE binding to *A. lumbricoides* antigens was still present in sera from some individuals **(Figure 5.6)**.

We sought to investigate the possibility of co-sensitization to multiple parasites in our cohort and if there were any differences in anti-alpha-gal IgE related to polysensitization. Using an ELISA, we assessed for the presence of IgE to *A. hebraeum* proteins. Three-dimensional analysis showed a trend for increased IgE to *A. lumbricoides*, *A. hebraeum* and alpha-gal in the alpha-gal allergic group **(Figure 5.7b)**. However, this trend may be due to the presence of alpha-gal in both parasite antigens. We then grouped our cohort into four groups based on IgE to either *A. lumbricoides, or A. hebraeum*, both or none **(Figure 5.8a and b)**. Participants with IgE to both *A. lumbricoides* and *A. hebraeum* proteins had significantly higher levels of anti-alpha-gal IgE than the control group and individuals with only anti-*A. hebraeum* IgE **(Figure 5.8c).** The levels of anti-alpha-gal IgE were not different in the groups with either anti-*A. lumbricoides* IgE or anti-*A. hebraeum* IgE only **(Figure 5.8c)**. Individuals with no IgE to both *A. hebraeum* or *A. lumbricoides* showed no raised IgE to alpha-gal.

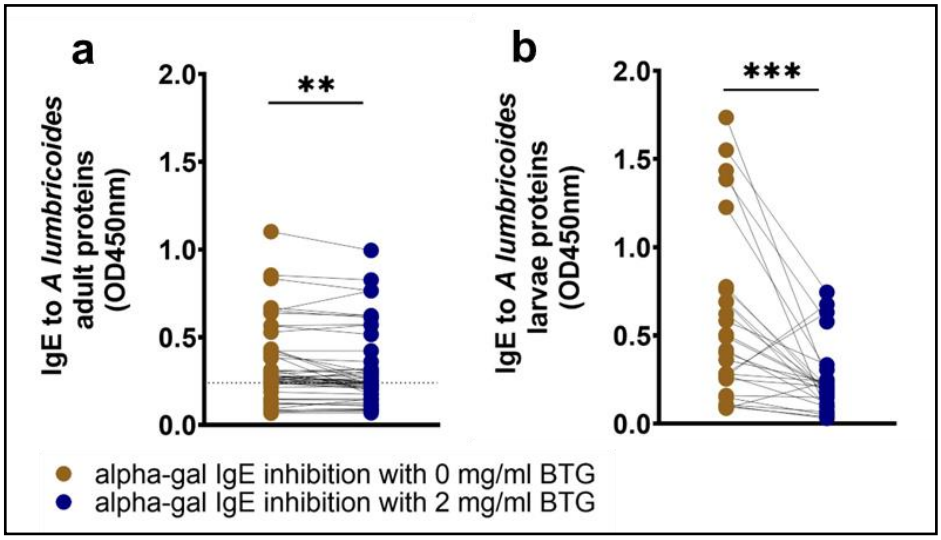


Figure 5.6: IgE binding to *A. lumbricoides* is reduced after competitive inhibition of anti-alpha-gal IgE with BTG. Sera (n = 51) was incubated with and without 2mg/ml of alpha-gal rich bovine thyroglobulin (BTG) onto ELISA plates coated with 50µg/ml of **a)** adult and **b)** larval

Ascaris lumbricoides protein. Experiment was carried out in triplicates (mean±sem). Statistical significance was calculated by the Wilcoxon test. ** p ≤ 0.01. Dotted line indicates the cut-off for background binding.

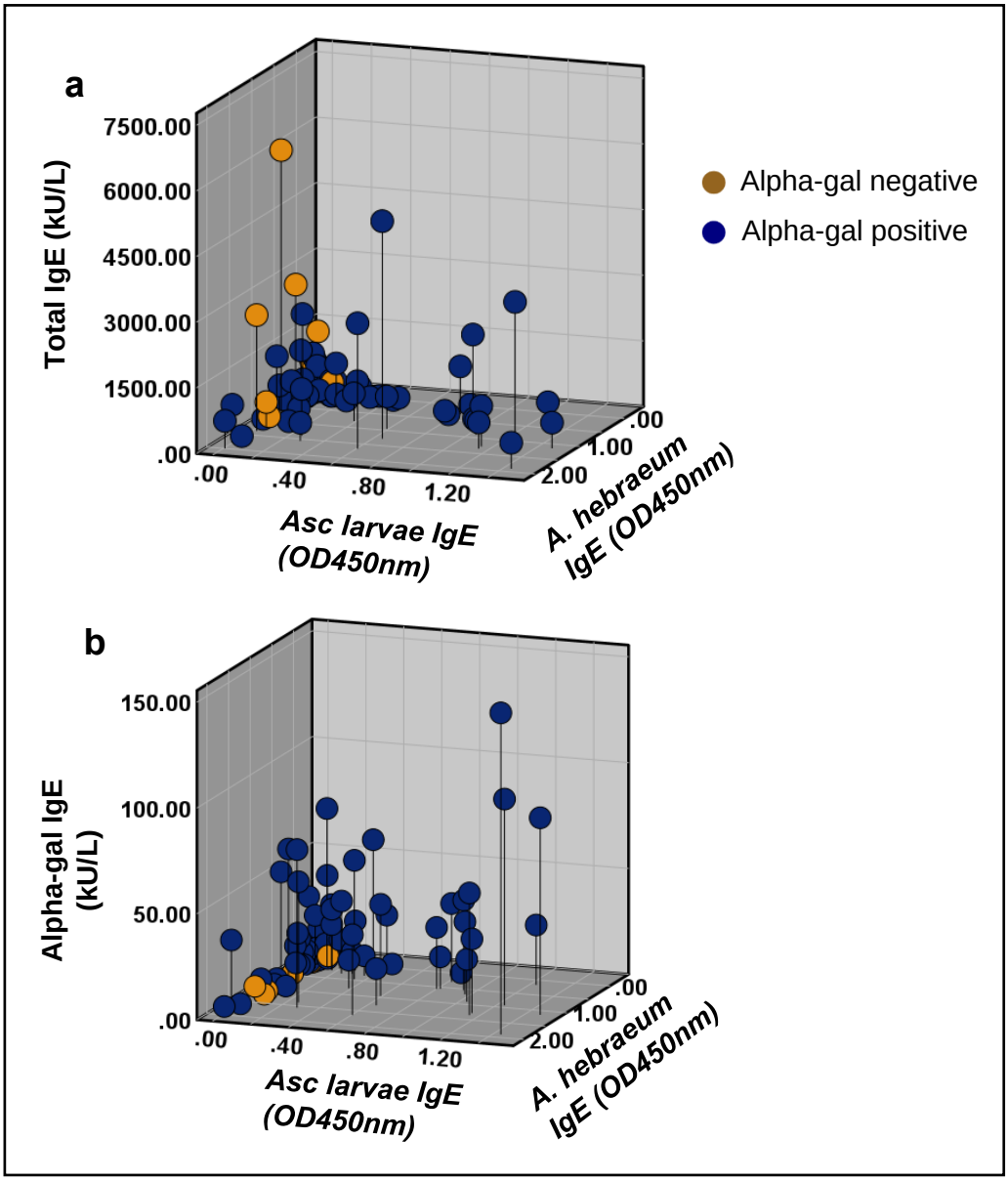


Figure 5.7: Alpha-gal allergic Individuals have elevated IgE to alpha-gal, *A. hebraeum* and *A. lumbricoides* larval antigens. Three-dimensional analysis of the relationship between IgE to *A. lumbricoides* and *A. hebraeum* together with **a)** total IgE and **b)** anti-alpha-gal IgE in sera from alpha allergic (n = 72) and non-allergic (n =21) patients. Experiment was carried out in triplicates (mean±sem).

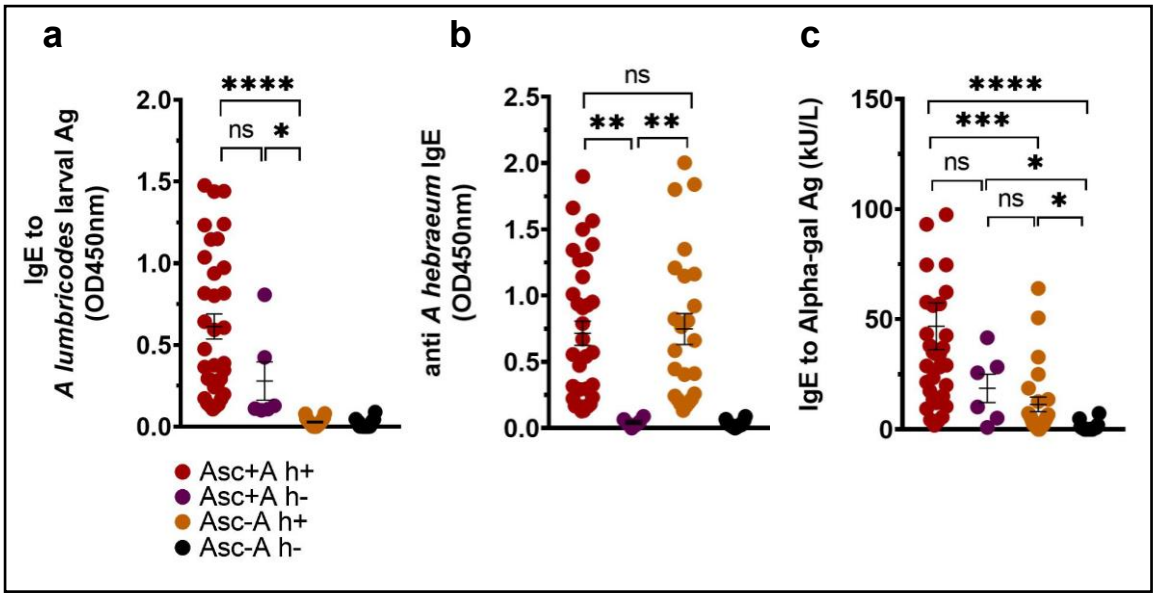


Figure 5.8: Patients with *A. lumbricoides* and *A. hebraeum* co-infections have significantly elevated anti-alpha-gal IgE while single infections have significant anti-alpha-gal IgE levels in comparison to the non-infected group. To assess the effect of exposure to a parasite and sensitization to alpha-gal we stratified our cohort based on IgE to **a)** *A. lumbricoides* larvae (Asc) and **b)** *A. hebraeum* (Ah) into four groups Asc+Ah+ (n=34), Asc+Ah- (n=6), Asc-Ah+(n=24) and Asc-Ah- (n=16). We then assessed IgE to **c)** Alpha-gal in these groups. Experiment was carried out in triplicates (mean±sem). Statistical significance was calculated by the Mann-Whitney U test. * $p \leq 0.05$,*** $p \leq 0.001$, **** $p \leq 0.0001$.

5.2.5. *A. lumbricoides* non-alpha-gal glycosylated antigens activates the RS-ATL8 IgE reporter cell line

Using a RS-ATL8 IgE reporter cell assay we investigated the potential of both native *A. lumbricoides* E/S and recombinant antigens to induce an allergic reaction. As expected, there was no difference between the allergic and non-allergic groups in the negative unstimulated cells **(Figure 5.9a)** and the positive controls stimulated with concanavalin A and anti-human IgE **(Figure 5.9b and c)**. Stimulating sensitized cells with *A. lumbricoides* E/S, rABA-1 and rtropomyosin resulted in elevated luciferase activity in cells sensitized with sera from the alpha-gal allergic group **(Figure 5.9d, e and f)**. This was more significant after treatments with *A. lumbricoides* E/S and rABA-1. Sensitization with sera from alpha-gal non-allergic individuals resulted in no activity after stimulation with *A. lumbricoides* native and/or recombinant proteins.

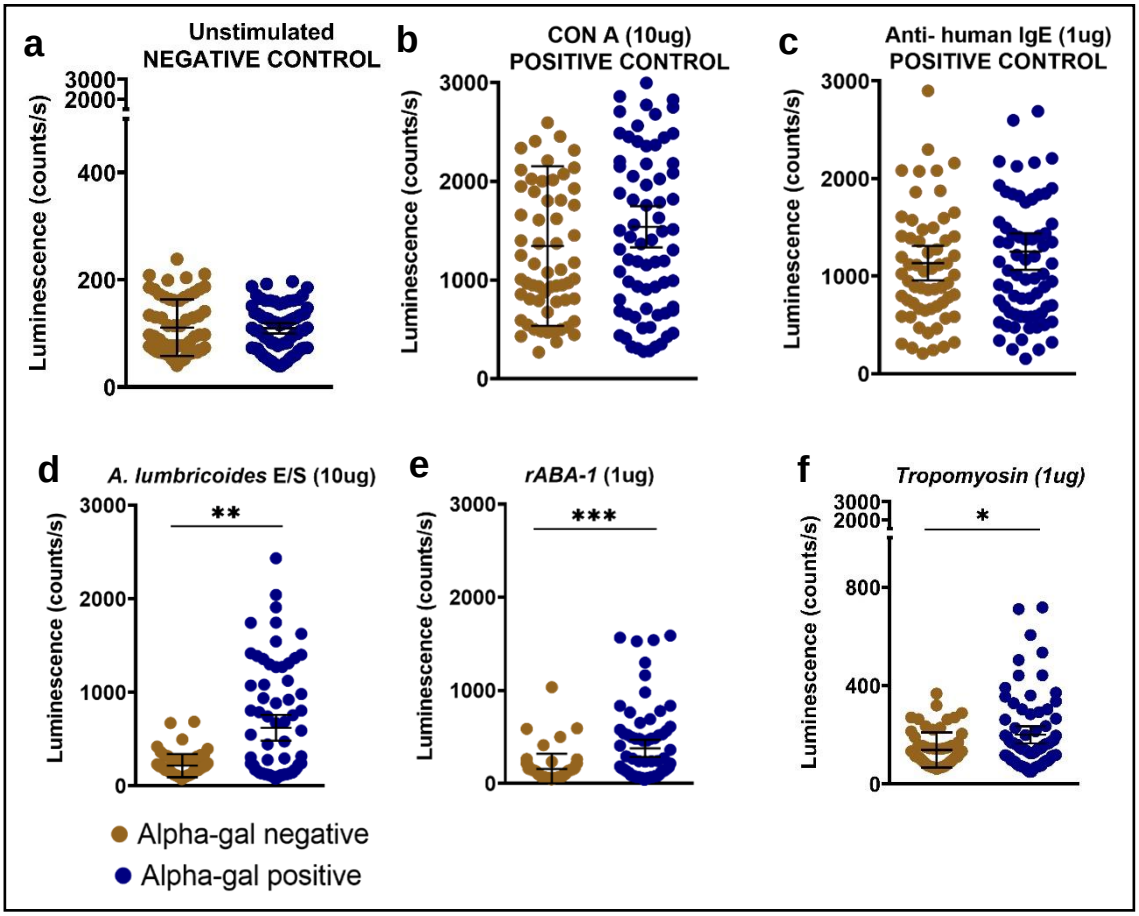


Figure 5.9: Non-alpha-gal glycosylated proteins stimulate IgE reporter cells sensitized with alpha-gal allergic serum. Luminescence from RS-ATL8 reporter cells after serum sensitization in the presence of subsequent treatments with **(a)** no stimulation as the negative control, **(b)** stimulation with the positive controls concanavalin A, and **(c)** anti-IgE, and test antigens **(d)** native *A. lumbricoides* E/S, **(e)** recombinant Asc l 1 and **(f)** recombinant Asc l 3.

Experiments were carried out in triplicates (mean±sem). Statistical significance was calculated by the Mann-Whitney U test. * $p \leq 0.05$,** $p \leq 0.00$, *** $p \leq 0.001$

5.3. Discussion

Helminth infections are strong inducers of allergic and immunomodulatory responses [610–612]. In this chapter, we look at the relationship between alpha-gal allergy and *Ascaris lumbricoides* infection. Firstly, IgE from alpha-gal positive individuals bound to mammalian meat and cat dander, but not cat serum albumin. The difference in response to cat dander and not cat serum albumin can be explained by the presence of an alpha-gal epitope on Fel d 5 antigen which is a part of cat dander [248,504,613]. Cat serum albumin on the other hand does not contain alpha-gal hence IgE from both allergic and non-allergic individuals does not bind to it (unless they have independent sensitization to cat serum albumin). The presence of alpha-gal in mammalian meat [260,301,614–617] is the driver of an IgE response towards this epitope in individuals with alpha-gal allergy hence the elevated IgE binding to mammalian meat antigens among the alpha-gal allergic group. However, for this allergic reaction to occur one has to first be sensitized to alpha-gal.

In our study, there was no difference in self-reported exposure to either ticks or worms in allergic and non-allergic patients, however, participant recall and covert infection make this an unreliable measure of prior exposure. IgG4 is a marker for chronic infection [450–453,502,618,619] and has been used in some studies to describe *A. lumbricoides* exposure [468,620]. Studies by Turner et al. (2005) demonstrated an increase in IgG4 to correlate with *A. lumbricoides* infection in children between the ages of 4-11 years [592]. We report elevated anti *A. lumbricoides* IgG4 in both alpha-gal allergic and non-allergic individuals although there is no significant difference between these groups. This suggests a high burden and chronic *A. lumbricoides* infection in our cohort as is common in an area with a low socio-economic status [621]. Our cohort is based in a rural environment with poor sanitation which encourages *A. lumbricoides* infection via oral faecal contamination. IgG4 is also associated with tolerance or mild symptoms depending on the IgG4/IgE ratio [251,502,618,622,623]. A study by Nkurunungi et al. (2021) demonstrates a low IgG4/IgE ratio in asthmatic children in comparison to the non-asthmatic group and also shows an inverse relationship between skin prick test and IgG4/IgE ratio [623]. This can be due to helminths being potent inducers of polyclonal IgE [611] which can modulate immediate hypersensitivity reactions by saturating high-affinity FcεRI receptors on mast cells and basophils [444,446,447] or due to reverse causation with atopic individuals differing in their response to parasite infestation. However, an increase in IgG4/IgE ratio in patients receiving immunotherapy for allergic rhinitis did not cause any change in clinical manifestations after 6 months in another study in Taiwan [624]. This may suggest IgG4/IgE relevance is dependent on

time as the study by Nkurunungi et al. (2021) was based on samples collected over three years in the rural cohort and one year for the urban cohort [623]. In our cohort, the alpha-gal negative group had a significantly higher *A. lumbricoides* IgG4/IgE ratio which suggests a tolerogenic phenotype in comparison to the alpha-gal allergic group.

Acute helminth infections have been suggested to cause allergic sensitization due to the initiation of a strong Th2 response which triggers the production of anti-Ascaris IgE. This anti-helminth response has been associated with the development of asthma and atopy [469–473,477,595,625–628], induction of an immune response to bystander antigens [596] and exacerbation of allergic reactions particularly HDM [627,628]. According to Ogilvie et al. (1982), it is the pulmonary phase of *A. lumbricoides* infection which causes IgE-mediated hypersensitivity [629]. As such, it is expected for antibodies from individuals with *A. lumbricoides* infection to bind *A. lumbricoides* larvae antigens. Hence, surface labelling of *A. lumbricoides* larvae with IgG4 by sera from alpha-gal allergic patients suggests a mechanism for alpha-gal sensitization. Interestingly, sera from non-alpha-gal allergic individuals did not show IgG4 surface labelling of *A. lumbricoides* larvae. We suggest raised IgG4 in ELISA by sera from non-alpha gal allergic individuals to be towards *A. lumbricoides* larval proteins that are not expressed on the larvae's surface. For example, ABA-1 found in the pseudocoelomic fluid and released by both adult and larval *A. lumbricoides* [440,630–632]. Studies by Xia et al. (2000) demonstrate ABA-1 mRNA to be confined to gut proteins and not in the body wall and/or reproductive tissues of adult *A. lumbricoides* [630]. Hence, we also demonstrate non-alpha-gal allergic individuals to have raised IgG4 towards rABA-1. Despite serum IgG4 surface labelling of *A. lumbricoides* larvae only happening after incubation with sera from the alpha-gal allergic group, surface labelling with IgG was present after incubation with sera from both groups. This is due to all humans naturally producing anti-alpha gal IgG against enteric bacteria such as *Escherichia coli* and *Klebsiella* [549]

We report significantly higher anti-*A. lumbricoides* IgE in the alpha-gal allergic group. Wilson et al. (2021) demonstrated anti-*A. lumbricoides* IgE to be more prevalent (80.5%In vs 40.7%; P <0.001) in alpha-gal sensitized children from Ecuador [503]. Arkestl et al. (2011) reported elevated anti-alpha-gal IgE in 85% of study participants from Zimbabwe with helminth exposure [504]. In Europe 3 out of 5 alpha-gal allergic patients also had elevated IgE to *A. lumbricoides* antigen [499]. Anti-alpha-gal IgE has been shown to positively correlate with anti-*A. lumbricoides* IgE [503]. This is the same as our results which show IgE to both larval and adult *A. lumbricoides* proteins to have a positive correlation with anti-alpha-gal IgE. Since we have previously shown the presence of alpha-gal epitopes in both the larval and adult stages of *A. lumbricoides*, we suggest subjects with primary sensitization to alpha-gal may show elevated IgE against *A. lumbricoides* during ELISA and ImmunoCAP testing as in the case with

mammalian meat and cat dander. However, this cannot account for individuals with elevated IgE to alpha-gal and with no crosslinking to *A. lumbricoides*. Competitive inhibition of anti-alpha-gal IgE with bovine thyroglobulin reduced IgE serum binding intensity to *A. lumbricoides* proteins. This shows that optimal binding to *A lumbricoides* antigen by sera from alpha-gal allergic patients requires alpha-gal. However, IgE against rABA-1, which is not alpha-gal glycosylated, was also significantly raised in the alpha-gal allergic group. This may suggest, a more complex interaction with antigen which could be due to other alpha-gal effects such as direct glycosylation of IgE.

Studies investigating the relevance of *A. lumbricoides* in causing alpha-gal sensitization negate the influence of possible co-sensitization in their cohorts [499,503]. Based on IgE levels we investigated the relevance of co-exposure to *A. lumbricoides* and *A. hebraeum* in causing alpha-gal sensitization. Interestingly, significantly higher levels of anti-alpha-gal IgE were present in individuals in the co-exposed group in comparison to participants with IgE to *A. hebraeum* only. This suggests that *A. lumbricoides* infection exacerbates alpha-gal sensitization in patients exposed to *A. hebraeum* and vice versa. We also noted significant anti-alpha-gal IgE levels in individuals with single parasite infections in comparison to the non-parasite exposed group. Thus, suggesting *A. lumbricoides* infection drives allergic sensitization to alpha-gal.

Lastly, we present data showing that *A. lumbricoides* E/S antigens and rABA-1 antigens are capable of inducing an allergic cellular response in the presence of serum from patients with alpha-gal allergy. The RS-ATL8 assay has high sensitivity which allows for the detection of allergen-specific IgE at a concentration of as little as 15pg/ml [516]. Unlike an ImmunoCAP test, which only detects serum IgE binding to allergens [633], an RS-ATL8 IgE reporter cell system cannot be stimulated by clinically irrelevant IgE which binds to cross-reactive carbohydrates [634–636]. To date, this assay has been used in studies investigating egg allergy, *S. mansoni* infection and screening for candidate vaccines [517,637,638]. Neither *A. lumbricoides* E/S nor recombinant proteins ABA-1 and tropomyosin are glycosylated with alpha-gal. The absence of alpha-gal in *A. lumbricoides* E/S is demonstrated through blotting experiments shown in Chapter 3. Recombinant allergens were produced in human HEK293-6E cells, which do not possess the enzymatic machinery to produce alpha-gal. Since rABA-1 is a marker of *A. lumbricoides* infection [639], RS-ATL8 luciferase reporter assay activation is indicating true *A. lumbricoides* exposure (ie, past and/or current infection) rather than cross-reactivity with alpha-gal in this cohort. This further strengthens the likelihood of a causal link between *A. lumbricoides* infection and sensitization to alpha-gal.

Together we show elevated IgE to *A. lumbricoides* proteins and surface labelling of *A. lumbricoides* larvae with IgG4 in sera from alpha-gal positive patients as well as the activation of RS-ATL8 IgE reporter cell line sensitized with sera from alpha-gal allergic patients after stimulation with *A. lumbricoides* native E/S and rABA-1. This presents infection with *A. lumbricoides* as a potential source of sensitization to alpha-gal via disruption of epithelial barriers during larval migration which skews the immune system towards a Th2 environment characterised by the production of anti-alpha-gal IgE.

CHAPTER 6: Evaluating the cross-reactivity between serum from alpha-gal allergic patients and ectoparasite antigens

Key findings

- Alpha-gal allergic individuals have raised IgE and IgG4 to *A. hebraeum*.
- There is a correlation between IgE to *A. hebraeum* and alpha-gal IgE in the allergic group.
- Inhibition of alpha-gal IgE binding decreases serum IgE binding to *A. hebraeum* and *R. evertsi* proteins.

6.1. Introduction

The disruption of host skin by tick mouthparts to allow for feeding initiates a cascade of events which culminates in the development of acquired tick resistance. This is initiated by the migration of AAM to the site of tick attachment which induces the upregulation of IL-10, IL-4, and TGF-β promoting a Th2 environment as seen in murine studies [332]. This Th2 phenotype is further augmented by the presence of immunomodulatory molecules in tick saliva such as PGE2 which can induce B cell isotype class switching to IgE-secreting plasma cells [338]. The infiltration of basophils coupled with anti-tick IgE at the site of tick attachment results in crosslinking with tick antigens which induces histamine release [329]. This together with other host homeostatic responses such as epidermal thickening and hyperplasia help in restricting tick attachment during a secondary exposure [329]. However, the strong Th2 response against tick antigens may result in allergic sensitization [231,330].

The morbidity associated with tick bite allergies has been linked to the direct effect of IgE responses to tick salivary antigens containing the alpha-gal epitope [246,256,272,302,343,500,605,640,641]. Subcutaneous exposure to tick salivary antigens induces the development of anti-alpha-gal IgE and is characterized by histamine release upon alpha-gal challenge in zebrafish and alpha-gal knockout mice [230,236,345].

In 1940, the first case of anaphylaxis caused by an allergy to the dog tick *I. holocylus* was reported in Australia [642]. An association between AGS and ticks has been established [234,235,267,309,343,500,508], with initial findings arising from an overlap between patients developing type I hypersensitivity reactions to a colorectal cancer drug, cetuximab and those presenting with delayed hypersensitivity reactions to red meat after bites from the tick *A. americanum* [270]. The induction of alpha-gal IgE in AGS patients after tick bites imply the adjuvant properties of tick saliva [643–645] capable of IgE production against previously harmless substances [266,309,500].

In Chapter 4, we demonstrated for the first time the presence of alpha-gal in local South African tick species *A. hebraeum* and *R. evertsi*. In this chapter, we investigate the relationship between local tick exposure and sensitization to alpha-gal in our cohort.

6.2. Results

6.2.1. Anti *A. hebraeum* IgE and IgG4 are raised in alpha-gal allergic patients.

Exposure to ticks in our cohort was assessed by detecting anti-*A. hebraeum* IgE and IgG4 in serum by ELISA. Anti-*A. hebraeum* IgE and IgG4 were significantly raised in sera from the alpha-gal allergic group in comparison to the non-allergic group **(Figure 6.1)**. There was a moderate level correlation between anti-*A. hebraeum IgE* and total IgE in the allergic group ($p<0.0001$) **(Figure 6.2a)**. Also, a positive correlation between anti-*A. hebraeum* IgE and anti-alpha-gal IgE was observed in the alpha-gal allergic group **(Figure 6.2b)**.

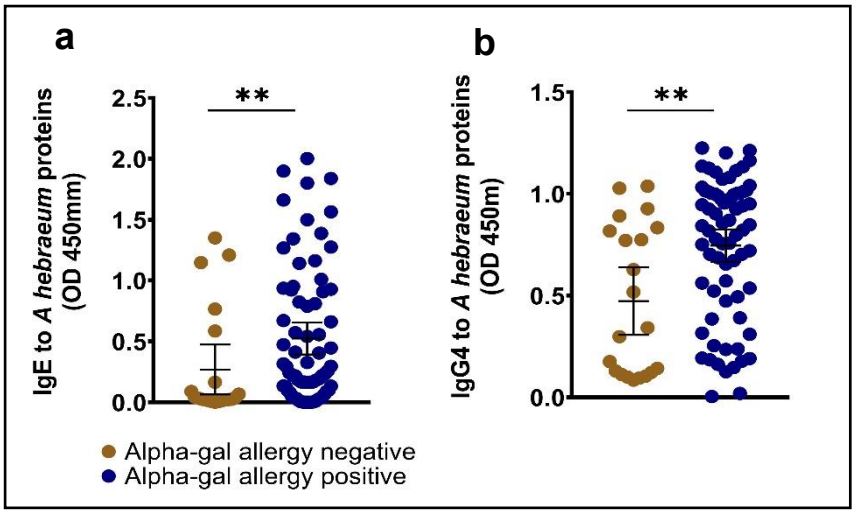


Figure 6.1: Alpha-gal allergic individuals have elevated IgE and IgG4 to *A. hebraeum* proteins. Sera from alpha-gal allergic (n = 72) and non-allergic patients (n = 21) were analyzed for **(a)** IgE and **(b)** IgG4 to *A. hebraeum* proteins by ELISA. Experiments were carried out in triplicates (mean±sem). Statistical significance was calculated by the Mann-Whitney U test. * $p \leq 0.05$, ** $p \leq 0.01$

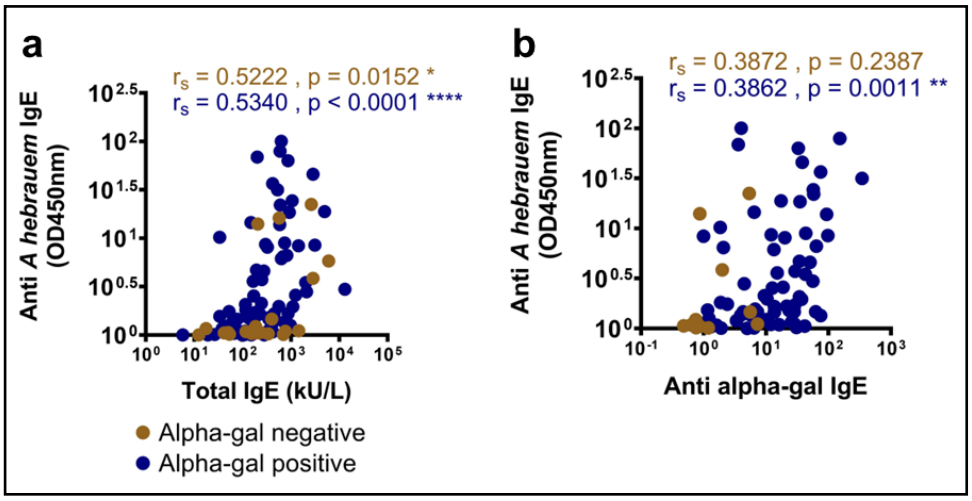


Figure 6.2: There is a correlation between total IgE, anti-alpha gal IgE and anti-*A. hebraeum* IgE in sera from alpha-gal allergic patients. Spearman correlation analysis of **(a)**Total IgE against anti-*A. hebraeum* IgE and **(b)** anti-*A. hebraeum* IgE against anti-alpha-gal IgE in sera from alpha allergic (n = 72) and non-allergic (n =21) patients. Experiments were carried out in triplicates (mean±sem). Statistical significance was calculated by Spearman rank correlation (rs). * $p \leq 0.05$, ** $p \leq 0.01$

6.2.2. Anti *A. hebraeum* IgE and anti-alpha-gal IgE correlation is dependent on age and sex

A positive moderate and high-level correlation between anti-*A. hebraeum* IgE and alpha-gal IgE was observed among patients in the age groups 4-14 years and 26-36 years respectively **(Figure 6.3a)**. Both male and female participants had a positive moderate correlation between *anti-A. hebraeum* IgE and anti-alpha-gal IgE although the significance was higher in females **(Figure 6.4a)**. Anti-*A. hebraeum* IgG4 correlation with anti-alpha-gal IgE was significant in females in comparison to males **(Figure 6.4b)**.

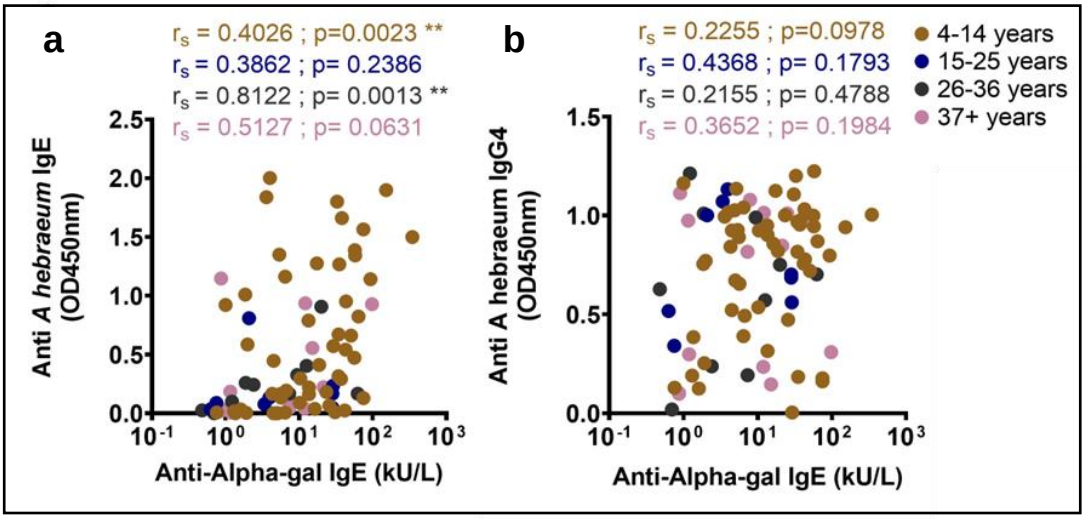


Figure 6.3: Correlation of anti-*A. hebraeum* IgE and anti-alpha-gal IgE is dependent on age. Spearman correlation analysis of anti *A. hebraeum* **(a)** IgE and **(b)** IgG4 against anti-alpha-gal IgE in sera from patients (n = 93) stratified by age. Experiment was carried out in triplicates (mean±sem). Statistical significance was calculated by Spearman rank correlation (rs). ** p ≤ 0.01

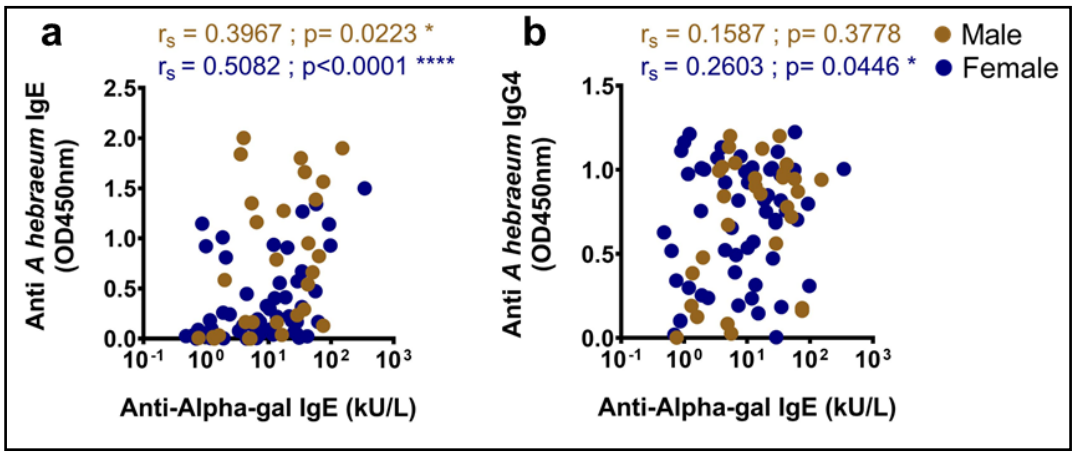


Figure 6.4: Sex influences correlations between anti-*A. hebraeum* IgE and anti-alpha gal IgE. Spearman correlation analysis of anti-*A. hebraeum* **(a)** IgE and **(b)** IgG4 against anti-alpha-gal IgE in sera from male (n = 33) and female (n = 60) patients. Experiments were

carried out in triplicates (mean±sem). Statistical significance was calculated by Spearman rank correlation (rs). * p ≤ 0.05, **** p ≤ 0.0001

6.2.3. IgE binding to *A. hebraeum* and *R. evertsi* proteins is reduced after alpha-gal IgE inhibition

To investigate if binding to *A. hebraeum* and *R. evertsi* proteins is independent of alpha-gal we competitively inhibited sera anti-alpha-gal IgE by incubation with bovine thyroglobulin. An established method for impairing anti-alpha-gal IgE in patient serum from binding to the alpha-gal antigen [242,255,272,609]. There was a decrease but no elimination in the binding to both *A. hebraeum* and *R. evertsi* proteins in sera treated with BTG in comparison to the untreated sera **(Figure 6.5)**.

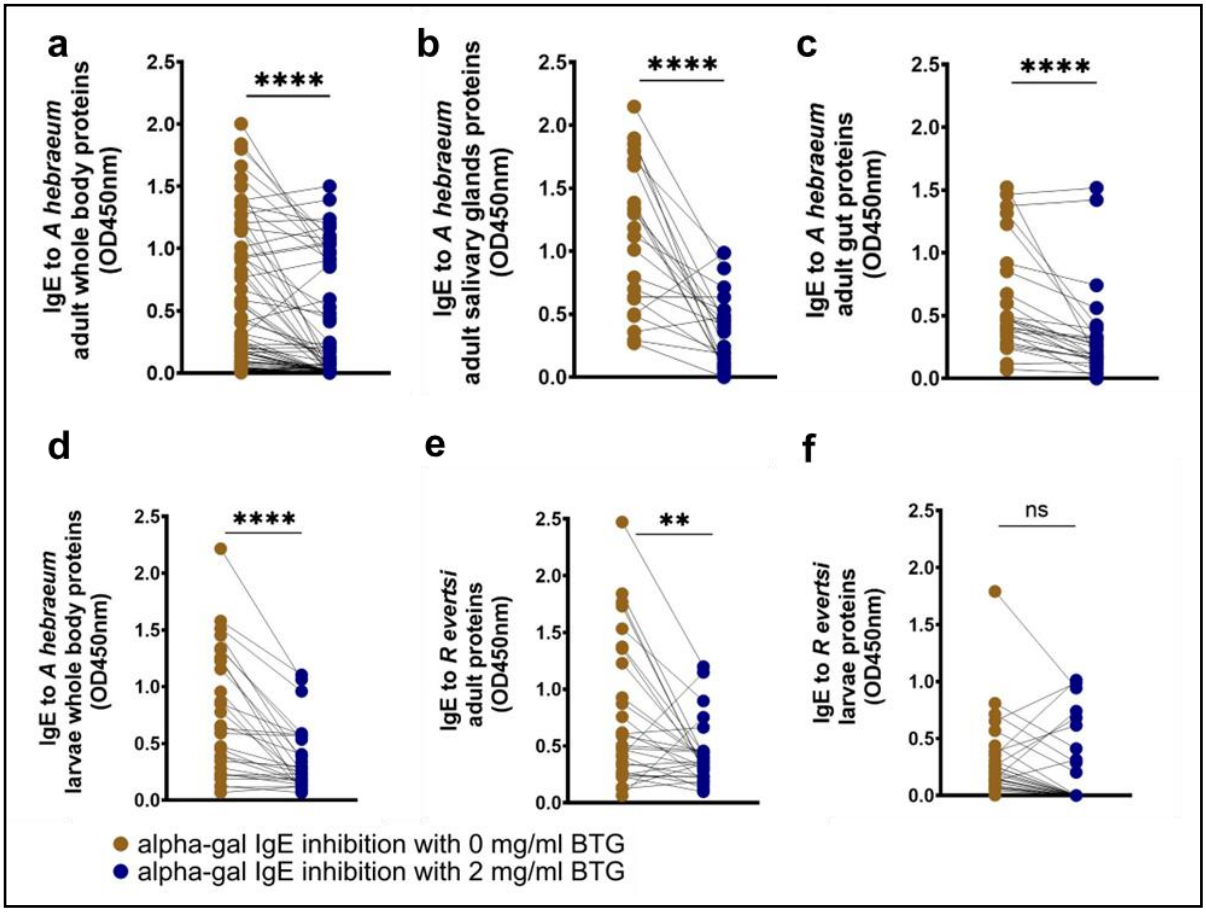


Figure 6.5: There is reduced binding to *A. hebraeum and R. evertsi* proteins by alpha-gal IgE-depleted sera. Sera (n = 80) was incubated with and without 2mg/ml of alpha-gal rich bovine thyroglobulin onto ELISA plates coated with 50µg/ml of *A. hebraeum* **a)** adult whole body protein **b)** unfed adult salivary gland protein **c)** unfed adult gut protein **d)** unfed larvae whole body proteins and R. evertsi whole body **e)** adult and **f)** unfed larvae protein. Experiment

was carried out in triplicates (mean±sem). Statistical significance was calculated by the Wilcoxon test. **** $p \leq 0.0001$

6.3. Discussion

In the previous chapter, we reported the presence of IgE towards *A. lumbricoides* antigens in our alpha-gal allergic cohort. However, the presence of alpha-gal on proteins from ticks and the overlap of tick distribution with alpha-gal allergy suggest ticks as the primary source of alpha-gal sensitization in many studies [500]. In this chapter, we sought to investigate the relationship between alpha-gal allergy and ticks particularly *A. hebraeum* due to its close proximity to our study cohort and our previous localization of alpha-gal in *A. hebraeum* salivary glands.

We report raised IgE to *A. hebraeum* in sera from the alpha-gal allergic group. We also demonstrate a positive moderate and low positive correlation to total IgE and anti-alpha gal IgE respectively. Inhibition of anti-alpha-gal IgE reduced serum IgE binding to *A. hebraeum* and *R. evertsi* proteins. This indicates the presence of alpha-gal on tick proteins and suggests the IgE response towards tick proteins to be in part driven by alpha-gal. Therefore, providing a link between ticks bites and the development of IgE to alpha-gal. Choudhary et al. (2021) and colleagues demonstrated the ability of tick salivary gland extract (TSGE) to induce an increase in total IgE at days 0, 7, 21, 28 and 56 after an intradermal injection in mice [646]. They also show the development of alpha-gal IgE at day 56 in the alpha-gal knockout (AGKO) mice treated with TSGE. Similarly, subcutaneous immunization with *A. americanum* whole tick extract has been seen to induce an increase in alpha-gal IgE [345]. The production of IgE to ticks has been attributed to CD4+ T cells within skin-draining lymph nodes as their depletion prior to alpha-gal challenge prevented IgE production in AGKO mice [345]. Apart from CD4+ T cells, toll-like receptor expression on B cells mediates IgE production to tick proteins through MyD88 signalling which is essential in driving sequential class switch recombination to IgE [647–650]. Studies show TSGE to initiate a recall response following antigen exposure by increasing CD69 expression on B220+CD19+ B cells [646]. Hence all this taken together suggests *A. hebraeum* bites as another source of sensitization in our cohort.

Anti-*A. hebraeum* IgG4 was significantly elevated in the alpha-gal allergic group as compared to the non-allergic group. Since IgG4 is a marker of chronic exposure it may suggest high frequencies of tick bites in the alpha-gal allergic group. Our cohort is located in a rural environment where there is constant interaction with vegetation and domesticated animals. Before attaching to their host, ticks wait on vegetation, thus a rural environment is ideal for increased host-tick interactions. Domestic animals being in close vicinity also make our cohort

easily accessible by ticks hence the raised *A. hebraeum* IgG4 in the allergic group. Studies by Hashizume et al. (2018) show that patients with 2 or more tick bites have higher levels of alpha-gal IgE, greater basophil and eosinophil infiltration at the site of tick bite and increased type 2 cytokine T cell infiltration in comparison to individuals with only 1 or no tick bites [651]. Other researchers show that alpha-gal IgE only develops after successive tick bites and the level of alpha-gal IgE seems to decrease after prolonged periods without tick bites [253,301,652]. Therefore, the elevated anti-*A. hebraeum* IgG4 in our study, suggests repeated bites with *A. hebraeum* which leads to the development of alpha-gal IgE in our alpha-gal allergic group.

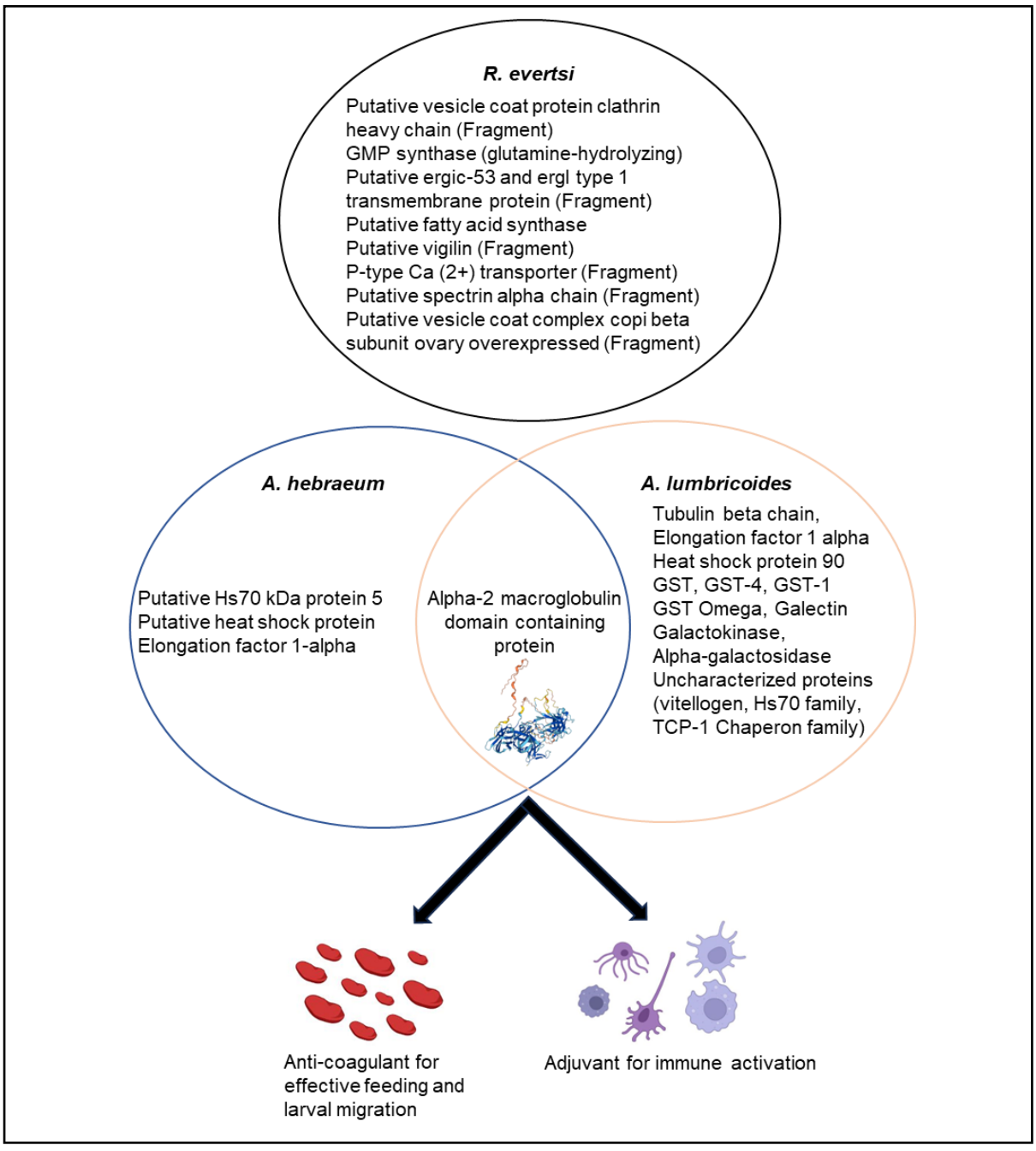
Our study shows the effect of age on the correlation of anti-*A. hebraeum* IgE and anti-alpha-gal IgE. There was a positive moderate level and a positive high correlation between anti-*A. hebraeum* IgE and anti-alpha-gal IgE in the age groups 4-14 years and 26-36 years respectively. Individuals in the 4–14 year range are associated with outside play and partaking in chores such as tending to livestock making them most likely to be bitten by ticks. However, the correlation in this age range may also be a result of recruitment bias since most of our participants were paediatric patients. The strong positive correlation to anti-*A. hebraeum* IgE and anti-alpha-gal IgE at the ages of 26-36 years may be indicative of the social-cultural norms of our cohort. This age group forms a hierarchy of young adults who are often highly involved in the economic activities in this community. Activities such as farming, and livestock rearing are common in the Eastern Cape and may put these individuals at great risk of tick bites. We also report a positive moderate level correlation of anti-*A. hebraeum* IgE and anti-alpha-gal IgE in males and females with the latter having a stronger significance. Correlations in both genders is indicative of both males and females being exposed to tick bites as they go about their daily chores. This is in contrast to studies in European cohorts which show elevated IgE to ticks to be more common in men due to their occupation, for example, forest workers [272,653,654]. Interestingly, AKGO male mice are 1,7 times more likely to have anaphylaxis than female mice during a challenge with mammalian meat [646].

Taken together, in this chapter we show that exposure to the tick *A. hebraeum* and *R. evertsi* is associated with sensitization to alpha-gal.

OBJECTIVE 3: Identification of possible alpha-gal glycosylated proteins in alpha-gal sensitizing parasites.

Rationale: General exposure to alpha-gal glycosylated products does not elicit a type I hypersensitivity reaction. To date, AGS is triggered after human parasite exposure. Therefore, we hypothesize that proteins from parasites which are glycosylated with alpha-gal have adjuvant properties which induce the production of allergen-specific IgE against alpha-gal.

Graphical abstract



CHAPTER 7: Identification and characterization of alpha-gal glycosylated proteins in *A. lumbricoides*, *A. hebraeum* and *R. evertsi*

Key findings

- Of all the possible alpha-gal glycosylated proteins we identified from *A. lumbricoides* (1D and 2D) and *A. hebraeum* (1D) excised gel pieces, alpha-2 macroglobulin was the only protein found in both *A. lumbricoides* and *A. hebraeum*.

7.1. Introduction

In chapters 3 and 4 we gave the first report of alpha-gal in the helminth *A. lumbricoides* and two South African ticks, *A. hebraeum* and *R. evertsi* [655]. We demonstrated an association between alpha-gal IgE and exposure to *A. lumbricoides*, *A. hebraeum* and *R. evertsi* among patients with AGS from Mqanduli district in the Eastern Cape. In this chapter, we sought to identify and characterize the alpha-gal glycosylated proteins from these parasites.

Alpha-gal glycosylation on beef proteins has been reported on creatine kinase M-type, aspartate aminotransferase, β-enolase, α-enolase triosephosphate isomerase, carbonic anhydrase 3, and lactate dehydrogenase A [243]. Studies on pork kidney identified two heat-stable alpha-gal glycosylated glycoproteins namely, angiotensin I-converting enzyme (ACE-1) and aminopeptidase N (AP-N) [245]. When subjected to simulated gastric digestion, AP-N remained stable even after 2 hours implying that alpha-gal would reach the small intestines attached to high molecular weight proteins, only to be broken down and made bioavailable after intestinal digestion, hence the delay in the AGS clinical reaction [656]. AP-N has been shown to cause positive basophil activation and skin prick test in alpha-gal allergic individuals. However, the relevance of glycoproteins in AGS has been previously questioned, as only alpha-gal that was attached to lipids and not proteins, was able to pass through a layer of Caco-2 cells in vitro and cause activation of basophils from a patient with alpha-gal allergy [241]. Contrary, other studies show both glycolipids and glycoproteins to sensitize and elicit an alpha-gal allergic reaction and suggest the relevance of a trigger molecule to be based on the abundance of alpha-gal epitopes and their stability [656,657].

Interestingly, Takahashi et al. (2014) showed low homology between alpha-gal glycosylated proteins, namely laminin and collagen (IV) chains from *B. taurus*, despite successful alpha-gal IgE inhibition with cetuximab [244]. Their study also showed the inability of alpha-gal IgE to bind to human laminin with high sequence identity to *B. taurus* laminin, but no alpha-gal glycosylation. Hence, IgE binding to alpha-gal is not dependent on the protein, but rather the alpha-gal moiety itself. However, the protein backbone of alpha-gal is a determinant for IgE recognition as binding to purified alpha-gal glycosylated AP-N, ACE-1 and alpha-gal HSA

yielded varying inhibition profiles after IgE inhibition with alpha-gal and pork kidney, respectively [245].

Proteins are efficient immunomodulators of both the innate and adaptive arms of the immune system [658]. This has resulted in their use as adjuvants in the development of therapeutics for influenza [659], pneumonic pestis [660] and Bechet's disease [661]. The development of IgE towards alpha-gal is only triggered after specific parasite exposure. Therefore, we hypothesize parasite proteins have an adjuvant effect which skews the immune system towards a Th2 phenotype against alpha-gal. The variability of alpha-gal expression among different tick species and our detection of this carbohydrate in the helminth *A. lumbricoides* warrants the need for us to investigate the identity of alpha-gal glycosylated proteins in *A. hebraeum, R. evertsi* and *A. lumbricoides*.

7.2. Results

7.2.1. Preliminary identification and characterization of alpha-gal glycosylated proteins in *A. lumbricoides*

To identify alpha-gal glycosylated proteins in *A. lumbricoides*, mass spectrometry (LC/MS-MS) analysis was carried out on excised bands and spots from 1D and 2D **(Figure 7.1)** gels which corresponded to anti-alpha gal immunoblotting. 2D immunoblotting with anti-alpha-gal chicken scFv antibody revealed 12 spots with possible alpha-gal glycosylation. Mass spectrometry analysis of these spots identified the following peptides: Tubulin beta chain, Elongation factor 1 alpha (EF1-a), Heat shock protein 90 (HSP90), alpha-2 macroglobulin domain-containing protein (A2M), Glutathione-S-transferase (GST), GST 4, 1 and -omega **(Table 7.1)**. We also identified enzymes and a lectin, namely galactokinase, alpha-galactosidase, and galectin **(Table 7.1)**. Mass spectrometry on *A. lumbricoides* 1D excised band identified peptides such as alpha-2 macroglobulin domain-containing protein, HATPase_c domain-containing protein (Heat shock 90 protein family), and 3 uncharacterized proteins belonging to the vitellogenin, heat shock protein 70, and TCP-1 chaperon families **(Table 7.2)**.

Previous studies have shown alpha-gal glycosylation to be through an N-linkage [234]. We evaluated identified peptides for the presence of putative N-linked glycosylation sites. A2M showed the presence of 5 possible N-linked glycosylation sites. Alpha-galactosidase had 4 possible N-linked glycosylation sites. The uncharacterized heat shock proteins, and tubulin beta chains have 3 such putative glycosylation sites. Lastly, elongation factor alpha 1, galactokinase, GST omega and GST only demonstrated 1 possible N-linked glycosylation site. However, other peptides such as GST-4, GST-1, galectin, uncharacterized vitellogenin and TCP-1 chaperone showed no possible N-linked glycosylation **(Table 7.1 and 7.2).** These

peptides were mostly predicted to localize within the cytoplasm, extracellular region, and cytoskeleton **(Table 7.1 and 7.2)**. A2M was the only peptide we found in both the 1D and 2D PAGE gel excisions. A2M has a signal peptide which suggests secretion of the peptide into the host by the *A. lumbricoides* (**Figure 7.2a)**. Phylogenetic analysis of A2M showed a close relationship between *A. lumbricoides* and *Anisakis simplex* A2M proteins (**Figure 7.2b).** Domain analysis predicted the presence of galactose binding domains on galectin and galactokinase **(Figure 7.3)**.

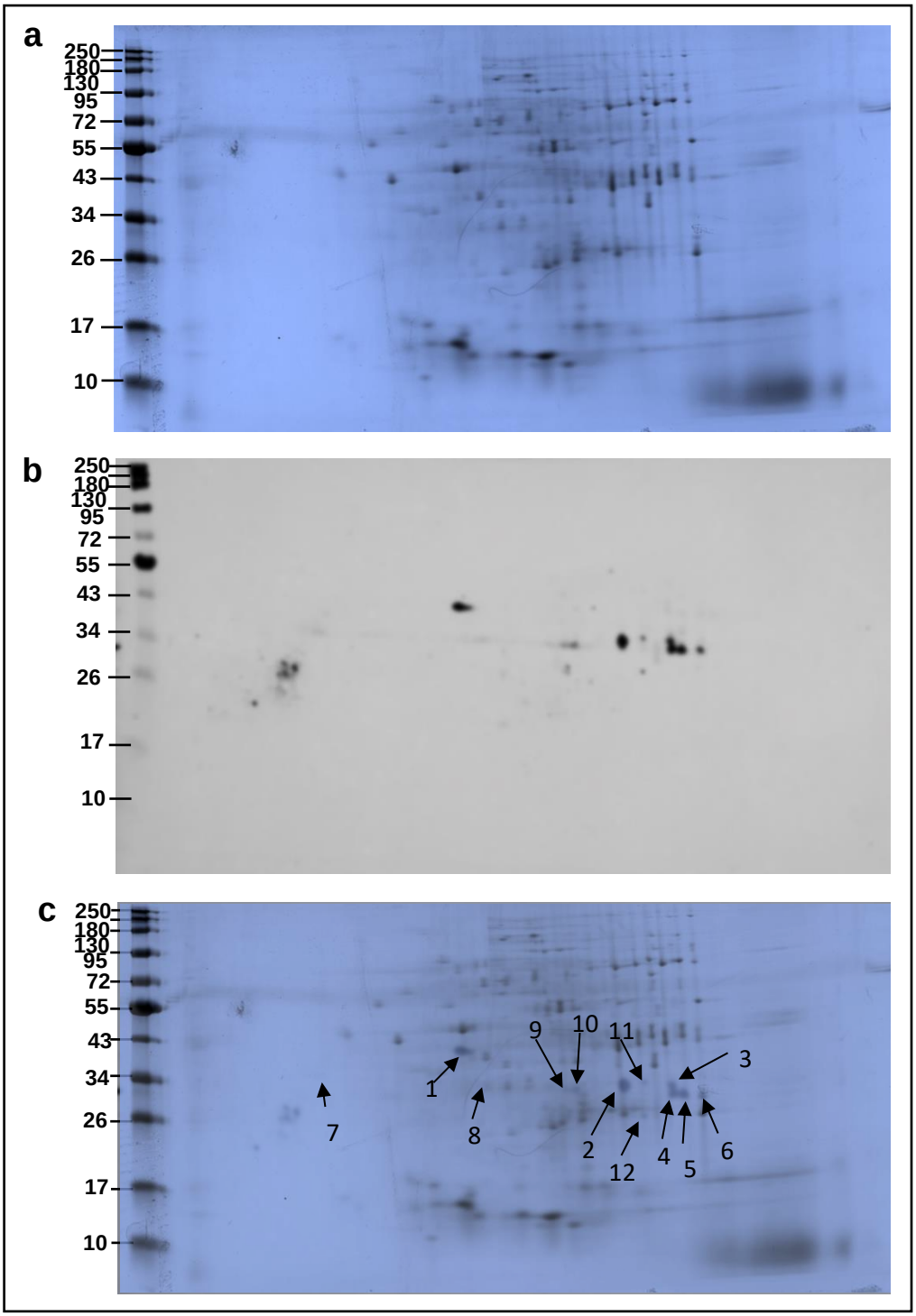


Figure 7.1: Detection of alpha-gal containing proteins in *A. lumbricoides.* 200µg of *A. lumbricoides* protein was run on a **(a)** 10% SDS PAGE 2D gel **(b)** Western blot using anti-alpha gal scFv chicken antibody. **(c)** An overlay image to show the position of the proteins bound to by the anti-alpha gal scFv chicken antibody was created indicating spots which were cut out for mass spectrometry analysis.

Table 7.1: Summary of probable alpha-gal glycosylated proteins identified in *A. lumbricoides* after 2D PAGE spots mass spec analysis.

Protein ID	Spots on 2D blot	Accession number	Function	Localization	Number of N-glycosylation site/s
Tubulin beta chain*	1, 8, 10	A0A0M3HZT2, C4MX32, A0A0M3I0Y5, F1L7U3	Structural constituent of cytoskeleton	Cytoplasm Cytoskeleton microtubule	3
Elongation factor 1 alpha	2, 4, 6, 8, 9, 11	A0A0M3HW34, B5RHZ9	translation elongation factor activity	Cytoplasm	1
Heat shock protein 90*	2, 8, 10	F1LFF3, C1KG49	Stress response, protein folding, Unfolded protein binding		3
A2M_N_2 domain-containing protein	9, 10	A0A0M3HPL0	Endopeptidase inhibitor activity	Secreted	5
Glutathione S-transferase	4, 5, 6, 8, 9, 11, 12	A0A0M3IA46, F1L8V4	glutathione transferase activity		1
Glutathione S-transferase 4	2, 3, 4, 5, 6, 9, 10, 11	F1LCY4	Transferase activity		0
Glutathione S-transferase 1	3, 4, 5, 9, 10	F1LCX2, F1LCT2	Transferase activity		0
Glutathione S-transferase omega	8, 10	F1LF49, F1LBW9	glutathione transferase activity	Cytoplasm	0 and 1 (2 isoforms detected)
Galectin	2, 3, 8, 9, 11	F1LAD2, F1KZZ8, F1L893, A0A0M3IMG8	Carbohydrate binding		0, 0 ,0 and 1 (4 isoforms detected)
Galactokinase	8	F1KV97	Galactokinase activity, galactose metabolism	Cytoplasm	1
Alpha-galactosidase	9, 10	F1L2I1	Hydrolase activity, Carbohydrate metabolic activity		4

Table 7.2: **Summary of probable alpha-gal glycosylated proteins identified in *A. lumbricoides* after 1D PAGE gel slices mass spectrometry analysis.**

PROTEIN IDENTIFIED	Accession number	Function	Location	N-glycosylation site/s
Alpha-2 macroglobulin_N_2 domain-containing protein	A0A0M3HPL0	endopeptidase inhibitor activity	Cytoplasm - secreted	5
HATPase_c domain-containing protein (Heat shock 90 protein family)	A0A0M3IEF2	Protein folding ATP binding		3
Uncharacterized protein (vitellogenin)	A0A0M3I246	Lipid transport	Extracellular region	0
Uncharacterized protein (heat shock protein 70 family)	A0A0M3IJC0	ATP binding		3
Uncharacterized protein (TCP-1 Chaperon family)	A0A0M3HLJ0	Protein refolding		0

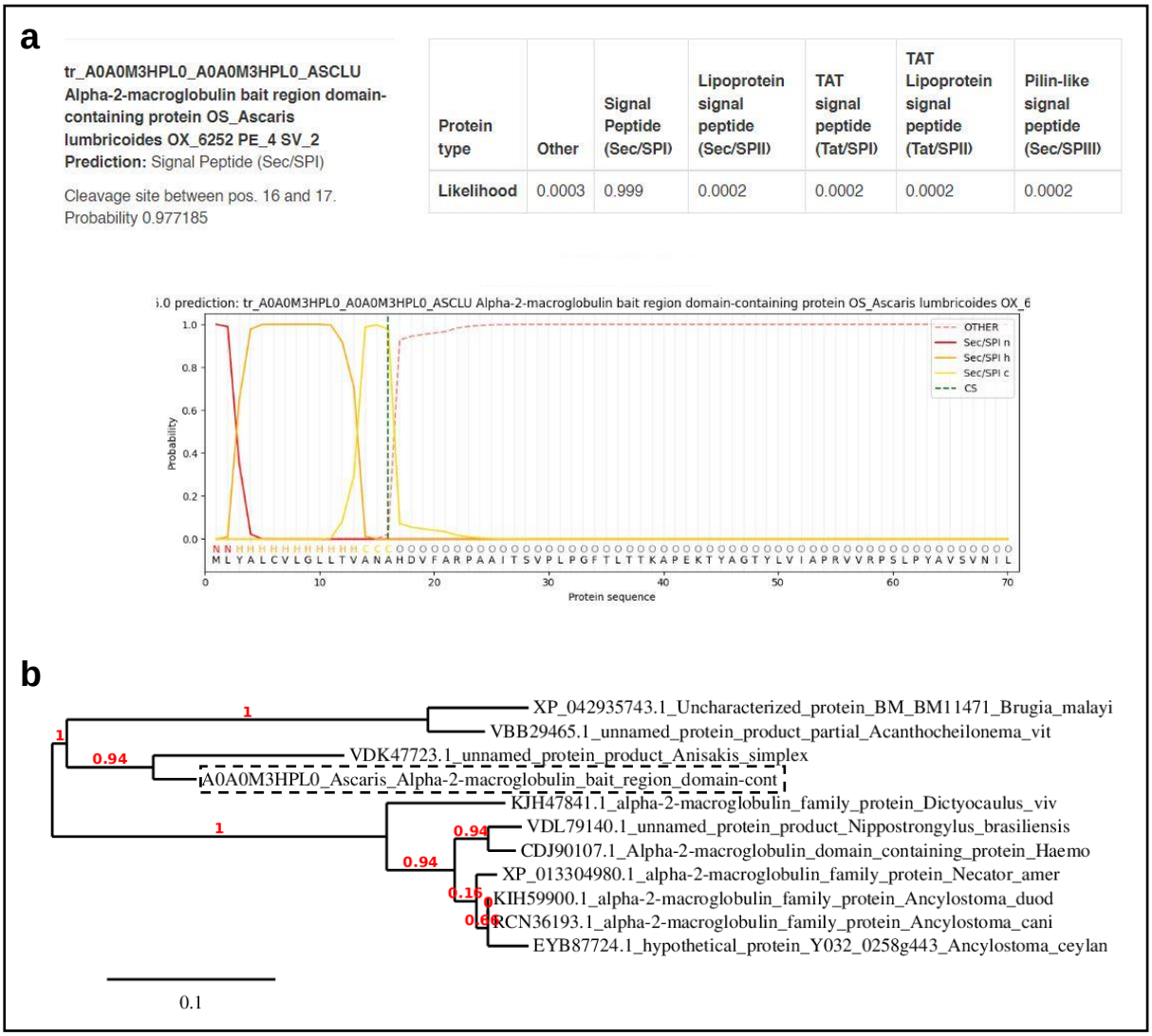


Figure 7.2: Characterization of *A. lumbricoides* alpha-2 macroglobulin_N_2 domain-containing protein. a) Signal peptide prediction using SignalP 6.0. *Key: Sec/SPI (Sec signal peptide), Sec/SPII (Lipoprotein signal peptide), Tat/SPI (Tat signal peptide), Sec/SPIII (Pilin signal peptide), Other (No signal peptide)* **b)** Phylogenetic tree comparing *A. lumbricoides* A2M with selected helminth A2M. Black dotted square showing *A. lumbricoides* A2M.

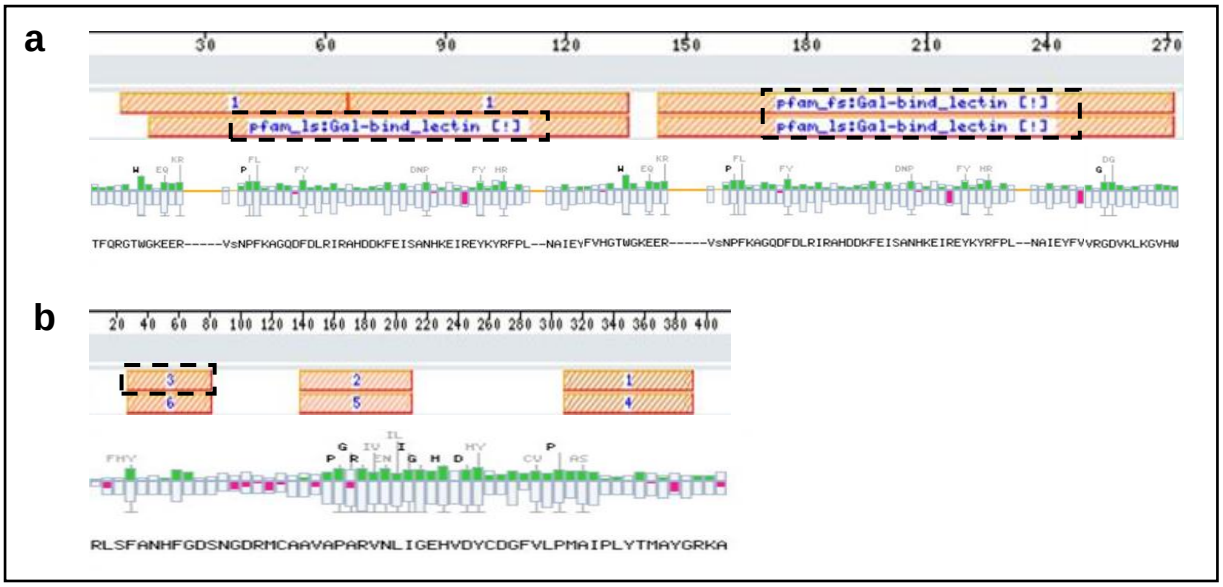


Figure 7.3: Identification of galactose binding domains in *A. lumbricoides* proteins. **a)** Galectin **b)** Galactokinase. Black dotted regions represent galactose binding domains predicted by Motif scan.

7.2.2. Preliminary Identification and characterization of alpha-gal glycosylated proteins in *A. hebraeum*

Identification of possible alpha-gal glycosylated proteins in *A. hebraeum* was carried out by conducting mass spectrometry analysis on 1D PAGE gel slices with *A. hebraeum* proteins which correspond with anti-alpha gal staining. Mass spectrometry analysis of the *A. hebraeum* gel slices showed peptides such as Putative heat shock proteins, Putative alpha-macroglobulin (A2M) and elongation factor 1-alpha **(Table 7.3)**.

Peptides identified associate with protein post-translation processes and protease inhibition **(Table 7.3)**. They are localized within the cytoplasm and the endoplasmic reticulum lumen. Possible N-linked glycosylation is present in A2M with 14 predicted sites. No possible N-linked glycosylation sites were detected in one of the putative heat shock proteins and elongation factor alpha-1. A2M shows the presence of a signal peptide and phylogenetic analysis demonstrates close relatedness to other tick A2M from *I. scapularis* and *I. ricinus* **(Figure 7.4).**

Table 7.3: Summary of probable alpha-gal glycosylated proteins identified in *A. hebraeum* after 1D PAGE gel slices mass spectrometry analysis.

PROTEIN IDENTIFIED	Accession number	Function	Location	N-glycosylation site/s
Putative heat shock 70 kDa protein 5	A0A1E1XF89	ATP binding	Endoplasmic reticulum lumen	0
Putative heat shock protein	A0A1E1XA94	ATP-dependent protein folding chaperone	Many cell components	6
Putative heat shock protein	A0A1E1X9V9	ATP-dependent protein folding chaperone		3
Putative alpha-macroglobulin (Fragment)	A0A1E1XEL3	Protease inhibitor	secreted	14
Elongation factor 1-alpha	A0A1E1XHQ1	Translation elongation	cytoplasm	0

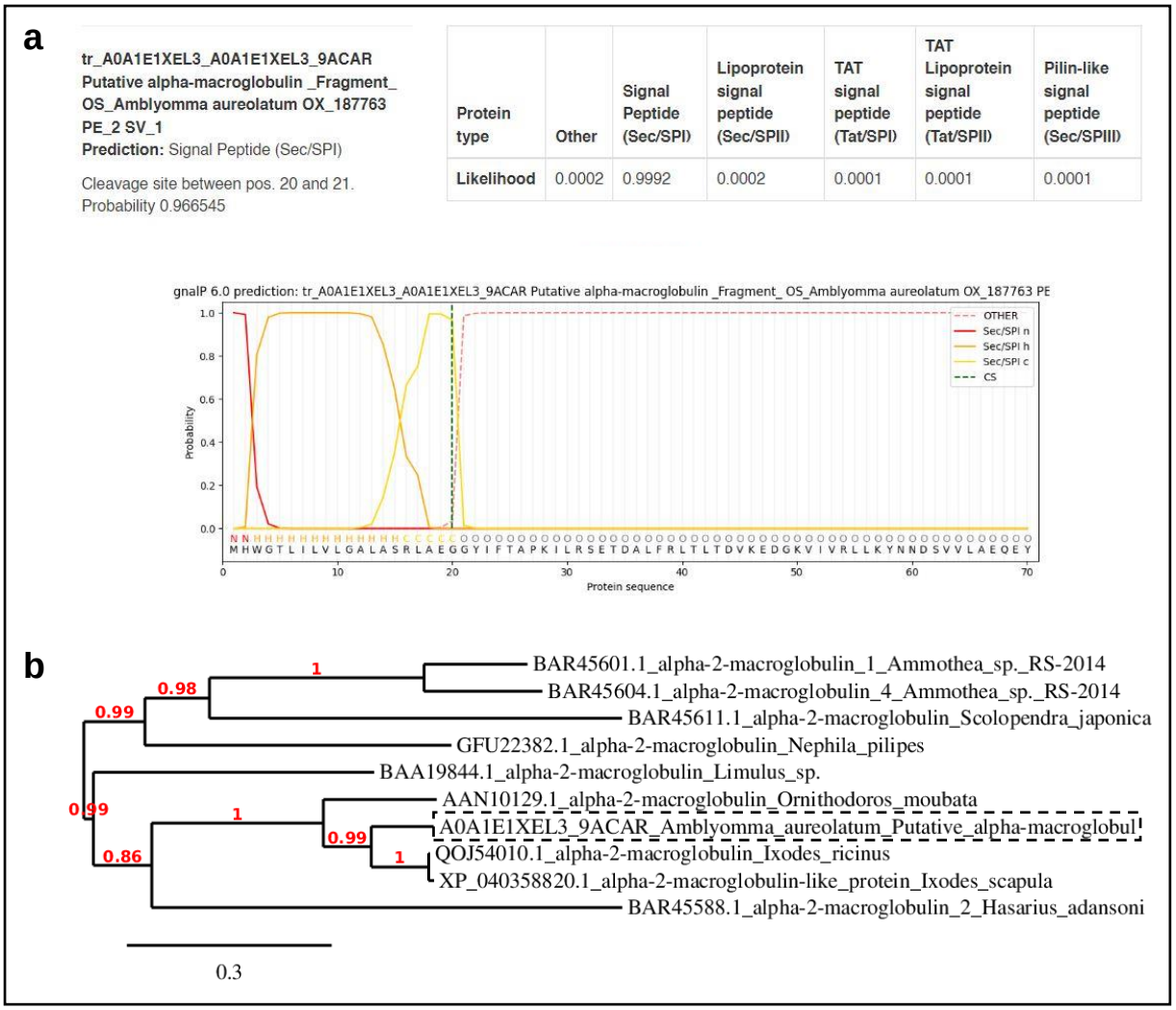


Figure 7.4: Characterization of *A. hebraeum* Putative alpha-macroglobulin. a) Signal peptide prediction by SignalP 6.0. *Key: Sec/SPI (Sec signal peptide), Sec/SPII (Lipoprotein signal peptide), Tat/SPI (Tat signal peptide), Sec/SPIII (Pilin signal peptide), Other (No signal peptide)* **b)** Phylogenetic tree comparing *A. hebraeum* putative alpha-macroglobulin with selected invertebrate putative alpha-macroglobulin. Black dotted square showing *A. hebraeum* A2M.

7.2.3. Preliminary identification and characterization of alpha-gal glycosylated proteins in *R. evertsi*

Mass spectrometry analysis of *R. evertsi* excised 1D gel slices corresponding to alpha-gal staining showed peptides such as putative vesicle coat protein clathrin heavy chain, GMP synthase (glutamine-hydrolyzing), putative ergic-53 and ergl type 1 transmembrane protein, putative fatty acid synthase, putative vigilin, P-type Ca (2+) transporter, putative spectrin alpha chain, and putative vesicle coat complex copi beta subunit ovary overexpressed **(Table 7.4)**.

Interestingly, peptides identified from *R. evertsi* are responsible for protein transportation within the cell and are localized in the clathrin complex, cytoplasm and membrane. Putative ergic-53 and ergl type 1 transmembrane protein showed the presence of a signal peptide and a carbohydrate-binding domain **(Figure 7.5)**, however, there were no possible N-linked glycosylation sites in this protein **(Table 7.4)**. On the other hand, all the other identified proteins showed possible N-linked glycosylation with putative vesicle coat protein clathrin heavy chain having the most N-glycan sites **(Table 7.4)**.

Table 7.4: Summary of probable alpha-gal glycosylated proteins identified in *R evertsi* after 1D PAGE gel slice mass spectrometry analysis.

PROTEIN IDENTIFIED	Accession number	Function	Location	N-glycosylation site/s
Putative vesicle coat protein clathrin heavy chain (Fragment)	A0A6G5A0K3	Intracellular protein transport; vesicle-mediated transport	Clathrin complex	5
GMP synthase (glutamine-hydrolyzing)	A0A6M2CJD2	Purine nucleotide biosynthetic process		3
Putative ergic-53 and ergl type 1 transmembrane protein (Fragment)	A0A6G4ZXX0		Cytoplasm and membrane	0
Putative fatty acid synthase	A0A6G4ZYV8	Fatty acid metabolism Transferase activity		2
Putative vigilin (Fragment)	A0A6G4ZZ00;	RNA and Nucleic acid binding		4
P-type Ca (2+) transporter (Fragment)	A0A6G5AC97	P-type calcium transporter activity	Membrane, Sarcoplasmic reticulum membrane	3
Putative spectrin alpha chain (Fragment)	A0A6G4ZV43; F0J9E3; A0A6G4ZUH7	Actin binding, Protein binding		2
Putative vesicle coat complex copi beta subunit ovary overexpressed (Fragment)	A0A6M2CXS5	Intracellular protein transport, vesicle-mediated transport	Membrane coat COPI vesicle coat	3

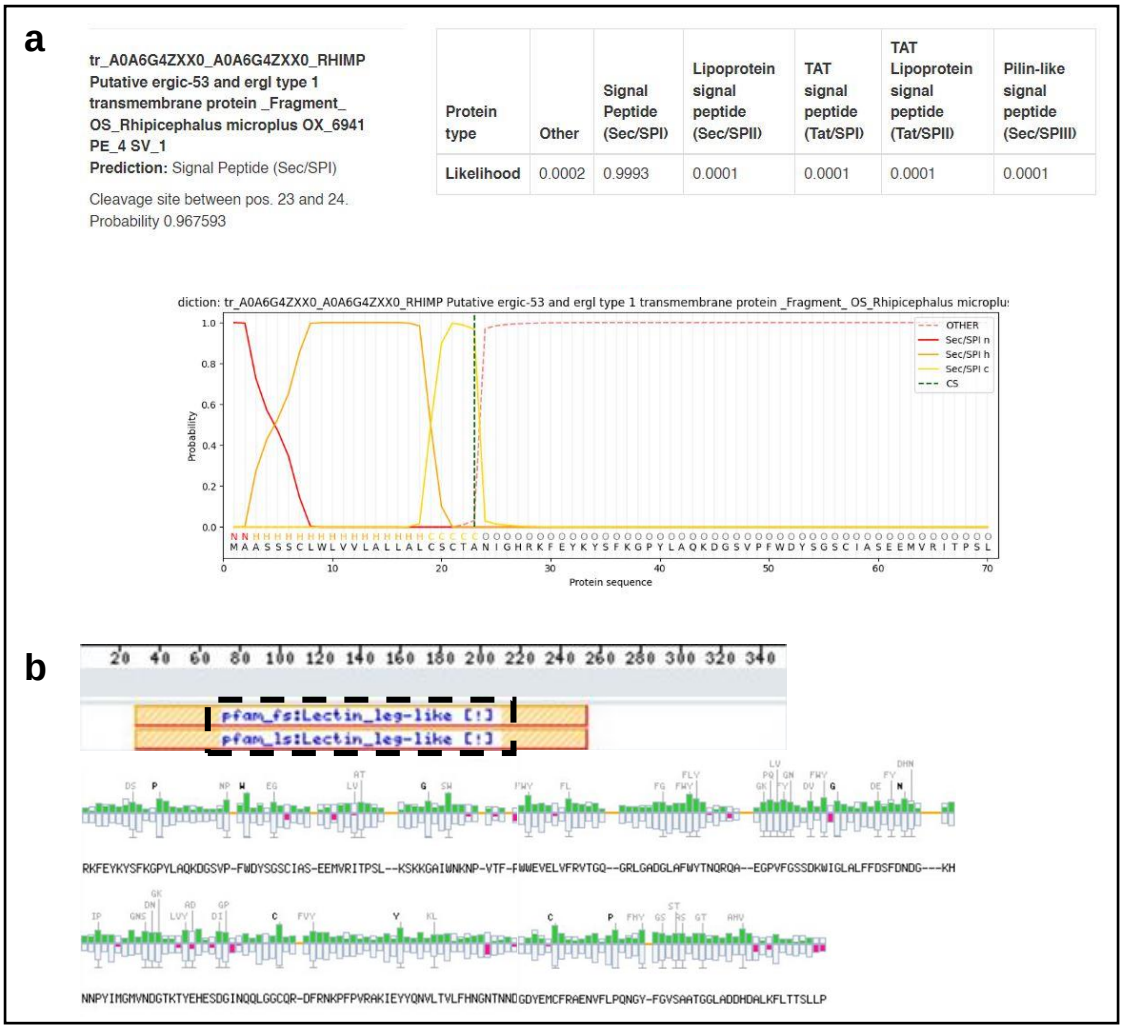


Figure 7.5: Characterization of *R. evertsi* putative ergic-53 and ergl type 1 transmembrane protein. a) Signal peptide prediction by SignalP 6.0. *Key: Sec/SPI (Sec signal peptide), Sec/SPII (Lipoprotein signal peptide), Tat/SPI (Tat signal peptide), Sec/SPIII (Pilin signal peptide), Other (No signal peptide)* **b)** Carbohydrate binding domain predicted by Motif scan. Black dotted square represents the carbohydrate-binding region.

7.3. Discussion

Our results suggest the presence of alpha-gal glycosylation on alpha-2 macroglobulin (A2M). This peptide was present in both 1D and 2D excised gel pieces with *A. lumbricoides* somatic antigen as well as 1D excised gels with *A. hebraeum* whole-body proteins.

A2M is a broad-range endopeptidase inhibitor [662] which forms a tetrameric cage [663] around active peptidases, thus rendering them inaccessible to large substrate reaction sites [664–666]. However, this endopeptidase inhibition is not complete as small molecules can still seep into the tetrameric cage and get digested [667]. A2M has been isolated from a wide range of organisms [668–671] and is reported to play an important role as a defence mechanism [668]. Given our results, the role of A2M in *A. lumbricoides* can be explained in two ways. Firstly, the host immune response towards helminths is associated with serine peptidases which break down collagen found in the nematode's cuticle [672]. Studies show the ability of *A. suum* and *A. lumbricoides* aqueous extracts to inhibit the activity of host peptidases such as trypsin [673], chymotrypsin [674], elastase [675], carboxypeptidase A and B [676], pepsin and gastricsin [677]. In the lungs, *A. lumbricoides* triggers the production of extracellular proteases by the host which may lead to delipidating inflammation in the lung [678]. Hence Jogi et al. (2022) shows a relationship between decreased lung function and *A. lumbricoides* infection in Norwegian subjects [468]. Studies on acute respiratory distress syndrome show the ability of A2M to bind to active neutrophil elastase thus reducing connective tissue injury in the lung [679,680]. Other studies demonstrate A2M inhibition of H_2O_2 production by polymorphonuclear leukocytes [681,682] and binding to cytokines such as basic fibroblast growth factor [683], platelet-derived growth factor [684], nerve growth factor [685], IL-1b [686], and IL-6 [687]. Hence, helminths use endopeptidase inhibitors such as A2M to bind to host serine peptidases and cytokines thus downregulating the host immune response [688–690]. Since *A. lumbricoides* can inhibit the activity of peptidases in the stomach, intestines, and lungs A2M is likely expressed in both the larval and adult stages of the nematode. However, this requires further investigation.

Secondly, studies suggest A2M plays a role in the reduction of blood clotting during tapeworm attachment and feeding [691]. This is due to its ability to bind to free and fibrin-bound thrombin [682,692]. Blood clotting is a host mechanism which has the potential to immobilise helminth larvae. Hence, the anti-coagulant properties of A2M ensure successful larval migration which is an essential part of *A. lumbricoides* life cycle.

Our results show the presence of a signal peptide on A2M. This suggests secretion of this alpha-gal glycosylated peptide by *A. lumbricoides*. Studies by Hawley et al. (1992) show that *A. suum* and *A. lumbricoides* inhibit host peptidases by absorbing them into their tissues such as the intestinal brush border, eggs, genital tract and in muscle/cuticle [693]. The co-localization

of host proteinases and *A. lumbricoides* protease inhibitors in parasite tissue may explain the staining pattern we previously demonstrated which shows the presence of alpha-gal in *A. lumbricoides* intestinal lining and ovaries [655]. Therefore, the presence of a signal peptide suggests internal secretion of A2M into *A. lumbricoides* tissues. Interestingly, phylogenetic analysis demonstrates a close relationship between *A. lumbricoides* and *Anisakis simplex* A2M. Considering how *Anisakis simplex* infection causes the development of type I hypersensitive reactions as a consequence of the immunomodulatory effects of *A. simplex* proteins including protease inhibitors [694]. *A. lumbricoides* A2M (with alpha-gal glycosylation) may have a Th2 skewing effect which results in the development of IgE to alpha-gal. Possible alpha-gal glycosylation of glutathione S-transferase (GST) detected in *A. lumbricoides* 2D spots can also result in the induction of a Th2 phenotype. Nematode GST is highly IgE cross-reactive with dust mite (Der p 8) and cockroach (Bla g 5) [695,696], thus alpha-gal glycosylation of *A. lumbricoides* GST may induce anti-alpha gal IgE production.

Our results show the presence of A2M in *A. hebraeum*. Studies by Diaz-Martin et al. (2013) show the presence of A2M in the tick *Ornithodoros moubata*, at the molecular weight of 100 and 140kDa [697]. However, this is dependent on whether the SDS PAGE is run under non-reducing or reducing conditions as these yield bands at either 190kDa and 92kDa respectively [698]. The alpha-gal-containing gel slices we excised for LC-MS/MS analysis were run under reducing conditions and corresponded to the range for A2M [697]. Mateos-Hernández et al. (2017) demonstrated the binding of an anti-alpha-gal antibody and IgE from alpha-gal allergic patients to A2M in *R. microplus* proteins [233]. Studies by Buresova et al. (2009) show the role of A2M in tick immune responses, as silencing A2M expression in tick haemocytes results in reduced phagocytosis of the tick pathogen, *Chryseobacterium indologenes* in *I. ricinus* [668]. Interestingly, screening for alpha-gal glycosylated proteins in *I. ricinus* by Apostolovic et al. (2021) identified A2M in both adult and larval extracts [699]. Phylogenetic analysis shows a close relationship among *A. hebraeum*, *O. moubata* and *I. ricinus* A2M proteins. Therefore, A2M is likely to be involved in the regulation of *A. hebraeum* immune responses against invading pathogens. Also as previously discussed, A2M has anti-coagulation properties hence it may be released into the human host together with salivary proteins by *A. hebraeum* during feeding. This ensures successful hematophagy and may result in alpha-gal sensitization through antigen presentation of alpha-gal bound to *A. hebraeum* A2M capable of priming a Th2 response.

Exposure to alpha-gal in meat without parasite exposure does not result in AGS. Thus, alpha-gal glycosylation of *A. hebraeum* and *A. lumbricoides* A2M may have adjuvant effects necessary for alpha-gal sensitization. Studies demonstrate A2M use as an antigen delivery system for vaccine candidates such as hepatitis B surface antigen [700,701] and the HIV envelope

gp120 C4-V3 peptide [702] which results in a 100 to 1000-fold increase in the antibody titres. When A2M encapsulates antigens, it becomes activated and changes its conformation to allow for receptor-mediated uptake by fibroblast cells, monocytes/macrophages, and syncytiotrophoblasts resulting in increased antigen processing and presentation [703–706]. Hence, alpha-gal sensitization may be due to the uptake and presentation of alpha-gal glycosylated *A. lumbricoides* and *A. hebraeum* A2M by host antigen-presenting cells.

2D results on *A. lumbricoides* proteins also show the presence of galectin. This is a carbohydrate-binding protein which mimics the action of host proteins and may serve as an immunomodulator during a helminth infection [707–710]. Studies suggest nematode galectins to have eosinophil chemokinetic properties [711–714]. Eosinophil-mediated mucosal damage creates an ideal microenvironment in the host due to reduced adsorption in the affected area which increases the nutrient supply for worm development. In ticks, studies suggest the involvement of galectin in galactose metabolism as well as N-glycan synthesis and maturation [715,716]. Our results also show the presence of the enzymes alpha-galactosidase and galactokinase. Alpha-galactosidase hydrolyses and cleaves galactosyl residues from glycoproteins and glycolipids resulting in free galactose molecules while galactokinase converts alpha-D-galactose to galactose 1-phosphate. Studies by Sharma et al. (2021) show a decrease in alpha-gal production after silencing alpha-galactosidase genes in *A. americanum* [508]. Hence, these enzymes may play a role in alpha-gal synthesis by *A. lumbricoides*. However, there is no report of alpha-1,3-galactosyltransferases in *A. lumbricoides* yet.

Mass spectrometry analysis of the excised *R. evertsi* 1D gel slices revealed peptides which mediate protein transportation. Fischer et al. (2020) previously demonstrated co-localization of alpha-gal and clathrin staining in *I. ricinus* [574]. Our identification of the peptide putative vesicle coat protein clathrin heavy chain suggests ticks have the ability to absorb alpha-gal from the blood meal and incorporate it into its proteins during endocytosis in clathrin pits. We also show another vesicle coat protein, i.e. putative vesicle coat complex copi beta subunit ovary overexpressed which we suggest helps in the absorption of alpha-gal from a host blood meal. Studies demonstrate the ability of these coat protein complexes to facilitate the sorting of lipids and proteins in the Golgi apparatus and the endoplasmic reticulum [717]. Hence, they may be involved in the sorting of host proteins and lipids after a blood meal. We also identified a carbohydrate-binding lectin, putative ergic-53 and ergl type 1 transmembrane protein which acts as a glycoprotein quality control and transport receptor [718]. Thus, this may facilitate the capture of host proteins by *R. evertsi*. These proteins may not lead to IgE responses but are critical in the production of alpha-gal by *R. evertsi* which in turn induces an anti-alpha-gal IgE response in humans after repetitive tick bites. However, this requires further study.

Our preliminary results suggest alpha-gal glycosylation on parasite proteins responsible for host immunomodulation and parasite homeostasis. We show the presence of enzymes and lectins which may be involved in galactose metabolism and host alpha-gal incorporation into the parasite protein repertoire. However, further studies are required to show the nature of N-glycans glycosylating these proteins and to ascertain their ability to cause allergic sensitization.

CHAPTER 8: Conclusion

8.1. Summary of results

We demonstrate the presence of alpha-gal in *A. lumbricoides* somatic antigens in both the adult and larval developmental stages. Evaluation of patient serum revealed raised IgE responses to *A. lumbricoides* somatic antigen in alpha-gal allergic individuals. This elevated IgE response positively correlated to alpha-gal IgE levels in the alpha-gal allergic group. Serum incubation with alpha-gal in the form of native bovine thyroglobulin caused a reduction in IgE binding to *A. lumbricoides*. In part, reduced IgE binding via alpha-gal inhibition assays validates alpha-gal presence on *A. lumbricoides* somatic antigen and suggests helminth IgE to be directed towards alpha-gal glycosylated helminth antigens. However, we report individuals with elevated alpha-gal IgE and no cross-reactivity to *A. lumbricoides.* Non-alpha-gal glycosylated *A. lumbricoides* native and recombinant proteins demonstrated activation of an RS-ATL8 IgE reporter basophil cell system indicating true *A. lumbricoides* exposure rather than cross-reactivity in the alpha-gal allergic group. Activation of RS-ATL8 IgE reporter cells also demonstrates the presence of clinically relevant IgE in the alpha-gal allergic group after stimulation with *A. lumbricoides* antigens. This indicates a possible role of *A. lumbricoides* in causing alpha-gal sensitization and elicitation of symptoms of the alpha-gal syndrome. Given the serum IgG4 surface labelling of *A. lumbricoides* larvae by serum from alpha-gal allergic individuals, we suggest epithelium disruption by helminth larval migration to provide a mechanism for alpha-gal sensitization. In line with our findings, other studies report elevated *A. lumbricoides* IgE to be a risk factor for the development of asthma [469–473] and *A. lumbricoides* infection to be responsible for oral tolerance disruption [600,601] thus likely resulting in allergic sensitization.

Our study also demonstrates the presence of alpha-gal in adult and larval developmental stages of local South African tick species namely *A. hebraeum* and *R. evertsi*. Our findings show differential alpha-gal expression in *A. hebraeum* and *R. evertsi* salivary glands and gut proteins during feeding, with increased crosslinking with anti-alpha-gal antibodies being proportional to feeding time in the salivary glands. Localization of alpha-gal was restricted to salivary acini in both unfed and partially fed *A. hebraeum* and *R. evertsi.* This suggests alpha-gal endogenous production via expression of previously described alpha-gal transferase genes b4galt7, a4galt-1, and a4galt-2 [238]. Sera from alpha-gal allergic individuals demonstrate raised IgE and IgG4 to *A. hebraeum* proteins and a positive correlation between *A. hebraeum* IgE and alpha-gal IgE. This indicates a possible role of repeated tick exposure in causing alpha-gal sensitization. Likewise, studies have shown alpha-gal knockout mice to develop alpha-gal IgE after exposure to tick salivary gland extract [345,646].

Mass spectrometry analysis of *A. lumbricoides* and *A. hebraeum* proteins identified A2M as a possible alpha-gal glycosylated protein in both parasites. A2M allows for parasite-host immune evasion and efficient parasite feeding through the inhibition of host serine peptidase activity and blood coagulation. We suggest A2M to have adjuvant properties which results in the skewing of host immune responses against *A. lumbricoides* and *A. hebraeum* towards a Th2 phenotype. This in turn results in the production of clinically relevant IgE against alpha-gal. Interestingly, individuals with elevated IgE to both *A. lumbricoides* and *A. hebraeum* demonstrated significantly higher alpha-gal IgE in comparison to individuals with IgE to *A. hebraeum* only.

Data presented in this thesis, therefore, suggest *A. lumbricoides*, *A. hebraeum* and *R. evertsi* to possess antigenic and immunological factors which may result in allergic sensitization to alpha-gal.

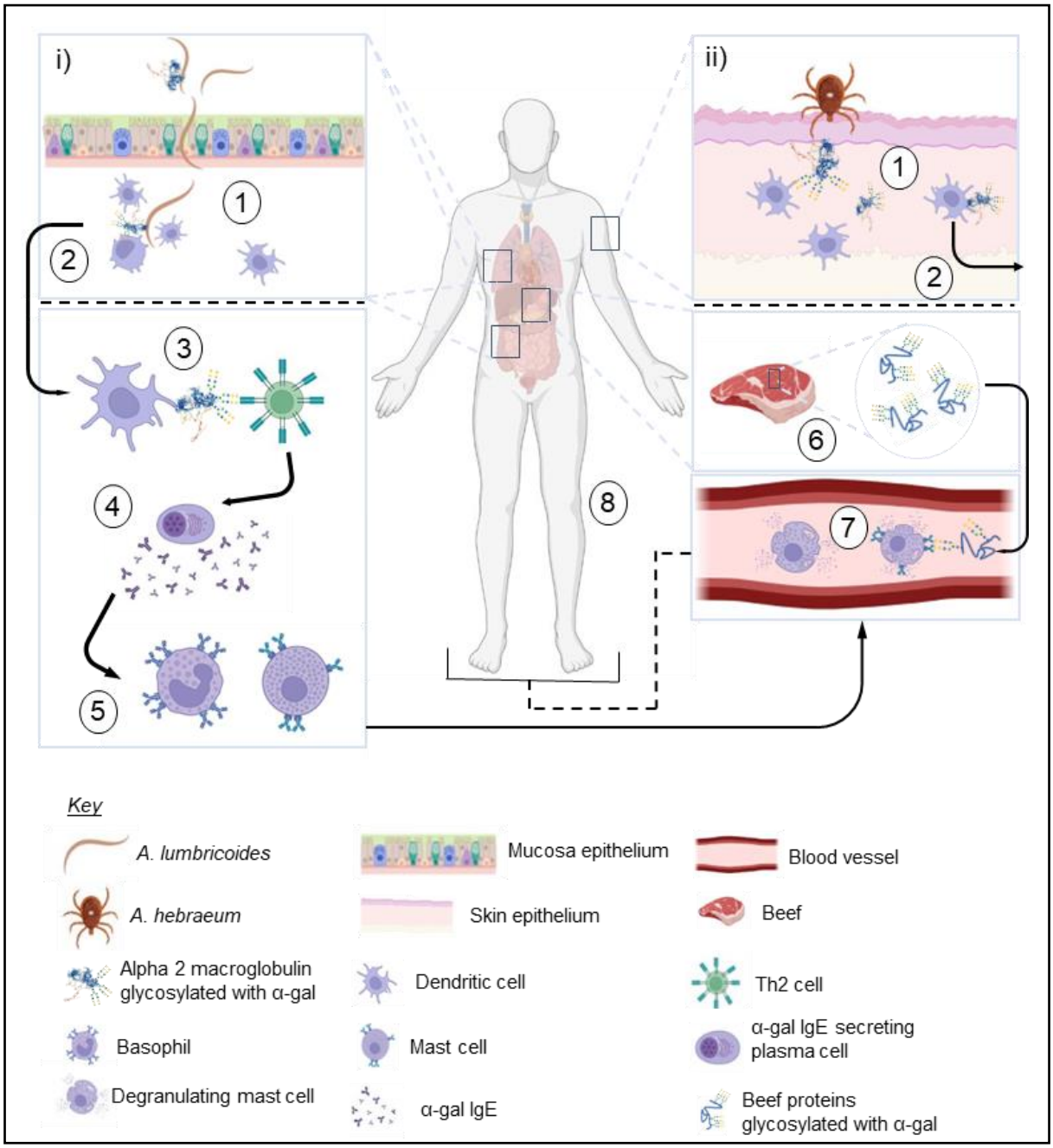


Figure 8.1: Hypothesized role of *A. lumbricoides*, *A. hebraeum* and *R. evertsi* in causing sensitization to alpha-gal. **1)** Epithelial disruption during **i)** *A. lumbricoides* larval migration and **ii)** tick mouth parts protrusion trigger the release of alarmins (IL-33, IL-25 and TLSP) by epithelial cells. Activated DCs at these sites take up alpha-gal glycosylated antigens (alpha-2 macroglobulin) from **i)** *A. lumbricoides* larval (these may be on the larval sheath) and **ii)** tick salivary antigens released at the site of the tick bite **2)** DCs migrate to draining lymph nodes **3)** Presentation of alpha-gal glycosylated proteins to naïve T-cells by DC expressing the co-stimulatory molecule OX40L induces T-cell differentiation into IL-4 secreting Th2 cells **4)** IL-4 induces B-cell class switching into anti-alpha-gal IgE plasma secreting cells **5)** Secreted anti-alpha gal IgE binds to the receptor of basophils and mast cells causing alpha-gal sensitization

6) Digestion of alpha-gal containing food products such as beef releases alpha-gal glycosylated peptides which can be absorbed into the circulatory system **7)** Released peptides cross-link with anti-alpha-gal IgE bound to mast cells and basophils and causes them to degranulate **8)** This results in the elicitation of an alpha-gal allergic response.

8.2. Future work

While this body of work presents significant evidence suggesting the role of *A. lumbricoides*, *A. hebraeum* and *R. evertsi* in the development of alpha-gal sensitization, a few questions are yet to be answered. Firstly, there is a need to investigate the direct induction of alpha-gal IgE development in vivo after *A. lumbricoides* (acute vs chronic), *A. hebraeum* and *R. evertsi* infection. Similar studies have been conducted using AGKO mice exposed to tick salivary gland extracts, particularly *A. americanum* [345,646]. These studies demonstrate the induction of alpha-gal IgE which is dependent on exposure to tick salivary gland extracts, however, this ability of alpha-gal glycosylated lysates to cause allergic sensitization is not dependent on the presence of alpha-gal alone as mammalian meat cannot cause primary sensitization but only elicit a reaction in sensitized individuals. Thus, the need to investigate the effect of *A. lumbricoides, A. hebraeum* and *R. evertsi* infection in the establishment of primary sensitization. We also present data suggesting *A. lumbricoides* and *A. hebraeum* coinfections drive significantly higher alpha-gal IgE outcomes. The development of an alpha-gal sensitization co-infection model would go a long way in understanding AGS development in low-middle income countries which have a high burden of helminth infections and ectoparasite exposure.

Secondly, despite anti-alpha-gal scFv chicken antibody crosslinking with homogenates from different life stages and feeding phases of *A. lumbricoides* and *A. hebraeum* the mass spectrometry data we presented focused on the adult stages of these parasites. Further work is required to identify whether A2M is expressed in all developmental phases of *A. lumbricoides* and *A. hebraeum* or if other proteins may be implicated as having adjuvant effects likely to result in allergic sensitization. This will be carried out through 2D western blotting and mass spectrometry techniques. Apart from only using anti-alpha chicken scFv antibody to detect probable alpha-gal glycosylated proteins, use of alpha-gal positive and alpha-gal negative patient serum in immunoblotting is essential. Spots stained by both anti-alpha-gal scFv chicken antibody and alpha-gal positive sera can reveal insights on the identity of proteins with alpha-gal glycosylation and bound by anti-alpha-gal IgE.

We also propose an investigation into the cross-reactivity of patient serum to A2M as well as probing into proteins that may be used as markers for parasite exposure. To note, Mateos-Hernández et al. (2017) demonstrated the ability of both anti-alpha-gal epitope IgM antibody and IgE from alpha-gal allergic patients to bind to A2M from the tick *Rhipicephalus microplus* [233]. Thus, validating the potential impact and relevance of our preliminary findings. Confirmation of alpha-gal presence on A2M may be carried out by using an anti-A2M commercial antibody to pull down Ascaris A2M proteins. These pull-down lysates may be probed for alpha-gal by anti-alpha-gal scFv chicken antibody via an ELISA or western blot. Secondly, N-glycan profiling of these Ascaris A2M lysates will reveal the possible structure of carbohydrates attached to them. To probe into the relevance of A2M in alpha-gal allergy, the use of this antigen as a stimulate in basophil activation tests will provide interesting insights.

Despite the need to carry out further investigations, the data presented in this thesis is reproducible and sets a foundation for future research which seek to establish and determine the role of *A. lumbricoides, A. hebraeum* and *R. evertsi* in the development of alpha-gal sensitization.

CHAPTER 9: Appendices

KW-2 medium

500 mL FCS

500 mL NCTC 135

1,125 g yeast extract

1,4 g Casein-Hydrolysate

1,4 g Glucose

1000 U-Ml Penicillin G, K-salt

1mg/ml Streptomycin sulphate

10µg/mL Amphotericin B

5-10 mM L-Cysteine

Adjust to pH 6.8. Aliquot media and store at 2-8^{o}C

Carbonate Buffer

2.9g $NaHCO_3$

1.6g Na_2CO_3

4.2g NaCl

Make up to 1L of distilled H_2O and adjust the pH to 9.5. Store at 4°C.

Paraformaldehyde (PFA, 2%)

473 mL 1x PBS

27 mL 37% Formaldehyde solution (Sigma-Aldrich®)

Mix and adjust pH to 7.2. Store at room temperature

Citrate buffer

2.1g Citric acid in 1 L distilled water

Adjust pH to 6.0

In-Gel protein digest buffer formulations

Ammonium bicarbonate (ABC) buffer (20 mM NH4HCO3 aq.; pH 8.0)

Acetonitrile (ACN): pure; HPLC grade

Dithiothreitol (DTT) stock solution: 1 M in 20 mM ABC buffer

Iodoacetamide (IAA) stock solution: 550 mM in 20 mM ABC buffer

Washing buffer: 5 mM ABC buffer in 50% v/v ACN

Extraction buffer A: 50% v/v ACN, 0.1% Formic acid (FA) aq.

Extraction buffer B: 80% v/v ACN, 0.1% FA v/v aq.

Loading buffer: 2x Laemmli buffer

4% SDS

10% 2-mercaptoethanol

20% glycerol

0.004% Bromophenol blue

0.125M Tris-HCl

Check the pH and adjust to pH 6.8.

CHAPTER 10: References

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