STUDIES ON IgE, IgG1 and IgG4 RESPONSE PATTERNS TO GLYCANS AND THEIR IMPLICATIONS ON ALLERGIES IN A SELECTION OF GHANAIAN SCHOOL CHILDREN FROM URBAN HIGH, URBAN LOW AND RURAL AREAS

By

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DECLARATION

I hereby declare that the work presented in this thesis is mine, and has not been presented to any other institution for the award of any degree. All citations made in the text have been duly acknowledged.

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DEDICATION

This work is lovingly dedicated to my sweetheart, Abigail D. O. Asuming-Brempong, and to our 1-year old son Perez Nana Kofi Asuming-Brempong.

This also goes to my wonderful parents, Apostle Prof. Samuel Asuming-Brempong and Dr. Mrs Stella Asuming-Brempong; Mr Carlos Quaynor, and Mrs. Diana Quaynor. Thanks for standing by us through it all.

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ADCC …….	Antibody dependent cell-mediated cytotoxicity
AIT……	Allergen-specific immunotherapy
APC……	Antigen-presenting cell
Ara h ……	*Arachis hypogaea*
AWA…….	Adult Worm Antigen
B……	Background fluorescence
Bla g …….	*Blatella germanica*
BSA……...	Bovine Serum Albumin
C region……	Constant region
CCD ……	Cross-reactive Carbohydrate determinant
ChS……..	Chondriotin Sulphate
Der p ……..	*Dermatophagoides pteronysinnus*
DNA……	Deoxyribonucleic Acid
DS……	Dermatan Sulphate
EDTA…….	Ethylenediaminetetraacetic acid
EIB…….	Exercise-induced bronchospasm
EU……..	European
F…….	Fluorescence
Fc……...	Fragment crystallizable domain
GAGs…….	Glycosaminoglycan
GalNAc……..	N-Acetyl galactosamine
GC……..	Germinal Centre
GlcNAc……..	N-Acetyl glucosamine
GLOFAL……	Global View of Food Allergy
GPI…….	Glycophosphatidylinositol
GSL……	Glycosphingolipid

HA……..	Hyaluronic Acid
HDM……..	House Dust Mite
HLA……	Human Leukocyte Antigen
HS……	Heparan Sulphate
IgA1……	Immunoglobulin A1
IgA2……	Immunoglobulin A2
IgD……..	Immunoglobulin D
IgE …….	Immunoglobulin E
IgG1 ……...	Immunoglobulin G1
IgG2……	Immunoglobulin G2
IgG3……	Immunoglobulin G3
IgG4 ……..	Immunoglobulin G4
IgM…….	Immunoglobulin M
IL-13……..	Interleukin 13
IL-4……	Interleukin 4
IL-5……	Interleukin 5
IRB…….	Institutional Review Board
J region……..	Joining region
KS……..	Keratan Sulphate
kU/L……..	kiloUnits/Litre
L region…….	Leader peptide region
MALT……	Mucosa-associated lymphoid tissue
MFI……	Median Fluorescence Intensity
MHC..……	Major Histocompatibility Complex
N-glycan……	Asparagine-linked glycan

NMIMR…….	Noguchi Memorial Institute for Medical Research
O-glycan……	Serine- or Threonine-linked glycan
PBS……	Phosphate Buffered Saline
pH……..	potential of Hydrogen
R……	Rural Area
RAST……	Radioallergosorbent Test
RT……	Room temperature
Ser……	Serine
sIgE…….	specific IgE
SPT……..	Skin Prick Test
T_{20}……	Tween 20
T_H1…….	T-Helper 1
T_H2…….	T-Helper 2
UH……	Urban High Area
UL……	Urban Low Area
V region……..	Variable region
WAO……...	World Allergy Organization
α-gal……	Galactose- α-1,3-galactose

ABSTRACT

Recent findings indicate many individuals in developing countries as not manifesting hypersensitivity reactions upon sensitization, due partly to the presence of cross-reactive Immunoglobulin E antibodies (IgEs) which recognize oligosaccharide epitopes with α3–fucose and β2-xylose core modifications; or galactose–α–1,3–galactose (α-gal). IgE, IgG1 and IgG4 response profiles were studied in a selection of pupils from urban and rural areas to printed glycan epitopes to compare glycan-associated antibody responses at different socio-economic levels in the Greater Accra Region, testing the hypothesis that cross-reactive antibody activity was more prevalent in the rural than urban areas. Out of an initial urban-rural recruitment of 2,331 participants who provided urine, stool, and blood samples, 20 each from an urban high (UH), urban low (UL), and rural (R) school, were randomly selected and their serum samples processed via a synthetic glycan microarray. With the ImmunoCAP, IgE titres in the 60 Ghanaian samples to a panel of allergens including bromelain, and α-gal were measured. For comparison, IgE, IgG1, and IgG4 response patterns to glycans were characterized for a selection of Europeans (N = 5) via the glycan microarray. Results indicated an area-associated trend, with higher IgE responses observed in the R than the urban areas towards α3-fucosylated N-glycans. Also, strong associations between *S. haematobium* positivity and IgE signal intensities to β2-xylose were observed. Glycan recognition patterns were similar for IgE and IgG1 across the 128 printed glycans, but differed for IgG4 which showed no clear pattern. High IgE and IgG1 signal responses were observed for the selected European subjects to be directed against core structures with either an α3-fucose, a β2-xylose, or both. IgG4 signal intensities were at threshold magnitudes, and did not yield any discernible pattern.

CHAPTER 1:

GENERAL INTRODUCTION

1.1 INTRODUCTION

Allergic disease, or allergy, is a hypersensitivity disorder of the immune system characterized by adverse clinical reactions to innocuous objects or environmental allergens including house-dust mites (HDMs), molds, plant pollen, and cat dander (reviewed by Kay, 2001; reviewed by Ring *et al.*, 2001). Findings from a number of studies point to the inadequate exposure of the immune system to environmental microbiota early in life, as the major risk factor to the development of allergy and autoimmune diseases (reviewed by Fishbein and Fuleihan, 2012).

According to the World Allergy Organization (WAO), the global incidence and prevalence of Allergic diseases have increased in recent decades (Pawankar 2014). Current estimates indicate 300 million sufferers of asthma, and up to 250 million sufferers of food allergies. Drug allergy and rhinitis cases are approximated to be 700 million and 400 million respectively (Masoli *et al.*, 2004; Pawankar, 2014). Research conducted in developing countries also show the prevalence of allergic hypersensitivity to have increased in the past 20 to 30 years (Stevens *et al.*, 2011; Haahtela *et al.*, 2013; Obeng *et al.*, 2014; Amoah *et al.*, 2014), due to the increased adoption of cultural patterns and nutritional habits from affluent countries; improved hygiene; as well as the reduction in infectious diseases (Haahtela *et al.*, 2013; Amoah *et al.*, 2014). Cases in point are two separate studies conducted in Ghana which observed certain clinical manifestations associated with allergic disease to be more prevalent among children from urban areas than among those in rural areas (Stevens *et al.*, 2011; Obeng *et al.*, 2013).

Nevertheless, there is growing evidence from studies conducted in developing countries, particularly helminth-endemic areas, that elevated serum immunoglobulin (Ig) E levels in individuals do not always translate into clinical outcomes (Amoah *et al.*, 2013, 2014). These findings among others have raised great research interest, as they present with a potential alternative in allergic disease therapeutics, particularly with regard to crossreactive IgEs, or IgEs that do not tend towards allergy. Recent studies have associated asparagine (N)-linked oligosaccharides (N-glycans) which possess α3-fucose and β2xylose core modifications in antigenic glycoproteins with induced IgE cross-reactivity (Stanley *et al.*, 2009; van Ree *et al.*, 2000). A classic source of these complex N-glycans is bromelain, a plant-derived glycoprotein and well-established marker for determining carbohydrate-associated cross-reactivity (Commins and Platts-Mills, 2010; Mari *et al.*, 2008; Commins and Platts-Mills, 2010; Amoah *et al.*, 2014). Studies in both high- and middle- to low-income countries have indicated that patients with IgE antibodies capable of recognizing this epitope do not present with clinical symptoms to food allergens (Mari *et al.*, 2008; Amoah *et al.*, 2013, 2014). Furthermore, these complex N-glycans have been identified in the eggs of *S. haematobium* (Hokke *et al.*, 2007; Jang-Lee *et al.*, 2007; reviewed by van Diepen *et al.*, 2012), the extracts of which was recently shown to almost completely inhibit peanut-specific IgE binding in the pooled sera of a subset of Ghanaian subjects (Amoah *et al.* 2013). Additionally, other known sources of these oligosaccharides of interest include the honey bee (Rendic *et al.*, 2008); the helminth *Haemonchus contortus*, which is a parasitic nematode to domestic ruminants and closely related to the human hookworm *N. americanus* (Haslam *et al.*, 1996); and molluscs

(Khoo *et al.*, 2001b).

Also noteworthy with regard to allergen-associated IgE-cross-reactivity, is the recent discovery of, and related studies involving galactose-α-1,3-galactose (α-gal), a major blood group oligosaccharide of non-primate mammals. In the southeastern United States where IgE response to this epitope was discovered, the associated clinical symptoms among patients were either an immediate-onset anaphylaxis following a first exposure to the anti-cancer agent cetuximab, or a delayed-onset anaphylaxis following the ingestion of such food products as beef and pork (Commins and Platts-Mills, 2010; Commins *et al.*, 2011). Further investigation indicated strong association between the generation of IgE antibody response to α-gal and a history of bites by ticks of the species *Ambylomma americanum*. Studies in Europe and Australia have confirmed this novel IgE response to the α-gal epitope with similar clinical symptoms, the induction of which has also been associated with tick bites, although of varying species (Commins *et al.*, 2011). However in a helminth-endemic rural community in Kenya where similar studies were carried out, detectable serum levels of IgE in participants were not paralleled with clinical cases or histories of anaphylaxis. This absence of clinical symptoms or histories in the 'presence' of measurable anti-α-gal IgE levels has further generated interest in cross-reactive IgErelated studies and its potential role both in allergy-associated diagnostics and therapeutics.

These cross-reactive IgE responses are however likely to occur against a backdrop of humoral activity that can be varied and complex. Hitherto, prevailing evidence from antibody-related studies in hypersensitivity reactions have suggested IgG1 to increase the potency of allergen-specific IgE (Aalberse 2011), and IgG4 antibodies to prevent the biological effects of complement-fixing antibodies by inhibiting complex formation (Hamilton, 2001; Aalberse, 2011). IgG4 has also been associated with the downmodulation

of allergic hypersensitivity (Aalberse *et al.*, 1983; van den Biggelaar *et al.*, 2000). However, little is known about IgG1 and IgG4 activity in association with IgE cross-reactivity, which is not only essential in increasing our understanding of the role of IgG antibodies in allergic reactions, but also in ensuring that IgE cross-reactivity is studied in a more holistic context.

Evidence from previous studies conducted in Ghana have demonstrated the presence of cross-reactive IgE to peanut or other allergens in our Ghanaian population, and its role in allergen-associated sensitization and reaction (Amoah *et al.*, 2013). With a welldeveloped array of synthetic glycans, as well as an ImmunoCAP assay, this work sought to characterize glycan epitopes in terms of responses to IgE, IgG1, and IgG4 against an urban-rural backdrop. Glycan-associated IgE responses were also compared between a selection of Ghanaian participants and a selection of Europeans previously found to have high serum levels of IgEs to bromelain (Mari *et al.*, 2008). Furthermore, work was done in this study to measure and explain observed anti-glycan IgG1 and IgG4 patterns in this selection of Ghanaian and European participants. That findings could have important implications both in allergy-related clinical diagnostics, and in the search for novel strategies in the treatment of adverse clinical cases of allergy cannot over-emphasized.

1.2 JUSTIFICATION AND OVERALL OBJECTIVE

The manipulation of the immune system in an attempt to mitigate clinical cases of allergic disease is a strategy increasingly being adopted by clinicians. In pursuit of more novel strategies, molecules from microorganisms or parasites, capable of inducing IgEs that do not tend towards allergic disease are being identified and characterized. High titres of such glycan-specific IgE antibodies in circulation could potentially reduce the likelihood of allergen-specific IgE binding by their Fc region to the Fcε receptors on mast and other

inflammation-inducing cells (Godfrey, 1975; Lynch *et al.*, 1993), hence considerably minimizing clinical outcomes. This study therefore sets out to characterize based on antibody responses, synthetic oligosaccharides which are similar in structure and functional properties to their counterparts in plants, invertebrates, and other environmental sources.

The overall objective of this work is to study the glycan-specific IgE, IgG1, and IgG4 antibody responses to printed synthetic glycans in a selection of serum samples of school children from urban, semi-urban, and rural areas in the Greater Accra Region of Ghana.

1.2.1 SPECIFIC OBJECTIVES

1. To determine, using the glycan microarray and ImmunoCAP assays, the existence of an urban-rural pattern in anti-glycan IgE, IgG1, and IgG4 responses
2. To identify glycans which are significantly associated with Area for each antibody studied.
3. To identify, with respect to Area, glycans or components of glycans, influencing recognition by IgE, IgG1, and IgG4 antibodies
4. To identify risk factors significantly associated with identified glycans for all three antibodies
5. To compare anti-glycan IgE, IgG1, and IgG4 responses between a selection of Ghanaian participants and European pollen allergics

CHAPTER 2:

LITERATURE REVIEW

2.1 Hyper-sensitivity reactions and Allergies

Generally, hypersensitivity reactions have been defined as the over-reaction of the adaptive immune system to innocuous objects following prior sensitization (Wu, 2005), which involves at best an irritation, and at worst, a threat to life.

The development of allergic disease or hypersensitivity reactions is known to involve a complicated relationship between environmental and genetic factors (Cookson, 1999). It can be deduced from Strachan's Hygiene Hypothesis (1989; reviewed by Fishbein and Fuleihan, 2012) that inadequate exposure of the immune system to infectious or environmental microorganisms and parasites during early stages of development could lead to insufficient development of the regulatory arm of the immune system (Rook, 2000; Ege *et al.*, 2011). Furthermore, studies currently indicate that biodiversity loss following human activities is strongly associated with the sharp rise in allergic disease globally (Haahtela *et al.*, 2013).

2.2 Epidemiology and worldwide distribution

The incidence and prevalence of allergic disease and asthma are increasing worldwide, becoming a major Public Health Concern. This is because patients with Allergic disease and asthma have a reduced quality of life. Asthma for instance, is estimated to affect 300 million people around the world, and this mostly among children and young adults (Masoli *et al.*, 2004; Bateman *et al.*, 2008; Pawankar, 2014). Mortality due to Asthma alone has reached over 250,000 annually (Pawankar, 2014). In Western Europe the incidence of

asthma has doubled in ten years. Around 8% of the Swiss population suffers from asthma as compared with only 2%, 25-30 years ago. In the United States, there were an estimated 20.3 million asthmatics in 2001, a 60% increase since the early 1980s. There are about 3 million asthmatics in Japan of whom 7% have severe asthma and 30% have moderate asthma. In Australia, one child in six under the age of 16 is affected (Kushnir and Kaliner, 2006). Current estimates also indicate up to 250 million and 400 million sufferers of food allergies and rhinitis respectively (Pawankar, 2014). For IgE-mediated drug allergies, the incidence and prevalence is currently unknown. It must be noted that current prevalence rates overlap as these allergic diseases, in many cases, occur together in the same individual (Pawanker, 2014).

2.2.1 Epidemiology in developing countries

Allergic disease and asthma is increasingly becoming a major Public Health concern in developing countries, as urbanization and hence, the adoption of cultural patterns and nutritional habits of affluent countries (Haahtela *et al.*, 2013) grows. Indeed, findings from a number of studies show the prevalence of allergic hypersensitivity to have increased in the past 20 to 30 years (Stevens *et al.*, 2011; Haahtela *et al.*, 2013; Obeng *et al.*, 2014; Amoah *et al.*, 2014). Current allergy data on developing countries is largely asthma-related, with great variations in the incidence of the disease. According to the 2002 report of the Global Burden of Disease (GBD) study, the Indian subcontinent, the Asia-Pacific, the eastern Mediterranean countries, as well as Northern and Eastern Europe, had a prevalence of less than 5%, whilst prevalences ranging between 10 and 20% were mostly observed for various parts of Africa (Kushnir and Kaliner, 2006; Bateman *et al.*, 2008).

2.2.2 Epidemiology in Sub-Saharan Africa and Ghana

Apart from South Africa, where the prevalence of asthma is estimated to be above 20% (Kushnir and Kaliner, 2006), there is very limited available data on the prevalence of asthma or other allergic diseases in most of the other countries in Sub-Saharan Africa including Ghana. A number of studies conducted in communities in Gambia, Ethiopia, South Africa, and in Ghana however indicate urban-rural differences in the prevalence of Asthma and food-related allergies (Perzanowski *et al.*, 2002; Stevens *et al.*, 2011; Amoah *et al.*, 2013). The paucity of information on allergic disease and Asthma in Ghana and many of the countries in Sub-Saharan Africa could be attributed to the low prioritization given to allergies in these countries. With governments saddled with numerous other health, political, and economic challenges, diseases associated with affluence usually receive little or no consideration.

2.3 Types of Hyper-sensitivity reactions

All hypersensitivity reactions are currently categorized into four major groups, namely Type-1, Type-2, Type-3, and Type-4 Hypersensitivity. Briefly, Type-1 hypersensitivity involves the binding of an allergen or particulate antigen to a minimum of two allergenspecific IgEs bound to their Fc receptors on mast cells, eosinophils or basophils. This cross-linking between two membrane-bound IgE antibodies, followed by a series of intracellular transductions (Parham, 2005) leads to the release of inflammatory mediators such as histamine, leukotrienes, prostaglandins, chemokines, and cytokines (Licona-Limon, *et al.*, 2013; Girgis *et al.*, 2013), hence engendering the clinical manifestations associated with allergies. It is worth noting that apart from allergies and asthma, some

drugassociated hypersensitivities are also IgE-mediated. A case in point is the hyper responsiveness that was observed in a number of patients from the South Eastern United

States after their first infusion of the anti-cancer agent Cetuximab (Commins *et al.*, 2011). The second type of hypersensitivity, or Type-2 hypersensitivity, involves the covalent bonding of small, harmless molecules such as penicillin to surface components of human cells. This leads to the formation of a complex, or the modification of the cell surface often leading to the sensitization of B-cells, and hence, the production of IgG antibodies against that complex. The binding of the IgG antibodies to the complex facilitates destruction of the self-cell via complement activation and phagocytosis (Aarndt *et al.*, 2005).

Type-3 hypersensitivity involves the binding of IgG antibodies to small, soluble protein antigens. The complexes formed are deposited in the walls of capillaries or in the alveoli of lungs leading to the activation of complement, and hence, facilitating the damage of surrounding tissues (Gamarra *et al.*, 2006; Murphy *et al.*, 2008). The introduction of an attenuated candidate antigen in a participant during a human vaccine trial is capable of inducing such a response.

The fourth and final category of hypersensitivity reactions currently known is the Type-4 hypersensitivity, which involves recognition of modified Human Leukocyte Antigen (HLA) I receptors by CD8 cytotoxic T-cells. This modification comes about when small, lipid-soluble molecules pass through the human cell membrane and bind to intracellular proteins. As a result, there is degradation of function of the affected protein and hence, the modification of secreted peptides. The peptides bind to the HLA I receptors leading to

the observed reaction with CD8 T-cells. A case in point is the chemical pentadecacatechol which is acquired on touching the leaves of the poison ivy (*Toxicodendron radicans*) plant (Kalish *et al.*, 1994).

Considering however, that much of the hypersensitivity reactions reported or observed in developing countries, and in Ghana in particular are allergies, our focus for this work will be primarily on the Type-1 hypersensitivity.

2.4 Allergy-associated Immunology

2.4.1 Immune mechanisms in an individual predisposed to allergy

Before an individual manifests hypersensitivity reactions to an allergen, there is first prior exposure to the allergen, a phase also known as sensitization (Parham 2005; Wu and Zarrin, 2014). During this phase, the allergen or innocuous object crosses the mucosal or skin barrier, and is taken up by antigen presenting cells (APCs) such as dendritic cells. These in turn process the antigen, presenting it on MHC II receptors. Interaction with naïve CD4+ T-cells leads to polarized differentiation to type-II helper T-cells. These TH2 cells next produce cytokines, including interleukin-4 (IL-4), IL-5, and IL-13, which induce B-cells to class-switch into IgE production. Once the antigen has been cleared, all naïve IgEs bind by their Fc regions to the FcεR1 receptors on mast cells, basophils, and eosinophils. Thus, in the event of subsequent exposure to the same allergen, the innocuous particle binds to the cell-bound IgEs leading to cross-linking and hence, cell degranulation (Pearlman, 1999; Wu and Zarrin, 2014).

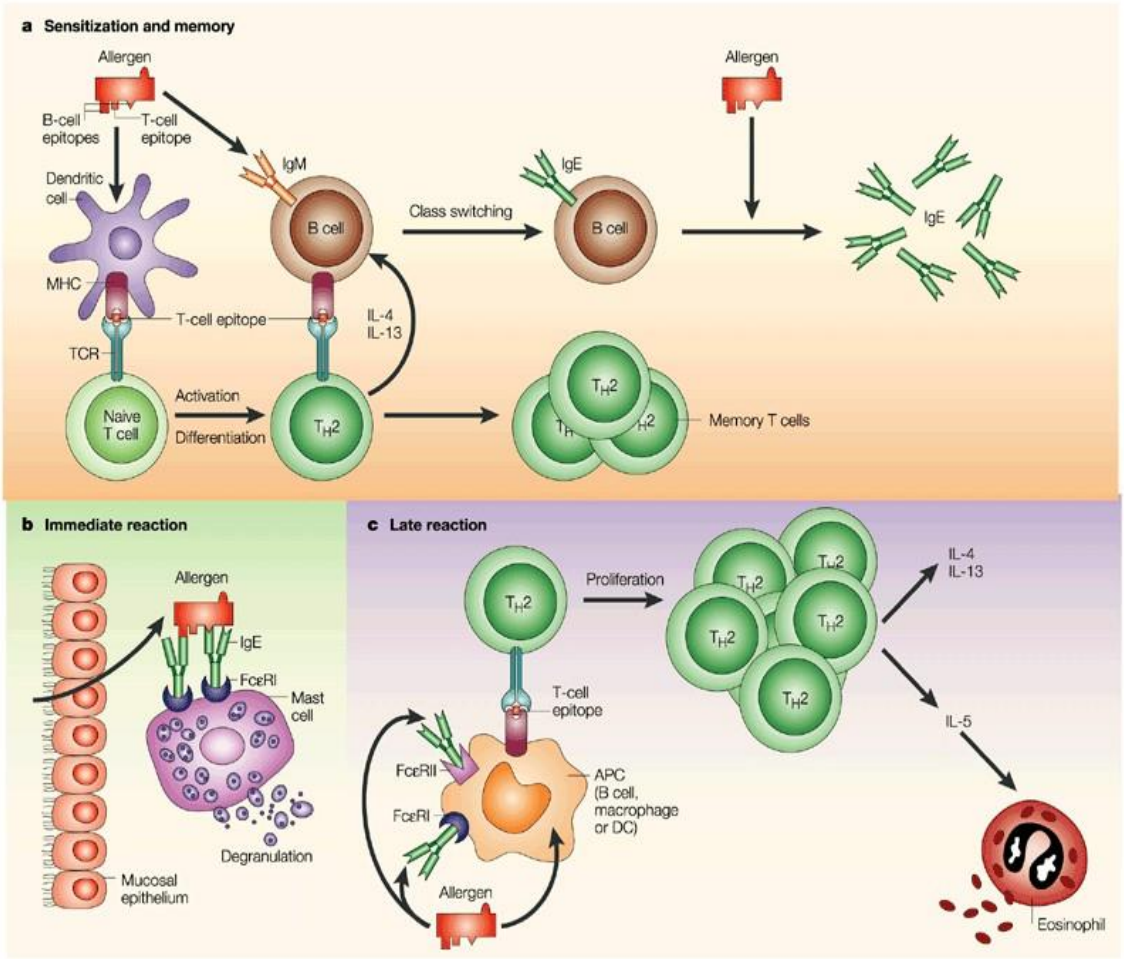


Figure 2.1: Immunological events leading to clinical allergic reactions: (a)

Sensitization and memory**:** Upon exposure to allergen for the first time, dendritic cells ingest, process and present the antigen on their MHC II receptors. Interaction with naïve CD4+ T-cells leads to polarized differentiation into T_H2 cells, and their production of cytokines such as IL-4 and IL-13 induces class-switch IgE production by B-cells. (b) Reinfection: Exposure to the same allergen at a later time leads to hyper-sensitivity responses involving mast cell-bound IgE. (*Courtesy: Nature Reviews, 2002*)

2.4.2 Immune mechanisms in an individual with helminth infections

For a helminth-infected individual however, prior exposure and hence sensitization may occur, leading eventually to class-switch IgE production by B-cells. But, a strong regulatory network is induced involving regulatory T-cells (Tregs), and alternatively activated macrophages. Increased IL-10 production leads to increased production of IgG4 by B-cells and the general suppression of the TH2-activated responses (Girgis *et al.*, 2013). Hence, re-exposure to the allergen does not elicit any hyper-responsiveness.

Studies have shown that during chronic helminth infections, production of IgG4 by Bcells is increased due to elevated levels of IL-10 production by Tregs and alternativelyactivated macrophages (De Moira *et al.*, 2012), as well as a concurrent decrease in total and allergen-specific IgE production (Akdis 2009).

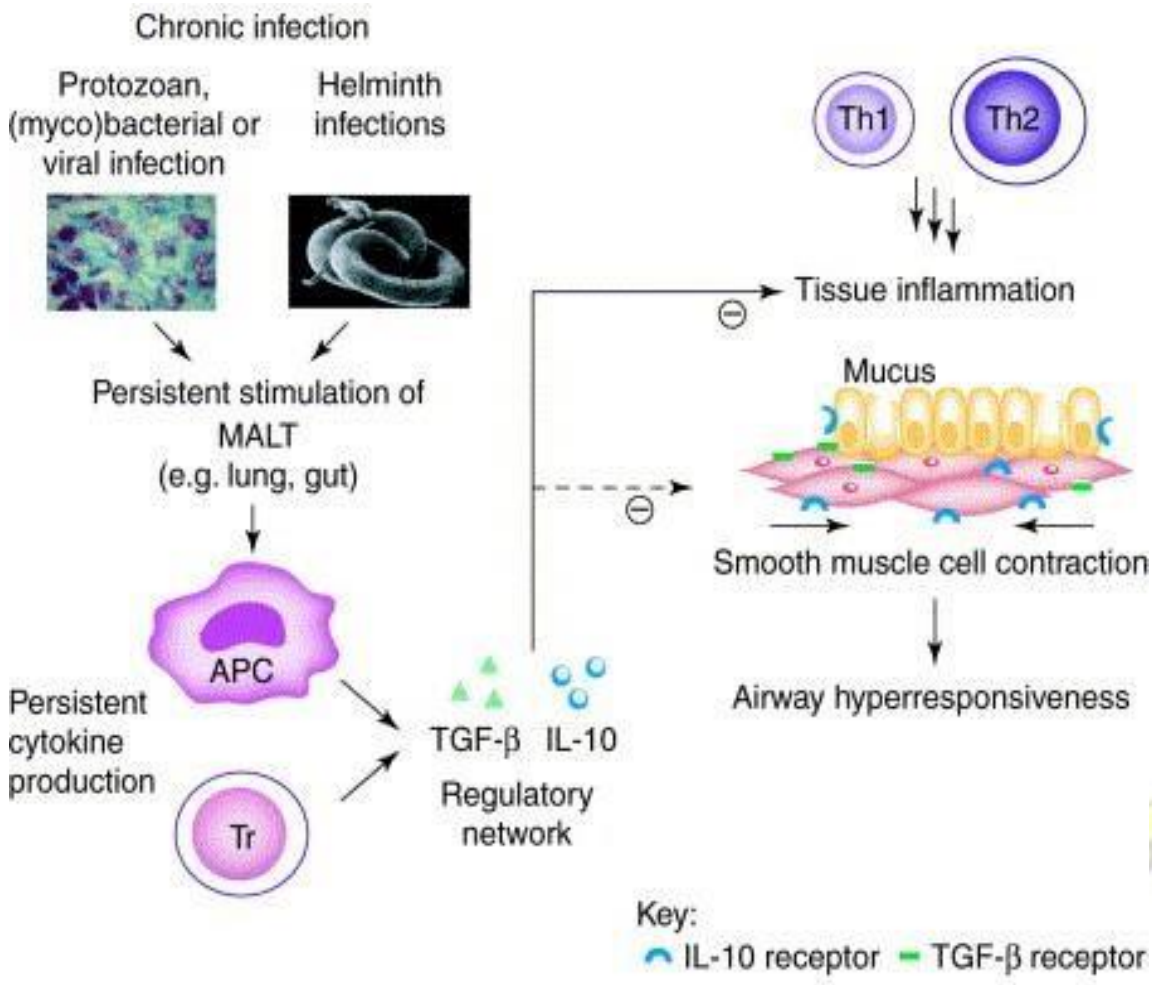


Figure 2.2: Allergen-associated immunity in the face of chronic helminth infections: Allergic sensitization in an individual chronically infected with helminths is possible.

However, due to the strong regulatory responses induced by the helminth, T_H2 effector mechanisms are suppressed. Hence, re-infection by the allergen doesn't induce any hypersensitivity response (*Courtesy: www.Immunology.com*).

2.5 Current therapeutic regimes for adverse allergic reactions

Until very recently, therapeutic measures in treating allergic disease either targeted the symptoms only, like the antihistamines; or served as general immune-suppressants, like the corticosteroids used for treatment of asthma and other chronic allergic diseases (Murphy *et al.*, 2008). However, new strategies have been developed in recent years which seek to manipulate particularly, the adaptive arm of immune responses. An overview of therapeutic strategies employed till date, as well as novel areas currently under exploration is provided in the next few sections.

2.5.1 Allergen Avoidance (Non-pharmacological)

This therapeutic strategy involves the adoption of measures (which are largely behavioral) aimed at minimizing as much as possible, contact or exposure to the allergen. This is with reference to indoor allergens which are more frequently encountered as compared to those associated with the outdoors. Such allergens as ragweed pollen which abound during the autumn season in certain parts of North America are often avoided by patients either remaining indoors or moving away from areas where contact with the allergen is likely. Also, such allergens cease to abound once the season is over. Indoor allergens such as cat dander have been more difficult to avoid, with considerable changes in the behavior of the patient required (Platts-Mills *et al.*, 1997). In this regard, two avoidance strategies have been extensively scrutinized, the first involving the movement of the patient to an allergen-free environment; and the second, the adoption of measures to decrease exposure to the allergen in the patient's own home (Platts-Mills, 2003). The allergen principally under consideration was the house dust mite. The first strategy was considered to be highly effective, and not surprisingly so, as this involved moving the patient to the hospital for a

period of time. The second strategy under scrutiny was more work intensive, as it involved such measures as regularly cleaning floors, removing carpets, washing mattresses, using mite impermeable mattress covers, along with an educational package on allergen avoidance (Platts-Mills, 2003; Barr *et al.*, 2014). Recent multi-centre, randomized control studies conducted to compare between the efficacy of this measure of allergen removal from the patient's immediate surroundings and a control group have indicated little difference between those who practice it and those who don't (Bjornsdottir *et al.*, 2003; Barr *et al.*, 2014). Nevertheless, it has been shown to be successful where pets are concerned, and is hence recommended for patients allergic to pet dander or hairs (Barr *et al.*, 2014; Platts-Mills, 2004).

2.5.2 Nasal irrigation

Useful in management of rhinitis and asthma, this technique involves the administration of saline to intranasal mucosal surfaces preferably via fine sprays (through the use of spray bottles) or douching (which in this context involves squirting saline solution into one nostril with a syringe or appropriate apparatus and allowing the fluid to flow out the other, bathing the nostril in the process). It is a widely adopted measure due to its affordability and ease of use. Although the exact mechanism of action of this technique is unknown, it has been suggested that saline might be crucial in restoring the normal protective nasal mucosal function which when lost, facilitates the contracting of various upper respiratory conditions, including rhinitis. Saline irrigation may therefore have such physiologic effects as the direct cleansing of nasal mucosal tissues (Kurtaran *et al.*, 2005), the removal of inflammatory mediators (Georgitis, 1994), and the improvement of muco-ciliary function

(Talbot *et al.*, 1997), as evidenced by the increase in muco-ciliary beat frequency following administration (Rabago and Zgierska, 2009;).

A randomized controlled trial conducted about a decade ago also indicated its effectiveness at minimizing symptoms following 2 weeks of continued treatment (Garavello *et al.*, 2003; Barr *et al.*, 2014).

2.5.3 Drug treatment

The three main categories of drugs used in the treatment or mitigation clinical allergic symptoms include the antihistamines, the corticosteroids, and the antileukotrienes. By way of action mechanism, antihistamines block the histamine H1 receptor on relevant cells including those comprising blood vessel walls and unmyelinated nerve fibres (Murphy *et al.*, 2008). This is because when Histamine binds to its H1 receptor on blood vessel walls, permeability of blood vessels is increased, leading to movement of fluids and immune cells to surrounding tissues and hence, swelling. This occurs locally at the site of allergen invasion (Murphy *et al.*, 2008). Anaphylactic shock is a more severe form, in which the movement of fluids and immune cells to surrounding tissues is systemic. Hence, so much fluid is lost to surrounding tissues from circulating blood that pressure drops, leading to hypotension. Additionally, tissues swell and, along with a number of internal organs, sustain injuries (Conner and Saini, 2005). In such instances, treatment with antihistamines is inadequate, and must be substituted with the intramuscular (the mid-portion of the outer thigh) injection of epinephrine (also known as adrenaline), using commercially-available auto-injectors (Simons *et al*., 2011; Mok et al.,

215).

Corticosteroids on the other hand, are topical or systemic variants used to suppress the chronic inflammatory changes observed in asthma, rhinitis or eczema (Heyman, 2000; Murphy *et al.*, 2008); whiles anti-leukotrienes are capable of blocking the action of leukotrienes, which are inflammatory mediators released by eosinophils during hypersensitivity responses. These have been found to be as effective as anti-histamines, but not corticosteroids, in improving nasal symptom scores and hence, the quality of life of asthma or allergic rhinitis patients (Barr *et al.*, 2014).

2.5.4 Allergen-specific immunotherapy or desensitization

This interestingly, is a therapeutic strategy that has been in use by clinicians for the last 100 years (Akdis and Akdis, 2010), and is now being better understood immunologically. It involves exposing the patient to increasing amounts of allergen to which he or she is sensitive, to induce immunological tolerance through the presumed induction of the regulatory arm of the immune system (Murphy *et al.*, 2008; Akdis, 2009; Barr *et al.*, 2014). Subcutaneous and Sublingual delivery of the allergen are currently the two modes of exposure used in this treatment strategy. While subcutaneous treatment involves the administration of the allergen via intradermal injections, sublingual treatment involves the administration of the allergen under the tongue. Sublingual treatment is increasingly becoming the preferred form of exposure among clinicians due to the less invasive nature of administration, and reviews and meta-analytical studies conducted have shown this mode of exposure to be just as effective in reducing disease burden in children and adolescents. It must be noted however, that accurate identification of the inducing allergen via clinical history and allergen testing is critical to the success of the therapy (Barr *et al.*, 2014).

2.5.5 Immunologically targeting allergy-inducing signaling pathways

Yet another strategy currently being explored involves targeting signaling pathways that enhance either IgE response in allergic disease; or the recruitment of eosinophils to the site of inflammation. Here, cytokines known to induce class-switch IgE production by Bcells such as IL-4, IL-5, or IL-13 are blocked, thus negatively influencing allergenspecific IgE titres and inhibiting eosinophil recruitment during a hypersensitivity reaction. The setback with this kind of treatment as discovered by studies is that, in spite of reduced numbers of inflammatory cells like eosinophils in blood, acute or late phase hyper-reactivity to inhaled allergen or histamine is not mitigated (Murphy *et al.*, 2008).

2.5.6 Inhibiting binding to the FcεR1 receptor on inflammatory cells by free unbound IgEs

This is a novel form of treatment that has recently been approved for use by clinicians in the United States and the European Union in 2014 (Busse *et al.*, 2001; Soler *et al.*, 2001). Briefly, the strategy seeks to limit the binding of IgE to its high-affinity Fcε receptor via a blocking agent such as a humanized mouse anti-IgE monoclonal antibody Omalizumab. This antibody binds to an antigenic epitope on the IgE which overlaps with the site with which it binds to the FcεR1 on the mast cell or eosinophil. The complex formed inhibits the binding of the IgE to the FcεR1 receptor, hence preventing eventual cross-linking and degranulation by mast cells, eosinophils and basophils (Chang *et al.*, 2007). Additionally, the reduced titres of free IgE has been found to down-regulate the FcεR1 receptors on basophils, and mast cells. Worth noting is the fact that the antibody is only effective on free unbound IgEs and IgEs on B-cell surfaces; and that this therapeutic measure is meant to

complement treatments of severe cases of asthma and allergies especially in situations where high doses of corticosteroids have not been effective.

2.5.7 The use of helminth products as therapies

An important immunological feature associated with helminths in general is the induction of CD4 TH2-like responses that mediate the increase in IgE titres in infected individuals. However, findings from numerous studies have shown a negative association between elevated IgE titres in helminth-infected participants and positive skin prick test results (Amoah *et al.*, 2013). Additionally, evidence from a number of studies suggest that these helminths and their products have immunomodulatory properties that could prove therapeutic in clinical allergy cases. A case in point was the use of eggs of the pig nematode *Trichuris suis* to treat the Th1-associated inflammatory bowel disease or Chron's disease (Summers *et al.*, 2003). Though patients involved in the study were treated, gains had to be maintained by the administration of *T. suis* eggs every 3 weeks for 28 weeks. There are currently a combined total of 13 completed and on-going clinical trials with *T. suis* eggs aimed at comparing the efficacy of treatment early or later in life. Hookworm larvae have also been used to infect human patients in order to treat against inflammatory gastro-intestinal diseases (Amoah *et al.*, 2014).

2.6 Cross-reactive IgE-inducing carbohydrates: potential agents in the treatment of clinical allergies

The possibility of being able to identify or characterize molecules from helminths or other environmental sources with immunomodulatory capability in mitigating allergic disease is currently an area under intense research. This strategy ultimately seeks to induce immune tolerance both in allergy-prone participants and clinical allergy cases without necessarily

serving them with whole helminths. In food allergy studies, these kinds of IgE have been found to have variable degrees of biological activity, but have never been convincingly shown to induce clinical symptoms (van Ree, 2002).

It must be noted that properties associated with the low biological activity of crossreactive IgEs are yet to be fully understood. Also, whether or not high titres of crossreactive IgE are capable of mitigating allergic hyper-reactivity remains unclear. However, previous studies (Smits *et al.*, 2010) have shown how in developing countries where there is exposure to higher ambient concentrations of environmental microorganisms and parasites, the tendency to hyper react to innocuous environmental particles is much less. In many of these studies as well, a negative association has been apparent between a number of helminth infections and allergies (Obeng *et al.*, 2014; reviewed by Amoah *et al.*, 2014). Hence remains the challenge of how to induce a similar type of hyporesponsiveness in populations where the incidence and prevalence of allergic disease are high.

Food allergy-related studies have identified certain glycan epitopes as likely inducers of these cross-reactive IgEs (van Ree, 2002; Amoah *et al.*, 2013). Hence, an overview of what carbohydrates are, the various kinds available in nature, and their usefulness in allergic disease immunotherapy would prove useful.

2.6.1 Carbohydrates: definition and main types found in nature

Carbohydrates or saccharides consist of the atoms carbon, hydrogen and oxygen (Whitney *et al.*, 1998; Smolin and Grosvenor, 1994). Apart from being a major food source and a key form of energy for most organisms, carbohydrates are capable of functioning as long-term storage molecules, as protective membranes for organisms and cells, and as the main structural support for plants, when combined together to form polymers. Additionally,

carbohydrates covalently bind with other bio-molecules during the post-translational process to form glycoconjugates such as proteoglycans, glycolipids, and glycoproteins, which are widely distributed in nature.

2.6.1.1 Proteoglycans

A proteoglycan is a glycoconjugate with one or more covalently attached glycosaminoglycan (GAG) chains. These GAG chains consist of repeating disaccharide units that are normally built on (or extend from) the tri- or tetrasaccharide -3Galβ13Galβ1-4Xylβ- or GlcNAcβ1-3Galβ1-3Galβ1-4Xylβ- linked to Serine (Ser) respectively (Figure 2.3). The GAGs, or proteoglycans, are classified into 6 main groups based on the nature of repeating disaccharide units extending from the tetrasaccharide linked to Ser. These are: hyaluronic acid (HA), chondroitin sulphate (ChS), dermatan sulphate (DS), heparan sulphate (HS), Heparin, and Keratan sulphate (KS) (Robertson and Cain, 1985; Hamed *et al.*, 1997).

Proteoglycans are an integral part of the extra-cellular matrix of animal cells as well as in connective tissues (Kjellen and Lindahl, 1991; Etzler, 2004). Hyaluronic acid occurs in synovial fluid, and is part of the extra-cellular matrix of loose connective tissue. Chondroitin sulphate is the most abundant glycosaminoglycan in animals. It occurs in cartilage, bone, and heart valves. Heparin is a component of intracellular granules of mast cells, whiles Heparan sulphate (with less sulphate groups) occurs in basement membranes, and on cell surfaces. Dermatan sulphate occurs in skin, blood vessels and heart valves, whiles Keratan sulphate occurs in the cornea, bone and cartilage (Kjellen and Lindahl, 1991; Hardingham and Fosang, 1992; Esko *et al.*, 2009).

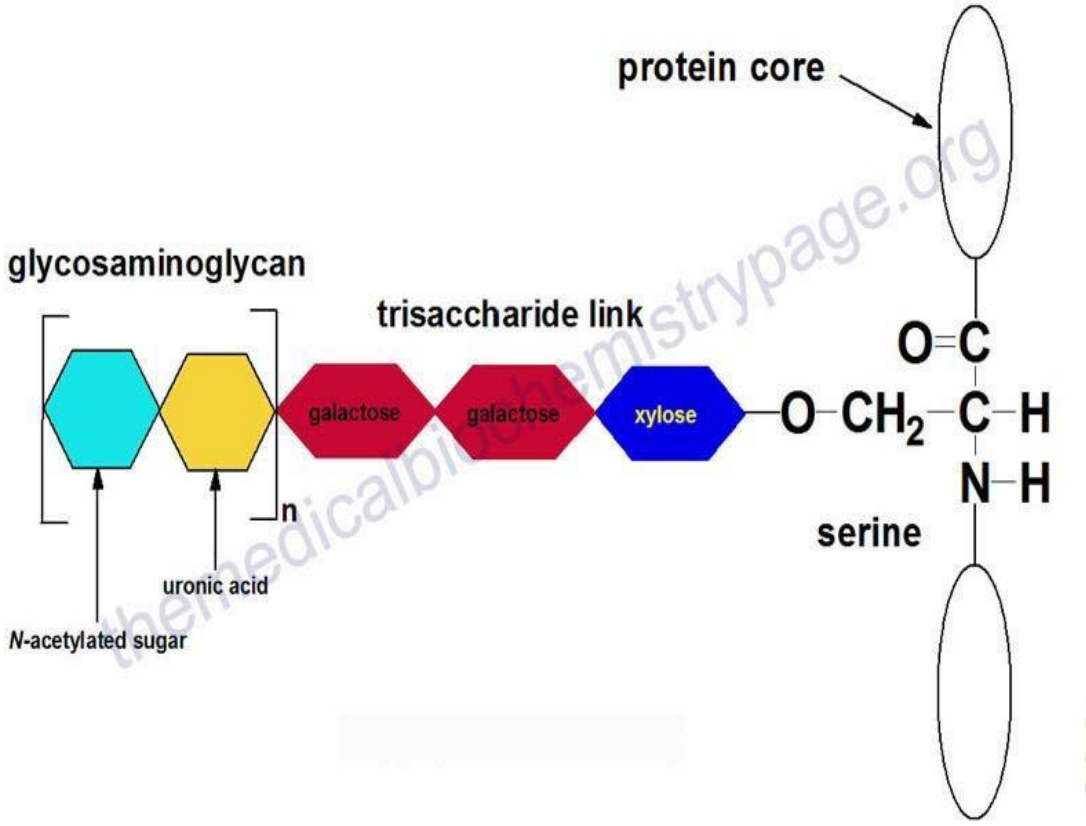


Figure 2.3: The general proteoglycans structure: The basic structure of the proteoglycans comprises a glycosaminoglycan (GAG; in square brackets) attached to the trisaccharide link extending from the protein core. The GAG is a repeating unit (*Courtesy: www.themedicalbiochemistrypage.org*)

Proteoglycans are also produced by invertebrates except that, hyaluronan/hyaluronic acid is absent, and chondroitin chains tend not to be sulphated (Esko *et al.*, 2009). Interestingly,

hyaluronic acid has been found in the tegument of adult worms of *S. mansoni* along with Heparin, and Heparan sulphate. Dermatan sulphate and chondroitin sulphate were found in *S. haematobium* (Robertson and Cain, 1985; Hamed *et al.*, 1997).

2.6.1.2 Glycolipids

Glycolipids refer to molecules comprising a glycan chain linked to a lipid unit via a ceramide unit (Makaaru *et al.*, 1992). Glycophosphatidylinositols and glycosphingolipids are the two main types of glycolipids in nature. In higher organisms or eukaryotes however, most glycolipids exist as glycosphingolipids (Schnaar *et al.*, 2009).

2.6.1.2.1 Glycophosphatidylinositols (GPIs)

Also referred to as glycophosphatidylinositol anchors, or GPI anchors (Schnaar *et al.*, 2009; Robijn, 2008), glycophosphatidylinositols (GPIs) are a distinct family of glycolipids that are found via their carboxy termini in the outer leaflet of the cell membrane lipid bi-layer. They serve as membrane anchors to attached glycoproteins. Proteins which are covalently bonded to GPIs face the extracellular environment.

The biosynthesis of the GPI anchor begins in the endoplasmic reticulum, where the preassembling of the GPI molecule involves the sequential adding of the oligosaccharides and phosophoethanolamine to the phosphatidylinositol precursor. Next, the molecule is transferred to the carboxy termini of a membrane-associated peptide, which is next transported to the Golgi complex for further modification (Hart, 1999).

2.6.1.2.2 Glycosphingolipids (GSLs)

They are found in cell membranes of organisms from bacteria to man and comprise over 80% of glycoconjugates found in the vertebrate brain (Schnaar *et al.*, 2009). Both the

ceramide and the attached glycan subunits have been recognized as the sources of diversity in this large family of glycans. The ceramide component of GSLs constitutes a long-chain amino alcohol (sphingosine) attached to a fatty acid via an amide linkage. The variations in length, hydroxylation, and saturation of both the sphingosine and fatty acid components are important in the observed structural and functional diversity of these membrane surface-bound glycans (Hakomori, 1990). Inspite of this however, classification of these glycans have been based traditionally on the glycans, which constitute glycan units that usually comprise -3Manβ1-4Glc-, 3Galβ1-4Glc-, 4Galβ14Glc-, and Gal-α-1,4-Gal-, each of which is linked to a lipid moiety, or ceramide (Cer) (Makaaru *et al.*, 1992). Essentially, these 4 grouping units can be further placed in two major categories depending on the sugar unit being attached to the ceramide: the GlcCer, and the GalCer categories.

The biosynthesis of GSLs generally commences at the cytoplasmic side of the endoplasmic reticulum (ER) with the synthesis of ceramide. The newly synthesized ceramide is then transferred by means not fully understood, into the luminal surface of the ER and transported to the early Golgi apparatus. At the cytoplasmic side of the Golgi apparatus, a monosaccharide unit is added to the ceramide, forming X-Cer (where X denotes either glucose (Glc) or Galactose (Gal)). The subsequent structure is 'flipped' into the lumen of the Golgi bodies, and the X-Cer compound is elongated with the aid of glycosyltransferases (Schnaar *et al.*, 2009).

Although the biological function of these membrane-bound structures is being intensively studied, current evidence suggest that they might act as intermediaries in the flow of information from the outside to the inside of cells (Schnaar *et al.*, 2009; Hakomori and Igarashi, 1995).

2.6.1.3 Glycoproteins

These comprise carbohydrates that are covalently attached to proteins either via the amide nitrogen in the side chain of Asparagine (N-linked), or to the oxygen atom in the side chain of serine or threonine (O-linked) (Berg *et al.*, 2002). The sugars in glycoproteins are much smaller than those in proteoglycans. Interestingly, most of the proteins in blood serum are glycosylated (Berg *et al.*, 2002; Ashwell and Morell, 2006); and glycoproteins are also prevalent in plants and invertebrates as secreted and cell surface factors.

2.6.1.3.1 N-Glycoprotein synthesis in eukaryotes

Protein glycosylation occurs in eukaryotic cells in the lumen of the endoplasmic reticulum (ER) and the Golgi Complex for N-glycans; and exclusively in the Golgi complex for O-glycans (Berg *et al.*, 2002). The biosynthesis of all N-glycans begins on the cytoplasmic side of the ER membrane. Here, the core N-glycan structure is formed by the adding first, of the 2 N-acetylglucosamine residues and then, the 5 mannose residues, to the ER membrane-attached Dolichol phosphate. The formed glycan core is next translocated or 'flipped' to the lumen of the ER via mechanisms not well understood. This event is matched with the concurrent synthesis of the peptide by the ribosomes on the surface of the endoplasmic reticulum. During synthesis, the peptide chain is inserted into the lumen of the ER as it grows, guided by a signal sequence of a certain number of amino acids at the amino terminus. Once the peptide chain grows into the lumen of the ER, the signal sequence at the amino terminus is cleaved.

In the ER lumen, additional sugars are added to the 5-glycan core to form a 14-sugar residue (still attached to dolichol phosphate) (Berg *et al.*, 2002; Gamblin *et al.*, 2008). This 14-sugar precursor is next transferred en bloc to the Asparagine in the concurrently growing

polypeptide with the consensus sequence Asn – X – Ser/Thr. This is followed by a series of processing reactions that trim the N-glycan (Stanley *et al.*, 2009). Next is the transfer of the glycopeptides to the medial- or trans- Golgi cisternae (van den Eijnden *et al.*, 1997), where they are further processed and 'packaged'

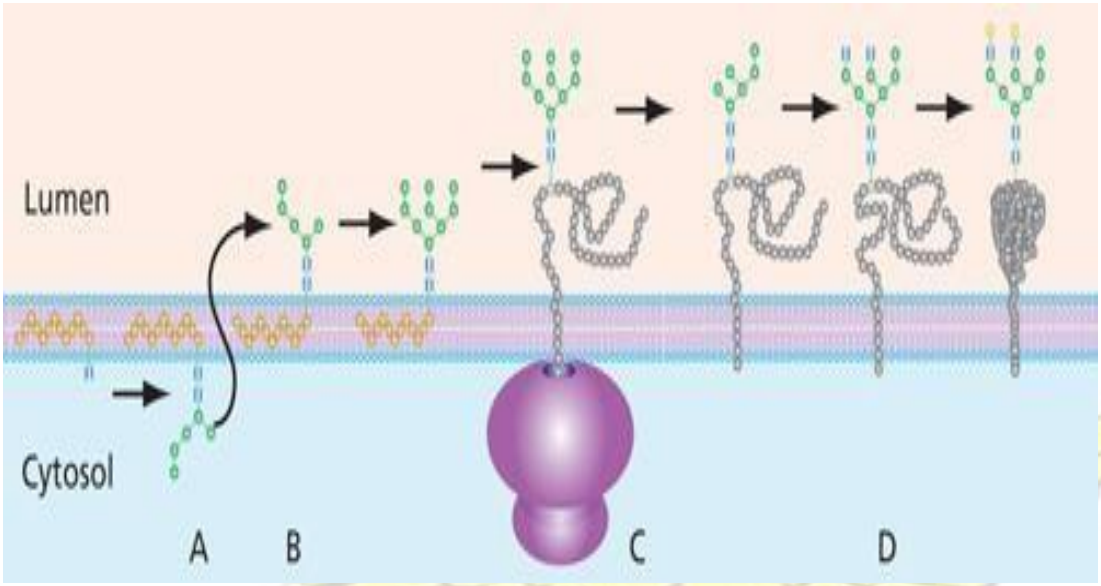


Figure 2.4: Glycoprotein synthesis in eukaryotic cells: (a) The core structure of the Nlinked glycan commences at the cytosol side of the endoplasmic reticulum (ER) with the glycan anchored to dolichol phosphate. (**b)** Via processes not fully understood, the entire core structure is 'flipped' into the lumen side of the ER and mannoses are further added to the core structure. (**c)** The N-glycan precursor is transferred en bloc to the growing peptide, attaching at an asparagine. (**d)** The glycan and peptide are further processed in preparation for transfer to the Golgi apparatus. (*Courtesy: www.Sigma-Aldrich.com*)

2.6.1.3.2 O-Glycoprotein synthesis

The biosynthesis of O-glycans in eukaryotic cells is however quite different for two key reasons. First, the process occurs exclusively in the Golgi bodies, following the transfer of peptide from the rough endoplasmic reticulum. Also with o-glycans, there is no precursor

structure and hence, no en bloc transfer (Berg *et al.*, 2002). In this instance, the sole transfer of a GalNAc to Ser or Thr of the polypeptide initiates o-glycosylation (Berg *et al.*, 2002).

2.6.1.3.3 Core modifications of N-glycans in vertebrates, invertebrates, and plants

Following the translocation of glycopeptides to the Golgi cisternae, further additions of sugars are made to the core N-glycan structure. However, N-glycan core modifications vary between plant, invertebrate, and vertebrate cells.

In mammals including humans, the major core modification that occurs in the Golgi cisternae is the addition of fucose in an α1-6 linkage to the N-acetylglucosamine (GlcNAc) adjacent to the asparagine of the peptide (terminal GlcNAc) (Stanley *et al.*, 2009). The addition of fucose in a α-1,3- linkage to the GlcNAc adjacent to the asparagine; or the addition of xylose in a β1-2 linkage to the β-mannose of the N-glycan core, are both absent in vertebrate cells. Apart from the α6-fucose core modification, Nglycans with no saccharide units added to the core structure are also abundant. Apart from the addition of fucose via an α-1,6 linkage to the terminal GlcNAc, α3fucosylation of the same GlcNAc is also possible in invertebrate cells (Stanley *et al.*, 2009). Hence, a common modification, particularly in insect glycoproteins, is the addition of two fucoses via both α-1,3 and α-1,6 linkages. These modifications have also been identified in plant and helminth glycoproteins (Amoah *et al.*, 2014). Another core modification common in helminths, particularly schistosomes, is the addition of xylose via a β-1,2 linkage to the β-mannose of the N-glycan core structure (Stanley *et al.*, 2009). In fact, the α3- and α6-difucosylated, and β2-xylosylated N-glycan is a unique combination described so far in egg glycoproteins of both *Schistosoma mansoni* and *S. japonicum* (Khoo *et al.*, 1997; Hokke *et al.*, 2007; van Diepen *et al.*, 2012; Smit *et al.*, 2015).

The core modifications of N-glycans in plant glycoproteins are similar particularly to those described for invertebrates. However, α6-fucosylation of the terminal GlcNAc is impossible in plant N-glycans (Mari *et al.*, 2008). Common variations of core-modified N-glycans observed in plant glycoproteins include β2-xylosylation; α3-fucosylation; and α3-fucosylation with β2-xylosylation.

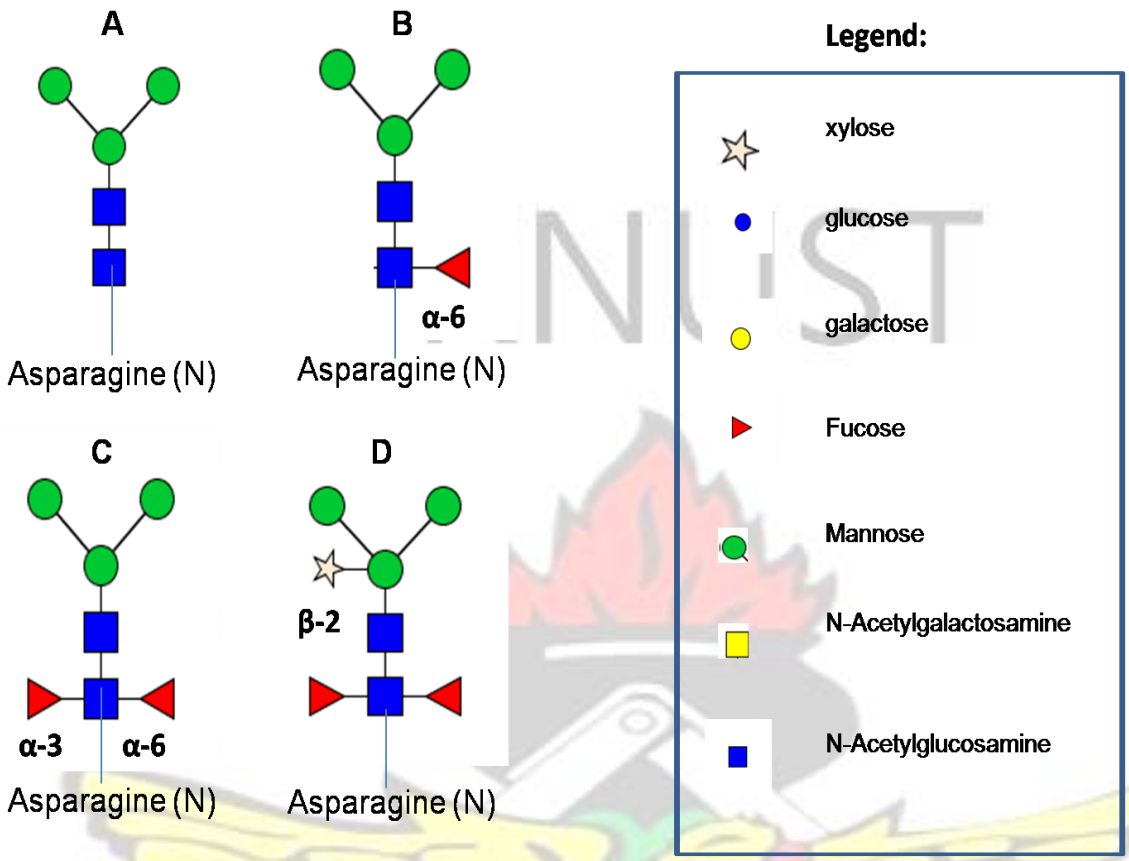


Figure 2.5: Major core-modifications of N-glycans: (a) This is representative of the Nglycan core in the absence of any core modifications. These are present in the glycoproteins of mammals and some invertebrates, particularly helminths. **(b)** The α6–fucosylated N-glycan is also found in the glycoproteins of mammals and Schistosomes. (**c)** The α3-/ α6-difucosylated core modification is found largely in the glycoproteins of insects and schistosomes. (**d)** The α3-/α6-difucose and β2-xylose core modification is found in plants and some invertebrates like the egg glycoproteins of schistosomes. (*Courtesy: Brzezicka et al., 2015*)

2.6.1.3.4 Core-modified N-glycans and the induction of cross-reactive IgE

The glycan epitopes α3-fucose and β2-xylose have been identified by a number of studies to play a major role in inducing cross-reactive IgEs, or IgEs with low biological activity (Amoah *et al.*, 2014). Additionally, findings from recent food allergy-related research implicate IgEs capable of recognizing such glycan epitopes to be of little or no clinical relevance, as they do not induce clinical symptoms (Mari *et al.*, 2008; van Ree, 2002). A case in point is a study conducted in Ghana, where although 17.5% participants were found to be sensitized to peanut (i.e had detectible levels of IgE to peanut in serum: >=0.35kU/L), more than 92% of these same participants did not show positive skin prick tests (SPT) results to the peanut allergen (Amoah *et al.*, 2014).

This raises the possibility of being able to identify or characterize molecules, including glycans, from helminths or other sources with immunomodulatory capability. This work therefore sought to determine patterns associated with serum IgE responses in a selection of children from the urban high, urban low, and rural areas to an array of glycans. It must be noted that, the glycans used in this work were not obtained from nature, but manufactured in the lab, and would hence be referred to as synthetic glycans for purposes of convenience. It is however expected that these synthetic structures would also provide a clear picture on anti-glycan IgE and IgG responses, especially regarding the epitopes influencing response patterns. One question of particular interest which this work sought to address was which glycan motifs (whether α3- fucose, or β2-xylose, or both) could potentially be influencing IgE, IgG1, and IgG4 response patterns in each of the Areas.

2.6.2 Associated IgG1 antibody responses in Allergic reactions and cross-reactive IgE positivity

Weighing approximately 150kDa, the immunoglobulin (Ig) G1 antibody comprises one of the four well characterized human IgG subclasses. It is also known to constitute the highest concentration of total IgG in blood and lymph (i.e. 5-12mg/ml) of adults (which is the reason for the number '1' in defining this isotype), followed with the IgG2 subclass (2-6mg/ml), the IgG3 (0.5-1.0mg/ml), and IgG4 (0.2-1.0mg/ml) subclasses respectively (Herrod, 1992; Hamilton, 2001; Murphy *et al.*, 2008). The IgG1 antibody, like other human immunoglobulins, is a glycoprotein with N-linked glycans attached to the Fc region/piece (Clark, 1997; Murphy *et al.*, 2008).

A key biological function of IgG1, following opsonization (or the binding of antigen by its variable or Fragment antigen binding (Fab) region to modify the surface of the antigen for phagocytosis), is the activation of the complement cascade by the classical pathway via its constant or Fragment crystallizable (Fc) domain. As a result, pathogens and antigens are either directly destroyed or engulfed by recruited and activated phagocytes (Ward and Ghetie, 1995). This process of complement-activation however, is physiologically active, leading sometimes to local, and sometimes, systemic tissue inflammation (Parham, 2005). Secondly IgG1, via its Fc region, is capable of binding to

Fcγ receptors on phagocytes, hence facilitating the clearance of antigen or immunecomplexes (Ward and Ghetie, 1995). Another important biological function of IgG1 is its ability to reach certain areas/compartments in the human body via the activation of a specific active transport receptor by its Fc domain. These areas/compartments include the mucous, lachrymal, and mammary glands, as well as foetal blood circulation through placental transfer. This is evidenced with the presence of these antibodies in mucous

secretions, tears, breast milk, and foetal blood respectively (Hamilton, 2001). Additionally, its functional role in Type-2 hypersensitivity reactions is wellcharacterized, involving recognition by its Fab regions of cell surfaces modified by such covalently-bound molecules as penicillin. This is followed by complement activation, and the eventual phagocytosis of self-cells (Clark, 1997). However, little is known with regards to its functional role in allergic hypersensitivity.

2.6.3 Associated IgG4 antibody responses in Allergic reactions

Immunoglobulin 4 (IgG), on the other hand, is quite an unusual antibody. Although similar in weight (i.e approximately 150kDa) to IgG1, it constitutes the lowest concentration of IgG in blood and lymph (0.2-1.0mg/ml), and is associated with biological activities that do not tend towards immune hyper-reactions (Parham, 2005; Ward and Ghetie, 1995; Hamilton, 2001).

First, among the IgG subclasses, IgG4 possesses the lowest complement activation capacity. This, it has been realized, is largely due to the monovalent nature of the binding sites on its Fab regions. Hence, one binding site recognizes an antigen, while the other binding site recognizes a different antigen altogether. The antibody at this point is said to be monovalent to one antigen, and thus cannot form immune complexes large enough to activate complement or induce immune inflammation (Hamilton, 2001; Aalberse and Schuurman, 2002; Aalberse 2011). Additionally, IgG4 has been found to be associated with comparably lower placental transfer than IgG1 and IgG3, implying lower concentrations in foetal blood circulation; and has also been found to exhibit much less affinity for the Fc receptors-1, and -2 present on the surfaces of phagocytes and B-cells

(Hamilton, 2001). Taken together, this is suggestive of the general anti-inflammatory activity associated with the IgG4 antibody. This is further buttressed by evidence from numerous parasite immuno-epidemiological studies demonstrating the induction of tolerance during chronic helminth infections via the activation of regulatory T-cells and the increased production of IL-10 and IgG4 by B-cells. Even in allergen-specific immunotherapy (AIT), the allergen of interest is administered in increasing doses to the patient with the purpose of activating the regulatory arm of the adaptive response system, characterized by increased IL-10 and IgG4 levels (Akdis and Akdis, 2011).

Extensive evidence from allergy-associated immunological studies implicates IgG4 with the capability of preventing the clinical symptoms associated with adverse allergy (De Moira, 2012). However, the role of IgG4 in glycoallergen-related responses is yet to be fully understood. Additionally, the mechanisms involving the production of IgG4 antibodies by B-cells during AIT regimens and glycan-associated IgE cross-reactivity would prove essential in our quest to develop new clinical strategies involving these 'clinically irrelevant' IgEs (Davies *et al.*, 2013).

2.6.4 IgG1 and IgG4 production by B-cells, class-switch theories, and implications in IgE cross-reactivity

In humans, antibody production by B lymphocytes begins in the nucleus, where the immunoglobulin genes are located at three chromosomal loci namely, the heavy chain locus on chromosome 14, the K light-chain locus on chromosome 2, and the λ light-chain locus on chromosome 22. With the exception of B-cells, these genes are in fragmented form in other human cells and hence, cannot be expressed. Essentially, these immunoglobulin genes comprise segments that encode a leader peptide (L), the lightchain Variable (V) and

Joining (J) regions, and the heavy-chain Constant (C) region within which the genes coding for the Fc regions of all the antibody isotypes are located

(Parham, 2005). Thus, the Cμ which encodes for the Fc piece of IgM, is upstream of the Cδ which encodes for the Fc region of the IgD antibody. This is also upstream of Cγ3, Cγ1, Cα1, Cγ2, Cγ4, Cε, and Cα2 which code for the Fc portions of IgG3, IgG1,IgA1, IgG2, IgG4, IgE, and IgA2 in that respective order (Jeffries *et al.*, 1995; Murphy *et al.*, 2008) (Figure 2.6).

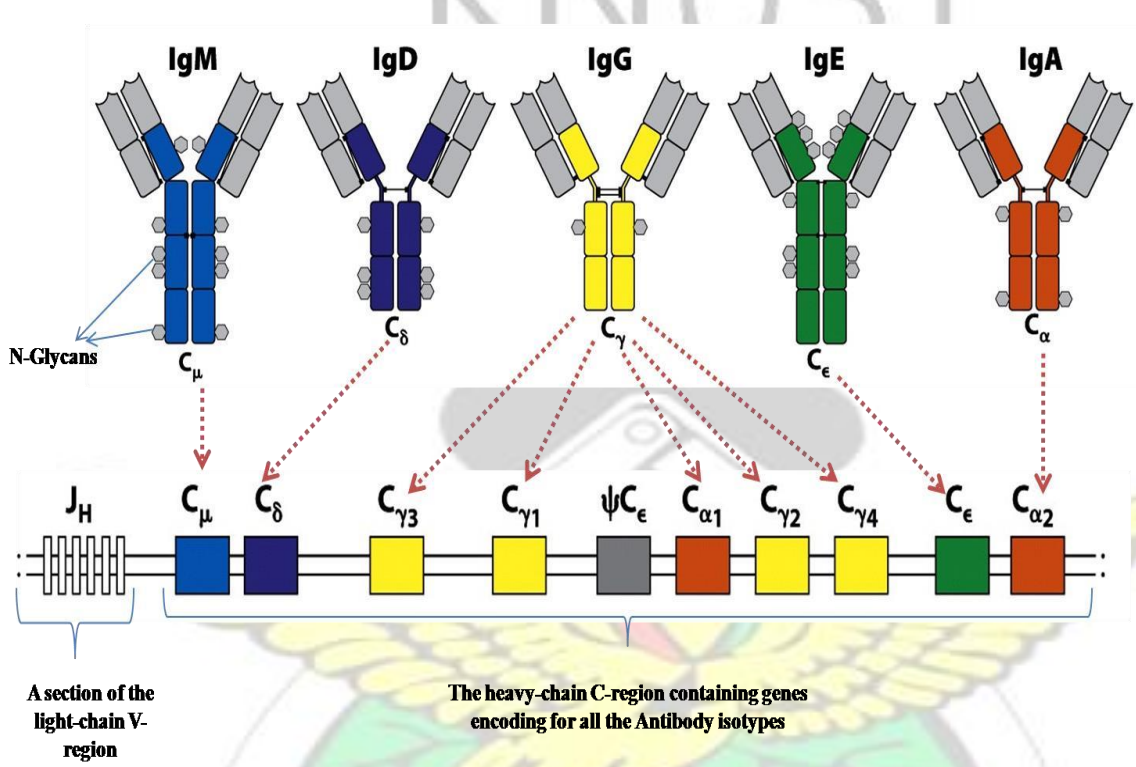


Figure 2.6: An illustration of the heavy-chain C region of the immunoglobulin genes in B-cells. The relative positions of the genes encoding the Fc pieces of the various antibody isotypes are indicated with colour codes. Note that: Cμ (blue) represents the region coding for the Fc piece of IgM; Cδ (navy blue) represents the region coding for the Fc piece of IgD; Cγ3 (yellow) = Fc piece for IgG3; Cγ1(yellow) = Fc piece for IgG1; Cα1(scarlet) = Fc piece for IgA1, Cγ2(yellow) = Fc piece for IgG2; Cγ4(yellow) = Fc piece for IgG4; Cε (green) = Fc piece for IgE; and Cα2(scarlet) = Fc piece for IgA2 (*Courtesy: Murphy et al., 2008*).

Interestingly, when a naïve B-cell (marked by the alternate expression of IgM and IgD on its surface) encounters antigen, it undergoes a tightly-controlled antibody-gene rearrangement process. As a result, antibodies of a particular isotype capable of recognizing only the particular antigen encountered are produced. Additionally, during isotype or class-switching by B-cells, further gene rearrangements occur within the heavy-chain C region, in which the C gene previously coding for the production of a particular antibody isotype is excised, and a different one downstream of the one removed is brought into close proximity with the light-chain V-region. This leads eventually to the production of the new isotype by the B lymphocytes. The process however is irreversible so that IgG1-producing B-cells that class-switch to producing IgG4 or IgE (the genes of which are downstream from the IgG1-encoding gene), may no more be capable of producing IgG1 (Parham, 2005; Aalberse, 2011).

With respect to IgE production by B lymphocytes, it has been generally accepted that the class-switch to production of this isotype may occur via two possible pathways: an indirect pathway in which the IgM and/or IgD-producing B-cell undergoes isotype switching to produce IgG, followed by further isotype switching to produce IgE antibodies; and a direct pathway in which the naïve B-cell class-switches directly into IgE production (Aalberse, 2011; Xiong *et al.*, 2012; Davies *et al.*, 2013). The pathway undertaken by each B-cell has been found to reflect essentially the amount of time spent in the germinal centre (GC) of the lymph node or the spleen. Thus, B-cells spending relatively little time in the GC neither undergo adequate somatic hypermutation/recombination, affinity maturation, nor acquire memory phenotypes; while the opposite is true for B-cells that remain relatively longer in the GC. Although still being debated, numerous findings indicate that most naïve B-cells

spend little time in the GC, and hence, are likely sources of circulating allergen-specific IgEs. Additionally, the relatively smaller amount of mature B-cells in the GC makes the production of IgEspecific memory B-cells rare in circulation (Davies *et al.*, 2013). Evidence provided by Xiong *et al.* (2012) on the other hand, point to these same pre-mature, IgE-producing Bcells as likely sources of cross-reactive IgE.

It is also unclear whether class-switching from the IgG1 or the IgG4 isotypes by B-cells has an effect on the binding affinity of the IgE produced. Work done by Aalberse (2011) indicated a propensity of IgG1 to increase the potency of IgE antibodies due largely to its ability to form precipitating antigen/antibody complexes. Evidence from the same study however suggested a blocking role for IgG4 due to its inability to form large enough antigen/antibody complexes that would induce a cascade of pro-inflammatory immune responses. Interestingly, it has been indicated elsewhere that the blocking activity of IgG might not necessarily be restricted to IgG4 alone but may include other IgG subclasses (Ejrnaes *et al.*, 2004). This has led to the hypothesis that the inability of glycans to induce potent inflammatory responses might be due to the generation of high titres of associated glycan-specific IgG antibodies, which in turn compete with the less abundant IgE antibodies for allergen molecules (Jin *et al.*, 2008). Much certainly remains to be understood with regards to the mechanisms involved in the development of cross-reactive IgE, and the involvement of IgG1 and IgG4 in the process. This however, is critical if we hope to ultimately control the immunomodulatory processes involved in ameliorating adverse allergic reactions.

2.7 Current techniques in the determination or measurement of glycan- antibody interactions

2.7.1 Enzyme-linked Immunosorbent Assay (ELISA)

This technique was originally described by Engvall and Perlmann in 1971, and has since revolutionized our understanding of immunology, particularly with regard to antigenantibody interactions (Thermo Fisher Scientific, 2010). It is also worth noting that, the other technologies herein described are essentially based on the principles governing this technique. Apart from protein-protein interaction studies, this technique is also essential in studying glycoprotein-antibody interactions, and as a result has been used extensively in allergy-associated and glycan-related research.

Four principal steps underline current variations of the assay, namely the coating or capture step, which involves the direct or indirect immobilization of the antigen of interest to the bottom surface of a polystyrene micro-titer plate well; the plate blocking step, which involves the administration of irrelevant proteins to cover all other surfaces of the well not colonized by the immobilized antigen or capture antibody-antigen complex; the probing/detection step, which involves the binding to the antigen by antigen-specific antibodies; and the signal measurement step involving the detection and quantification of the antigen-antibody complexes formed via interactions between the tag on the direct or secondary antibody probe and a colour-changing agent or substrate. In all ELISA variations, assay protocols are interspersed with washing steps which help to ensure that only bound antigen/antibody/probe complexes remain for signal detection and measurement in the final step (Murphy *et al.*, 2008; Thermo Fisher Scientific, 2010).

Additionally, current variations of ELISA can be categorized into three groups namely, the direct, the indirect, and the capture/sandwich assays. In the direct assay, the antigen is

immobilized onto the bottom surface of the micro-titer plate well followed with the conjugated primary antibody, and finally, a colour-changing substrate. The indirect assay is a slight modification of the direct ELISA in that, there is the administration of a primary detection antibody following antigen immobilization. Hence, incubation with a conjugate-labeled secondary antibody becomes a next step, followed with the addition of the colour-changing substrate. The capture/sandwich ELISA varies from the indirect assay only with regard to the way the antigen is immobilized. Here, the polystyrene well bottom of the micro-titer plate is coated with a capture antibody specific to the antigen of interest, which in turn immobilizes the antigen. The addition of the primary, then a conjugate-tagged secondary antibody, and finally a colour-changing substrate follows (ThermoFisher Scientific, 2010; Murphy *et al.*, 2008).

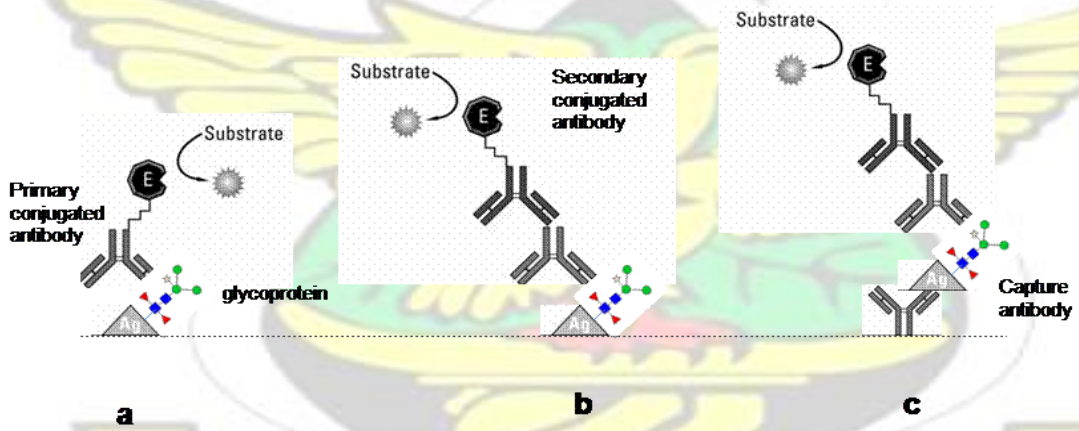


Figure 2.7: The three major ELISA types: The three main groups into which current variations of ELISA may be categorized are **(a)** the Direct Assay; **(b)** the Indirect Assay; and **(c)** the Capture/Sandwich Assay. For both the direct, and indirect assays, the antigen of interest (in this case, a glycoprotein) is immobilized onto the bottom surface of the well of the micro-titre plate. With the sandwich assay, a capture antibody is immobilized onto the bottom of the well of the plate, which in turn binds the antigen of interest (*Courtesy: Thermofisher Scientific,2010*).

2.7.2 Glycan microarray

Allergy-associated IgE cross-reactivity, as so far understood could be due to proteins or the glycans on glycoproteins. The immunomodulatory properties of the glycan components, or cross-reactive carbohydrate determinants (CCDs) may be understood to some extent by studying the humoral profiles they elicit, hence necessitating the use of the glycan microarray. This technology allows for the simultaneous study of glycanantibody interactions for a large number of glycans (synthesized or natural) immobilized on a solid phase or glass slide (Blixt *et al.*, 2004; Bochner *et al.*, 2005). Thus, with as little as 3µL of serum, a large volume of valuable data can be acquired.

The glycans immobilized on the solid phase may be derived from natural sources, or manufactured in the laboratory. Manufactured or synthetic glycans may differ from their natural counterparts in their stereochemistry, and composition. This is because, synthetic glycans are less complex than their natural counterparts which are often obtained from nature as glycoproteins or lipid glycan fractions (Smith *et al.*, 2010). One key advantage of using a synthetic glycan microarray is the absence of other bio-molecules or aglycones that may influence the conformation and hence, the binding of the antibody to the glycan. It must however be noted that there are many naturally-occurring glycans that have not yet been synthesized simply because they are yet to be structurally identified. Additionally, as synthesis of these glycans is laborious and time-consuming, only wellknown, relevant glycans and those that can be printed onto a glass slide, are synthesized.

2.7.3 Radioallergosorbent Test (RAST)

This technique preceded the ImmunoCAP assay by over a decade. Invented and marketed by Pharmacia Diagnostics AB (Phadia) in Uppsala, Sweden in 1974, the assay was designed to quantify the amount of IgE specific to a particular allergen in as little as 100µL of diluted patient serum (Berg *et al.*, 1974). As with other tests herein described, this assay provides information on allergen exposure, but not on reaction severity, or on the amount of cell-bound IgEs in circulation. Although the technology has been substituted since 1989 with the ImmunoCAP specific IgE blood test in most commercial clinical laboratories, the RAST test is still used in some labs for diagnostic and research purposes (Bousquet *et al.*, 1990).

Essentially, the serum obtained from the volar part of the patient's lower arm is diluted, and incubated for a period of time with a filter paper disc coated with the allergen of interest (or coupled to a specified quantity of sepharose) in a polystyrene tube. After washing off unbound IgEs, the radio-labeled anti-human IgE is added, binding to the allergen-IgE complex on the filter paper disc. The intensity of radioactivity is measured and the amount of bound allergen-specific IgE is quantified. Results are finally interpreted using established reference scores to estimate the amount of allergen-specific IgE present in the patient's serum (Berg *et al.*, 1974; Bousquet *et al.*, 1990; Hamid *et al.*, 2015).

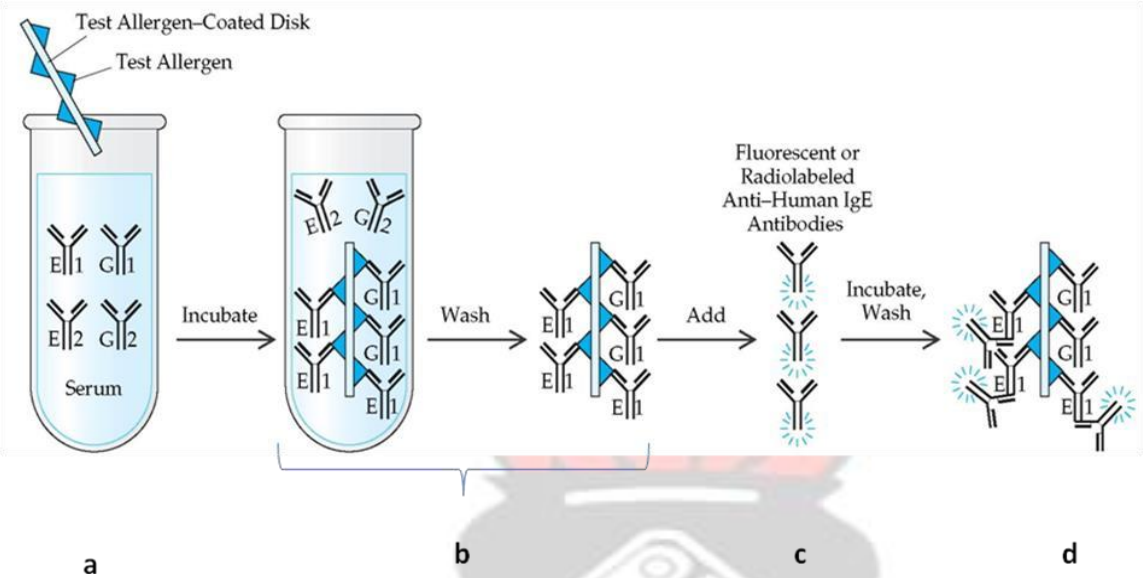


Figure 2.8: An illustration of the steps in a RAST assay. (a) The Test Allergen-coated filter paper/sepharose disc is placed in tube containing patient serum. **(b)** Antibodies in serum bind to Test Allergen on disc. **(c)** IgE-specific fluorescent probes are administered. **(d)** Fluorescent probes bind only to Fc region of IgEs bound to Test Allergen on disc (Courtesy: https://www.lookfordiagnosis.com/mesh_info.php?term=radioallergosorbent%20test&lang=1).

2.7.4 ImmunoCAP Assay

This is a fully mechanized test which was developed to facilitate diagnosis by measuring serum titres of IgE to a specific allergen or allergenic component. The test follows the same

principle as the sandwich Enzyme-linked Immunosorbent Assay (sandwich ELISA). The solid phase consists of a cellulose derivative enclosed in a capsule. The highly-branched, hydrophilic polymer ensures irreversible binding of antigens, while maintaining their native structure (Wang *et al.*, 2008). The use of allergenic components in testing, as against whole allergen extracts, is essentially referred to as componentresolved diagnostics (CRDs). This method of allergic assessment is increasingly being adopted by clinicians as it enhances the detection of active or immunogenic components of allergens. Access to these allergenic components is however possible due to recent improvements in molecular techniques and DNA technology, ensuring the sequencing, synthesizing, and cloning of allergenic proteins, also referred to as recombinant allergens (Gadisseur *et al.*, 2011). Apart from recombinant allergens, the ImmunoCAP assay has been used to determine IgE titres to such oligosaccharides as galactose-α1,3-galactose and bromelain (a marker for the detection of cross-reactive IgE responses to carbohydrate epitopes in allergens).

2.7.4.1 Titrated ImmunoCAP Inhibition Assays

This is another technique involving the use of the ImmunoCAP assay apparatus (Figure 2.7), and is employed where the degree of specificity of serum IgE of a patient to a particular allergen (or allergenic component) must be determined. A case in point is the study conducted in Ghana by Amoah *et al.* (2013) on IgE sensitivity to peanut. Although ImmunoCAP measurements of serum samples of Ghanaian subjects indicated high titres of IgE to whole peanut, further investigation was necessary to determine why skin prick results were not commensurate with serum results. Prior to measurement with ImmunoCAP, the serum sample (or sera pool) is pre-incubated with inhibiting agents, or antigens that are suspected to be sources of IgE cross-reactivity. Hence, such agents as the

schistosome egg antigen (SEA), the schistosome adult worm antigen (AWA), the *Ascaris* worm antigen, and bromelain are employed. During pre-incubation, the IgE antibodies bind to epitopes on the cross-reactive antigens, thus reducing the amount of IgE antibodies available for binding. The serum sample is next administered onto the cellulose polymer phase of the ImmunoCAP assay apparatus bearing the peanut antigens. Binding of the remaining antibodies to the peanut allergen is measured, and the percentage (%) inhibition determined. Hence, for the Ghanaian subjects, a near-complete inhibition of peanut-specific IgE was realized with *S. haematobium* egg antigens and bromelain (Amoah *et al.*, 2013).

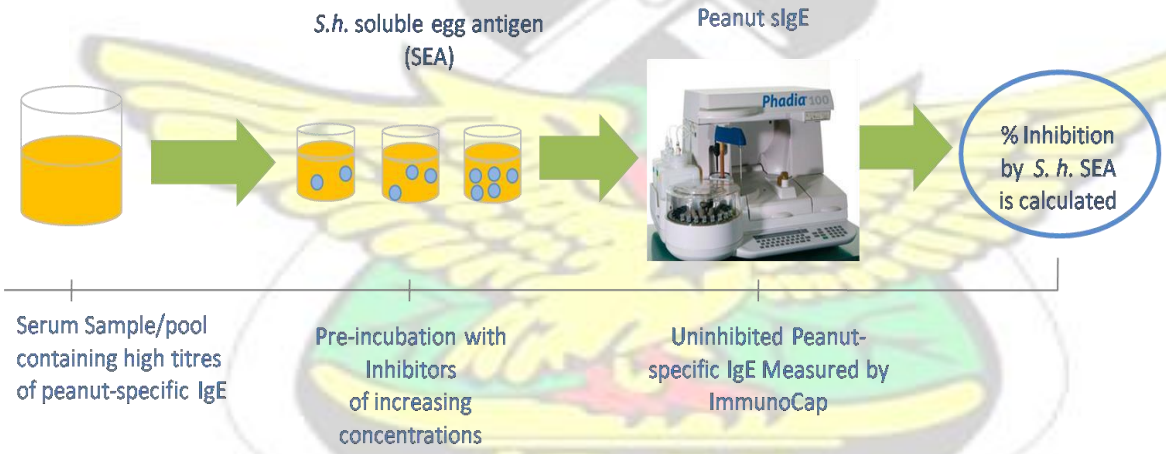


Figure 2.9: A step-wise illustration of the procedures in titrated ImmunoCAP inhibition assays. (*Courtesy: Abena Amoah, 2013*)

2.7.5 ImmunoCAP ISAC

This is a solid-phase immunoassay, and it differs slightly from the ImmunoCAP assay in terms of the number of allergenic components that can be tested at a time. Hence, while the ImmunoCAP assay may test one component at a time, this assay is designed to test 4 components per serum sample. Additionally, this immunoassay employs the use of a

biochip, or solid substrate on which the allergenic components are immobilized, as well as a microarray scanner to measure fluorescence intensities of surface-bound antibodyfluorescent probe complexes (Gadisseur *et al.*, 2011).

By way of procedure, the test serum is administered onto demarcated reaction sites on the biochip. Binding of serum IgEs to specific epitopes on printed allergenic components occurs, and this is detected by the administration of a secondary fluorescently-labeled anti-human IgE antibody. Next, a fluorescence scan image is acquired and analyzed using appropriate software (MIA – Microarray Image Analysis software) (Phadia, 2009).

CHAPTER 3:

MATERIALS AND METHODS

3.1 Study Design

This work sought to characterize antibody responses to glycan epitopes in the serum samples of a subset of subjects who had previously been recruited into a larger crosssectional study conducted in Southern Ghana under the auspices of the European Union Commission-funded EuroPrevall and GLOFAL projects (Amoah *et al.*, 2013). The study, which aimed at investigating the allergic sensitization and parasitic infections in school children in the Greater Accra Region, was conducted between 2006 and 2008 involving thirteen randomly-selected schools from four districts within the region, namely, Ga East, Ga West, Dangme East, and the Accra Metropolis. Seven of the selected schools were situated in rural communities located in the first three above-mentioned districts, while six were within the Accra Metropolis. In order to distinguish between urban low (UL) and urban high (UH) subjects, government-funded public schools assumed to cater towards subjects from low-income settings; and private, fee-paying schools thought to cater towards middle- to high-income individuals respectively, were selected. The study was approved by the Noguchi Memorial Institute for Medical Research Institutional Review Board, Ghana, and involved school children between the ages of 5 and 16 years. Parameters measured per participant included IgE serology; skin prick test (SPT) reactivity; the presence of *Schistosoma sp.* and intestinal helminths in urine and stool samples; and malaria parasitaemia in blood films. Information on risk factors associated with the development of allergy was also obtained via questionnaire administration.

3.2 Study Area and Population

This study was conducted in the Greater Accra Region in rural communities and in the national capital within the coordinates Longitudes 000.35377°W, 000.42752°E; and latitudes 005.72647°N, 005.53550°S. Participating rural communities within the region included Pantang in the Ga-East district; Mayera and Ayikai Doblo in the Ga West District; Anyaman, Goi, Toflokpo, Agbedrafor and Koluedor in the Dangme East District. The prevailing economic activities in these communities were farming and fishing as at the commencement of this work. Also, these communities were endemic for intestinal helminths, *Schistosoma sp.*, and malaria. Within the Accra metropolis, participating Urban Low (UL) schools included James Town, Immanuel Presbyterian, and Nii Okai Basic Schools, while Green Hill and the University of Ghana Basic School (formerly University Primary) were categorized as Urban High.

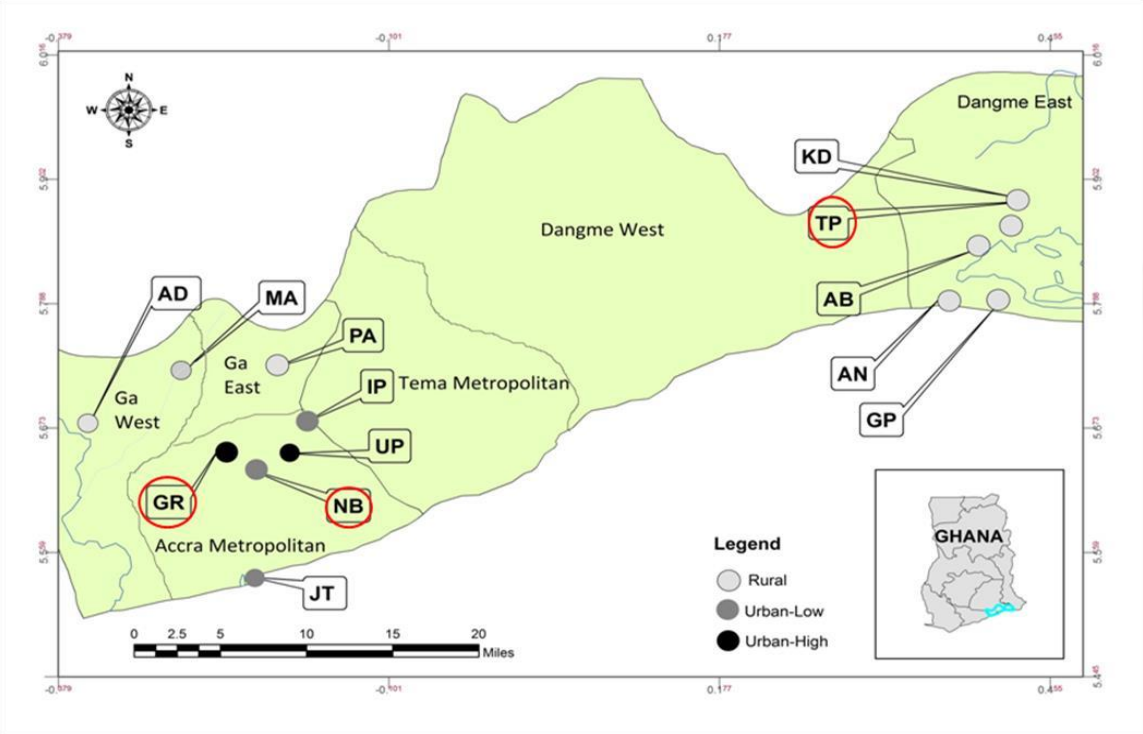


Figure 3.1: A map of the Greater Accra Region showing the locations of the schools selected for this study. The selected schools are circled in red.

3.3 Ethical Approval and Subject Recruitment

Prior to subject recruitment in the various communities within the region, ethical approval for the study was obtained from the Institutional Review Board (IRB) of the Noguchi Memorial Institute for Medical Research (NMIMR-IRB CPN 012/04-05). Permission was also granted by the Director of Education and the various district offices and school authorities for the study to be conducted in the various districts and schools respectively. Additionally, parents and guardians signed or thumb-printed informed consents after the study had been explained to them. Children also provided assent by thumb-printing on forms before samples were obtained from them. In all, 2,331 participants were recruited from the above-mentioned communities and provided samples for analysis.

3.4 Skin Prick Test

The panel of allergens used in the test included commercial preparations of peanut, house dust mite (*Dermatophagoides* mix), and cockroach (*Blatella germanica*) (all kind courtesy of ALK Abello, Madrid, Spain), along with fresh fruits obtained from the local market such as apple, pineapple, banana, mango, orange, and pawpaw. Selection of these fruits was done based largely on availability. These fruits were prepared for testing by cutting up into small, bite-size pieces. For the positive and negative controls, Histamine chloride (10mg/ml) and saline (ALK-Abello, Madrid, Spain) were used respectively. The test was performed on the volar side of the lower arm of consenting participants following sterilization of the area with ethanol. Briefly, puncture points 2 cm apart, were demarcated

on the same area using a permanent marker. Pricks were made using 1mm standardized lancets, the number of which was commensurate with the number of allergens to be tested. Each prick was exclusive to an allergen. There were however slight differences between the administration of the commercially-produced allergens and the local preparations of fruits. For the commercially-produced allergens, pricks were made, and a drop of each allergen administered to the puncture created. Regarding the panel of fruits, the tip of a lancet was used to prick a piece of fruit of interest, after which the same tip was used to puncture the skin. Approximately 15 minutes elapsed after which the pricked area was examined and considered positive for a particular allergen if the average of the length of the longest wheal diameter (D1) and its perpendicular length (D2) was

3mm or greater, along with the positive control (histamine).

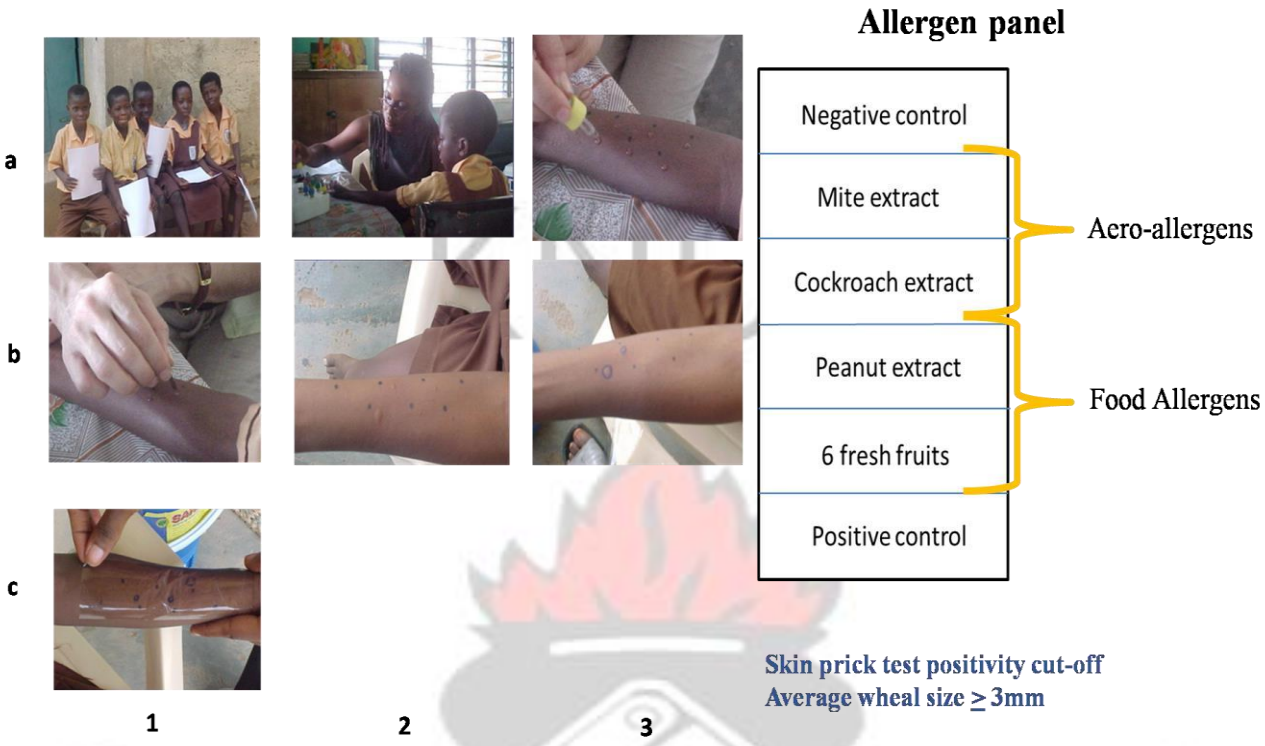


Figure 3.2: Allergen sensitization by skin prick testing (SPT): Having received consent from parents and assent from the participants themselves (1a), the volar part of the participant's arm is sterilized with a piece of cotton wool dabbed in ethanol (2a). A marker is used to demarcate the area to be pricked (2 cm apart), and a drop of the test allergen applied on the demarcated area (3a). Pricks are made with sterilized lancets (1b), and the participant is made to wait for 15 minutes for signs of sensitization (2b, 3b). The wheal generated is measured (1c).

3.5 Parasitology

3.5.1 Urine Filtration

Each of the participants recruited for the study provided a single urine sample between 10:00 am and 2:00 pm. Between 30 ml to 50 ml of urine was collected in labeled 50-ml Greiner centrifuge tubes provided for the study. Testing for hematuria, proteinuria, glucose level and pH was conducted using reagent strips (courtesy: Ames Hemastix). This was

followed with the shaking of each tube to re-suspend the eggs, after which 10 ml urine was drawn into a syringe and forced across a 25 mm Millipore filter membrane of pore size 12 µm secured in a Swinnex support chamber. Using forceps, each membrane was carefully removed from the chamber and inverted onto a microscope slide to ensure that trapped eggs were situated between the membrane and the slide. A single slide was prepared per participant, and examination by microscopy was conducted following the application of a drop of saline on the membrane. Prepared slides were examined for the presence and number of *S. haematobium* eggs, and a mean egg count determined. Values obtained were expressed as eggs per 10 ml of urine.

3.5.2 Kato-Katz technique

Single stool samples were provided by each participant in labeled containers with wide mouth screw cups and were processed for microscopy using the Kato-Katz technique. Briefly, mixing was done with the aid of a wooden spatula to ensure homogeneity of the sample. Next, a small amount (approximately 1 g) of stool was scraped through a plastic mesh of pore size 105 µm to remove particulate and fibrous material. Sieved faecal matter was then collected and applied to a hole in the centre of a plastic template situated on a glass slide. The template was removed carefully, and the stool specimen (~42 mg) cast on the slide was covered with a 25x35mm cellophane impregnated with 50% (v/v) glycerol in water containing 3% malachite green. The slide was turned upside down on a flat surface and pressed gently but firmly to spread the stool specimen evenly under the cellophane. Two slides were prepared per sample and left for approximately 30 minutes (30 min) at room temperature to clear before being examined microscopically for the ova of such soil-

transmitted helminths as *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworm (*Necator americanus* and *Ancylostoma duodenale*); as well as *S. mansoni* eggs.

3.6 Blood Samples

3.6.1 Thick Blood Smears

Malaria infection status was determined using the thick blood smear technique on glass examination slides. Briefly, a puncture was made in the thumb of the participant, and a large drop of blood was directed onto one side of the examination slide. A second glass slide, slanted at 45º, and the tip of which was touching the slide and the blood droplet, was used to smear the droplet across the surface of the slide in a single, quick, and deft motion. The smeared slide was left to dry, after which the Giemsa stain was applied and left to dry. The stained slides were washed and left to dry. Microscopic examination was done with the aid of immersion oil.

3.6.2 Serum samples

Approximately 2 ml of blood was collected per participant in plain vacutainer tubes, which were transferred to the laboratories of the Parasitology Department of the Noguchi Memorial Institute for Medical Research. Centrifugation was done, allowing the blood sample to separate into two components: serum and blood cells. The serum was obtained with the aid of a Pasteur pipette and transferred to a sterile eppendorf tube. Samples were stored at -20ºC until required.

3.7 Selection of serum samples for glycan study

In order to investigate urban-rural differences in serum IgE, IgG1, and IgG4 response patterns to printed synthetic glycans, stored serum samples from schools involved in the

study described were used. After stratifying by SES, one school was randomly selected from each of the UH, UL, and R groups. The UH (Green Hill International school) and UL (Nii Okine Basic School) schools were located in the Accra Metropolis, whilst the R school (Toflokpo Presbyterian Primary) was located in Toflokpo, a rural community in the Dangme East district (Figure 3.1). From each of the selected schools, serum samples of 20 subjects were randomly picked, making a total of 60 samples.

3.7.1 European samples with high IgE titres to cross-reactive carbohydrate determinants (CCDs)

For comparison purposes, glycan-associated IgE, IgG1, and IgG4 responses were measured in stored serum samples of European participants obtained from a separate study (Mari *et al.*, 2008) in which they were found to have significantly detectable serum levels of IgE to the cross-reactive carbohydrate bromelain (median response = 13.2 kU/L), and horseradish peroxidase (HRP). They comprised 3 males and 2 females (Mari *et al.*, 2008).

3.8 IgE antibody measurements to allergen extracts by ImmunoCAP

Serum levels of specific IgE (kU/L) to a panel of allergen extracts including bromelain (a well-characterized cross-reactive carbohydrate marker with core α-1,3-fucose and β2xylose modifications), galactose-α-1,3-galactose (α-gal; extracted from bovine thyroglobulin), house dust mite (*Dermatophagoides pteronyssinus*), cockroach (*Blattella germanica*), and peanut (*Arachis hypogaea*), were determined for this selection of Ghanaian subjects using the ImmunoCAP assay (Phadia AB, Uppsala, Sweden). Although fully automated, the test was designed in principle as a sandwich immunoassay. Hence the extract, or test allergen was covalently bound to the solid phase comprising a hydrophilic,

highly-branched cellulose polymer. Next, incubation with the subject serum sample ensured the binding of specific IgE to the cellulose-bound component of interest. After washing away unbound antibodies, there was further incubation with enzymelabeled anti-IgE antibodies, which bound to the allergen-antibody complexes. Following the washing away of unbound anti-IgE antibodies, a developing agent was applied, followed by a stop reagent. The fluorescence of the eluate was measured according to the manufacturer's instructions, and 0.35 kU/L was used as the cut-off level below which a sample was considered negative both for bromelain-specific and α-gal-specific IgE.

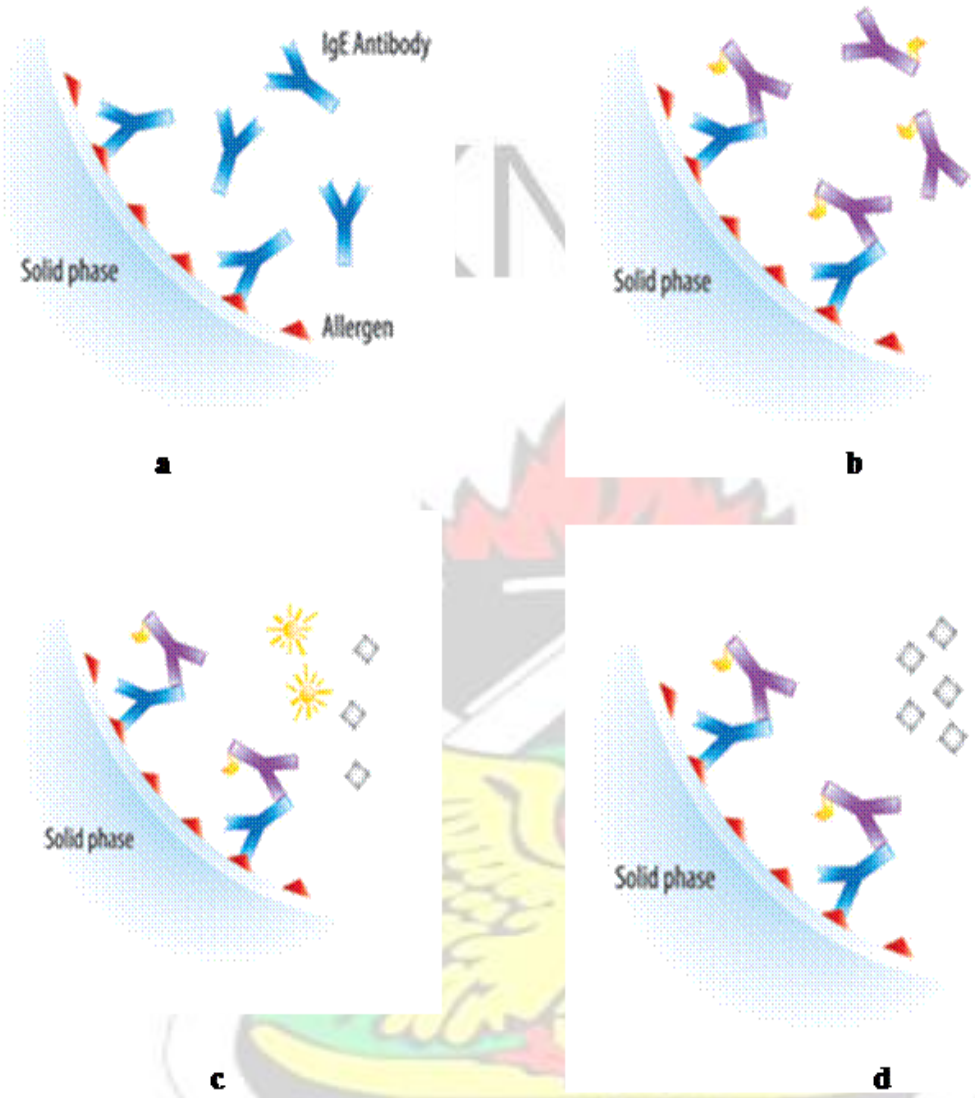


Figure 3.3: The ImmunoCAP assay: (a) Incubation of solid-phase bound allergen with patient serum leading to IgE binding. **(b)** Incubation with conjugate-bound anti-IgE antibody leads to formation of complex with seral IgE. **(c)** Incubation with fluorogenic substrate leads to reaction with already-formed antibody complexes, followed with **(d)** reaction stoppage (*Courtesy: www.phadia.com*).

3.9 Anti-glycan IgE, IgG1, and IgG4 binding Assays

As has been described elsewhere (Brzezicka *et al.*, 2015), seven arrays, each comprising quadruplicates of 128 synthetic glycans (Figure 3.4) were printed on each glass slide. Glass slides were then blocked with 50 mM ethanolamine in sodium borate buffer (pH = 9.0) for 1h, washed, and stored at -20ºC. At the commencement of the experiment, slides were placed at RT to thaw and dry. Next, a 7-well incubation chamber was placed onto the glass slide to separate the seven printed arrays and to hold incubation solutions. The array was incubated with serum diluted 1:30 in PBS, with 1% bovine serum albumin (BSA), and 0.01% Tween 20 (T_{20}) at RT for 1h. Washing was done first in PBS with 0.05% T_{20} and then in PBS. A 30-min incubation in the dark with Cy3-labelled antihuman IgG (Sigma Life Sciences, St. Louis, MO, USA; diluted 1:1000 in PBS with 1% BSA and 0.01% T_{20}) and Promofluor647 (PromoCell GnbH, Heidelberg, Germany)labelled anti-human IgE (Sanquin reagents, Amsterdam, the Netherlands; diluted 1:400 in PBS with 0.01% T_{20}) followed. Next the washing step was repeated, followed by a final wash in MilliQ water. The slide was then dried and kept in the dark until scanning.

Measurement of glycan-associated IgG1 and IgG4 antibody responses was conducted similarly, however with a few modifications. Following the placement of the 7-well incubation chamber on the glass slide, the arrays were incubated with serum diluted 1:100 in PBS with 5% BSA and 0.01% T_{20} at RT with shaking for 1h. This was followed with a washing step and incubation for an hour in the dark with PromoFluor 555-labelled anti-human IgG4 diluted 1:200 in PBS - 0.01% T_{20} (Sigma Life Sciences, St. Louis, MO, USA); and PromoFluor 647-labelled anti-human IgG1 diluted 1:200 in PBS - 0.01% T_{20}

(Sanquin reagents, Amsterdam, the Netherlands). Here as well, PromoFluor probelabeling was carried out on both antibodies using Promokine kits (PromoCell GnbH, Heidelberg, Germany). The glycan-associated IgE, IgG1, and IgG4 binding assays were performed for both the selected Ghanaian urban-rural, and European CCD-positive participants.

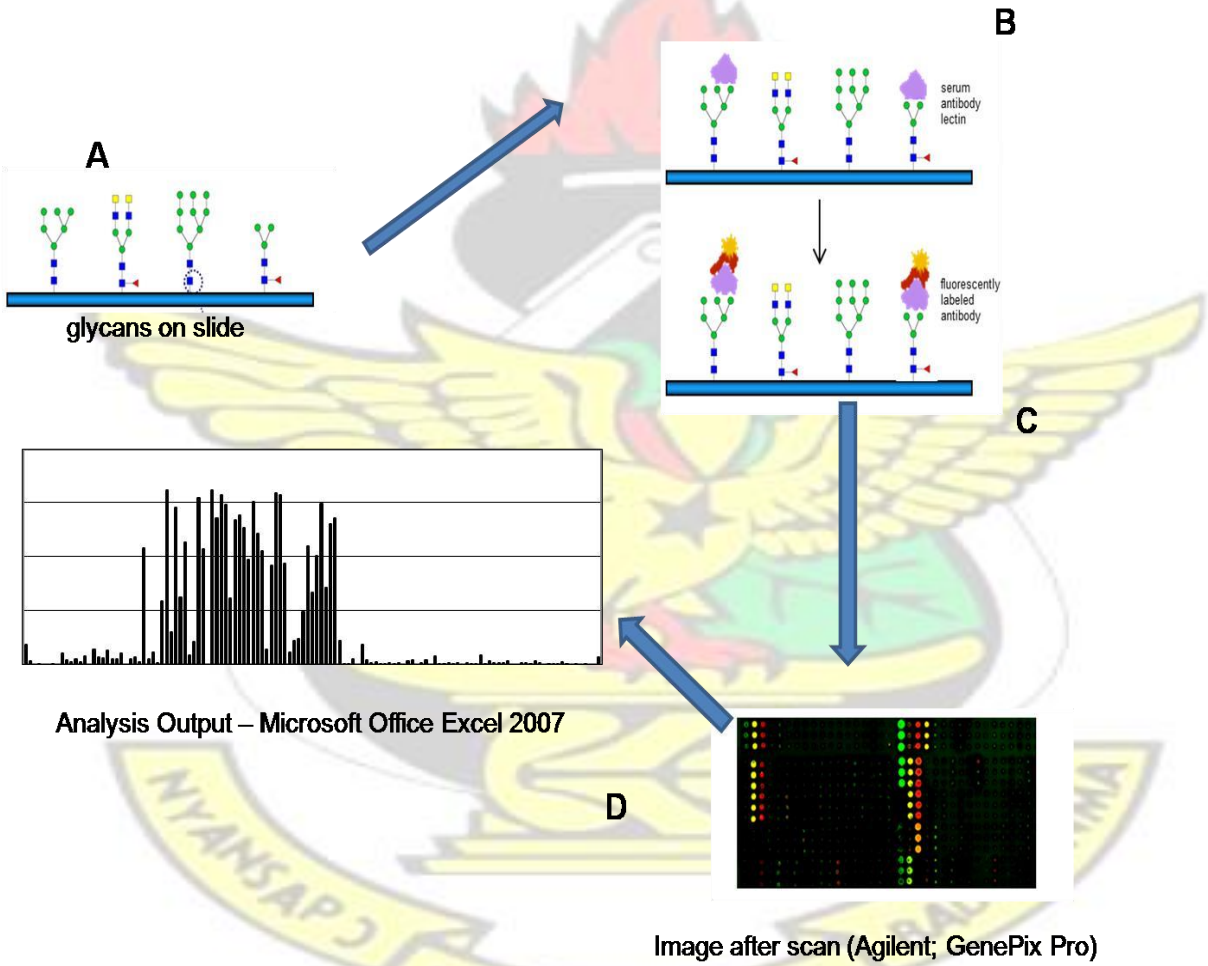


Figure 3.4: The glycan microarray technique: (a) Glycans are immobilized on the solid phase (glass slide). **(b)** This is followed by incubation with patient serum for antibody binding. **(c)** Next is the incubation with fluorescent antibodies or probes. **(d)** The solid

phase is scanned, and data is analyzed using GenePixPro, Microsoft Office Excel, and other statistical softwares.

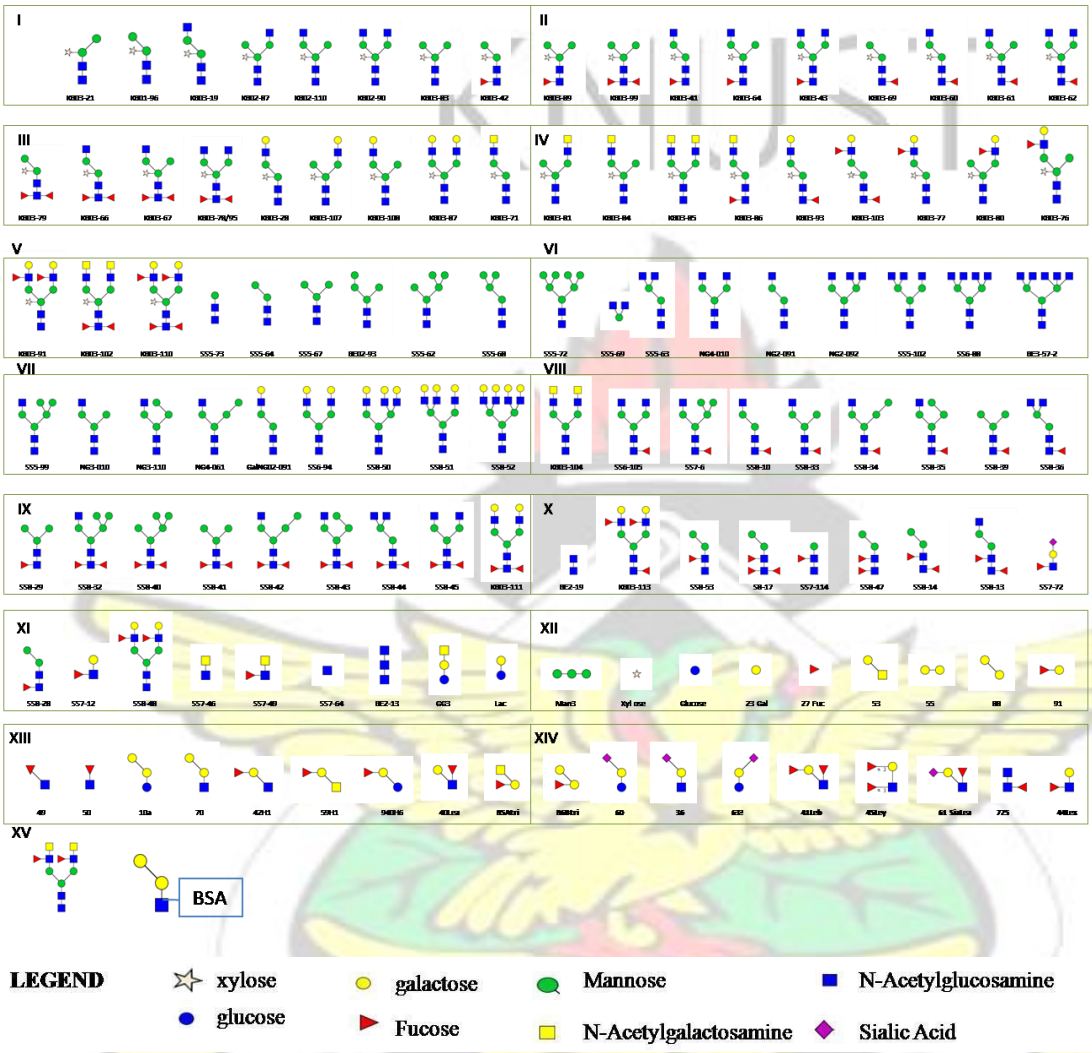


Figure 3.5: An illustration of the array of 128 printed glycans (Courtesy: Brzezidka *et al.,* 2015; Appendix III)**.**

3.10 Scanning of slides and processing of acquired data

All slides were scanned using a G2565BA fluorescence scanner (Agilent Technologies, Santa Clara California, USA) at 10 μm resolution using 2 lasers (532nm and 633nm). The GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, California) was used to align the spots using circular features with a composite pixel intensity (CPI) threshold of 10. The resulting data was exported to Microsoft Office Excel 2007 where the backgroundsubtracted median fluorescence intensities were first adjusted, and then averaged for each glycan. Datasets were further processed as described by Oyelaran *et al.*, (2009) and normalized to correct for low fluorescence intensity signals. Normalization was carried out by first calculating the ratio of the fluorescence intensity (F) to the background fluorescence (B) of each spot and multiplied by the mean background fluorescence (B avg.) calculated for all the spots in that particular array (Figure 3.5 is a mathematical illustration of the procedure). The mean over 4 spots (representing a glycan) was next calculated, and finally log-2 transformed prior to statistical analyses.

$Log_2[[\{(F_{532}/B_{532})xBavg.\}_1+\{(F_{532}/B_{532})xBavg.\}_2+\ldots+\{(F_{532}/B_{532})xBavg.\}_4]/4$ spots

Where:

F_{532} = The measured fluorescence intensity of a spot at laser wavelength (λ) = 532nm

B_{532} = The measured background fluorescence for a spot at laser wavelength (λ) = 532nm

B avg. = The mean of fluorescence backgrounds for all the spots in a particular array

Figure 3.6: Mathematical illustration of the normalization procedure carried out after scanning to correct for weak signals, or fluorescence intensities.

3.11 Statistical Analyses

Urban-rural differences in demographic characteristics of selected subjects were performed in SPSS version 16.0 (IBM Corp., Armonk, New York, USA) using the Pearson's χ^2 test (two degrees of freedom). Threshold fluorescence intensity values were determined for IgG1, IgG4, and IgE datasets using the medians of fluorescence values of glycans to the respective antibodies. Hence, measurements above the determined threshold value were considered responders, and vice versa. The Mann Whitney U test was used to determine glycans significantly associated with Area by comparing between two Areas at a time. Due to the large number of glycans generated as a result, only those showing clear Area-associated trends were selected for further analyses. Again, the Mann Whitney U test was used for the IgG1 and IgG4 datasets, to identify glycans significantly associated with Area. Also where necessary, the Mann Whitney U and Kruskal-Wallis tests were used to determine associations between each selected glycan and a number of factors considered to be *a priori* confounders to the main glycan-Area relationship. Correlations between covariates were determined using the Spearman's rank correlation
(ρ). All graphs were generated using Graph Pad Prism version 5.04 (La Jolla, California, USA).

CHAPTER 4:

ASSESSMENT OF GLYCAN-ASSOCIATED IGE RESPONSES

4.1 Introduction

Evidence provided by numerous studies since 1970 have indicated cross-reactive IgE activity especially in food-related allergies (Anderson *et al.*, 1970; Kato and Sasaki, 1974; Baur, 1979; Bahna *et al.*, 1980). However, following RAST experiments with serum samples from selected patients, Aalberse, Koshte, and Clemens in their publication in 1981 proposed for the first time, the role of certain antigenic determinants in allergenassociated cross-reactive IgE binding (Aalberse *et al.*, 1981, 1983; van Ree *et al,*, 2000). They described these determinants to comprise carbohydrate side-chains present mainly on the glycoproteins of plants, which were likely to be of little clinical relevance due to the near absence of mast cell or basophil activation when they complexed with cellbound IgEs (van Ree, 2002).

However, with more than one billion people estimated to be affected by allergic diseases currently (Akdis, 2015), research into these 'irrelevant' IgEs is growing, as they present with a potential alternative in allergic disease therapy. It is established that type-1 hypersensitivity reactions are induced by innocuous antigens, or allergens (Gauchat *et al.*, 1993; Geha *et al.*, 2003; Pease, 2006). However, with glycan-associated cross-reactive IgEs, a different inducing agent is implicated. In *Schistosoma*-infected individuals, for instance, eggs laid by the adult worms and trapped in host tissues have been suspected to secrete soluble molecules such as glycoproteins through pores in the eggshell (Meevissen *et al.*, 2012), which in turn skew immune response mechanisms to a Th2 isotype. These IgE antibodies, although induced by parasitic molecules, may likely be capable of

recognizing allergens crossing host skin and/or mucosal barriers. It was evidenced by Faveeuw *et al.* (2003) through murine model studies that *Schistosoma mansoni* eggderived glycoconjugates bearing N-glycans with α3-fucose and β2-xylose core modifications, were associated with the increased production of such Th2 cytokines as IL-4, IL-5, IL-10, and IL-13. Also, Tretter *et al.* (1993) provided evidence suggesting that IgEs from bee venom allergic patients could cross-react with bromelain, a glycoprotein derived from pineapple stems and bearing complex-type N-glycans with α3-fucose and β2-xylose.

Considering that helminth infections, and encounters with biting insects are more prevalent in rural areas of low- to middle-income counties, it is anticipated with respect to this study that more evidence would be put forth suggesting IgE cross-reactivity in the rural area as compared to the urban areas under scrutiny. Hence, with the aid of a welldeveloped array of synthetic glycans and an ImmunoCAP assay, this work sought to first determine the existence of urban-rural patterns in anti-glycan IgE response signals; to secondly identify the glycans or glycan epitopes significantly associated with measured IgE responses from the selected participants in the urban high, urban low and rural areas, along with potential risk factors associated with the anti-glycan IgE responses recognized; and to finally compare glycan-associated IgE responses between the Ghanaian participants and a selection of Europeans previously found to have high serum levels of IgEs to the plant-derived cross-reactive carbohydrate determinant (CCD) bromelain.

4.2 Results

4.2.1 General characteristics and Skin Prick Test (SPT) results of selected participants

Urban-rural distributions of basic demographics, parasitic infections, observed glycanassociated and allergen-associated IgE response profiles for the 60 selected participants are shown in Table 4.1, and in Figures 4.1 and 4.2. There was significant variation in gender, especially in the Rural (R) area, where among the 20 subjects selected, only 5 were female (25%, $p = 0.038$). Age, on the other hand, was more normally distributed in all three areas with no reported significance in differences ($p = 0.057$; Table 4.1).

Apart from *Necator americanus*, the prevalence distribution of helminth infections in the three areas were not significant (Table 4.1), although it is worth noting that the highest prevalence of *S. haematobium* infection was reported in the Urban Low (UL) area for this selection (25.0%). Prevalence of hookworm (*N. americanus*) was however highest in the R area (45.0%), compared to the UL (0.0%), and UH (0.0%) areas ($p < 0.001$). Put together however, prevalence of infection for any of the helminths was highest in the R (50.0%), followed with the UL (25.0%), and UH (5.0%) areas (Table 4.1). There was also considerable variation in the prevalence distribution by Area for *Plasmodium sp.* parasitaemia, with the highest prevalence reported in the R area (40.0%), followed with the UL (15.0%), and the UH (0.0%) areas ($p = 0.004$; Table 4.1).

Skin prick test (SPT) positivity results also followed an urban-rural trend, except for SPT positivity to peanut, which recorded a single positive case in the UL area. Hence, the prevalence of SPT positivity to cockroach (*Blatella germanica*) increased from no recorded result in the UH area, to 2 (10.0%), and 6 (30.0%) observations in the UL, and R areas

respectively (p = 0.018). This was similarly realized for the prevalence of SPT positivity to house dust mite (*Dermatophagoides pteronyssinus*), which also increased from no recorded observation in the UH area, to 1(5.0%), and 5 (25.0%) observations in the UL, and R areas respectively (p = 0.02).

4.2.2 Urban-rural differences in measured IgE responses to allergens, α-gal, and Bromelain by ImmunoCAP

The reported prevalences for measured levels of allergen specific IgE (sIgE) by ImmunoCAP (i.e. sIgE > 0.35 kU/L) to bromelain, galactose-α-1,3-galactose (α-gal), cockroach (*Blatella germanica* – Bla g), house dust mite (*Dermatophagoides pteronysinnus* - Der p), and peanut (*Arachis hypogaea* - Ara h), are also shown in Table 4.1. Figure 4.1 comprises graphical illustrations of the urban-rural trends observed for galactose-α-1,3-galactose (α-gal) (Figure 4.1a) and bromelain (Figure 4.1b). Similar urban-rural trends were observed for all the allergens tested, with the R area reporting the highest prevalence of positive cases in all instances, followed with the UL, and UH areas.

Significant differences were however only observed for α-gal (p < 0.001), cockroach (Bla g, p = 0.009), and house dust mite (Der p, p = 0.007) (Table 4.1).

Table 4.1: Characteristics of selected participants stratified by area

Characteristics of participants selected for study

Factor	Urban High [n/N(%)]	Urban low [n/N (%)]	Rural [n/N(%)]	p-value
Gender				
Male	8/20 (40.0)	8/20 (40.0)	15/20 (75.0)	**0.038**
Female	12/20 (60.0)	12/20 (60.0)	5/20 (25.0)	
Age				
< 11 years	2/20 (10.0)	8/20 (40.0)	8/20 (40.0)	0.057
>= 11 years	18/20 (90.0)	12/20 (60.0)	12/20 (60.0)	
Parasitic infections (infected)				
Schistosoma haematobium	1 /20(5.0)	5/20 (25.0)	1 /20(5.0)	0.075
Schistosoma mansoni	0/20 (0.0)	1 /20(5.0)	0/20 (0.0)	0.38
Ascaris lumbricoides	0/20 (0.0)	0/20 (0.0)	1 /20(5.0)	0.38
Trichuris sp.	0/20 (0.0)	1 /20(5.0)	2/20 (10.0)	0.38
Necator americanus	0/20 (0.0)	0/20 (0.0)	9/20 (45.0)	**<0.001**
§Any Helminth	1 /20(5.0)	5/20 (25.0)	10/20 (50.0)	**0.006**
Plasmodium sp.	0/20 (0.0)	3/20 (15.0)	8/20 (40.0)	**0.004**
Skin Prick Test (SPT) positives				
Peanut	0/20 (0.0)	1/20 (5.0)	0/20 (0.0)	0.36
Cockroach	0/20 (0.0)	2/20 (10.0)	6/20 (30.0)	**0.018**
House Dust Mite	0/20 (0.0)	1/20 (5.0)	5/20 (25.0)	**0.020**
Measured IgE levels to allergens by ImmunoCAP (≥0.35kU/L)				
Galactose-α-1,3-galactose (α-gal)	0/20 (0.0)	4/20 (20.0)	14/20 (70.0)	**<0.001**
Cockroach (*Blatella germanica* (Bla g))	4/20 (20.0)	6/20 (30.0)	13/20 (65.0)	**0.009**
House Dust Mite (*Dermatophagoides pteronyssinus* (Der p)	0/20 (0.0)	4/20 (20.0)	8/20 (40.0)	**0.007**
Peanut (*Arachis hypogaea* (Ara h)	1 /20(5.0)	3/20 (15.0)	5/20 (25.0)	0.208
Bromelain	1 /20(5.0)	4/20 (20.0)	7/20 (35.0)	0.06

§Any Helminth refers to participants infected with at least one or more of the following: *S. haematobium*, *S. mansoni*, *A. lumbricoides*, *Trichuris trichiura*, and *Necator americanus*. Associations were determined using the χ^2 test (with 2 degrees of freedom), and were considered significant at $p < 0.05$ (in boldface).

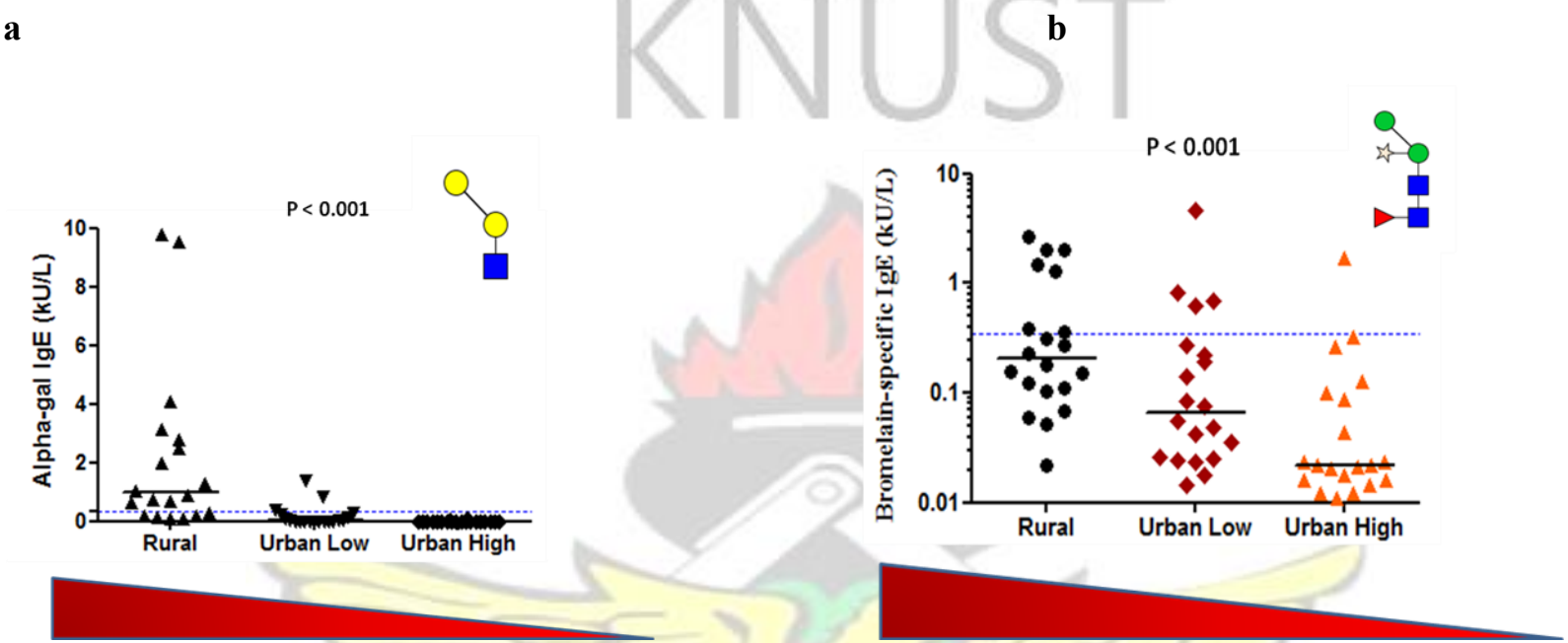


Figure 4.1: Measurement of serum titres (kU/L) of (a) galactose-α-1,3-galactose-specific IgE and (b) Bromelain-specific IgE by ImmunoCAP. Comparisons were made using the Kruskal Wallis test. Red triangle is indicative of the observed urbanrural gradient in IgE response to α-gal and bromelain, where the upright side (left) is representative of the higher prevalence of response in the R area, while the vertex (right) is representative of the lower prevalence of response in the UH Area. Blue dashed line is set at the assay threshold value of 0.35kU/L for both.

4.2.3 Urban-rural differences in measured IgE responses to synthetic glycans by glycan microarray

Area-stratified bar graphs depicting the mean of the measured IgE signal intensities for the selected participants per glycan were constructed, with all the 128 glycans on the microarray being represented. Each bar therefore represented the mean of IgE fluorescence intensities measured for 20 participants within a particular area for a particular glycan (Figure 4.2).

It was observed that response signals were largely restricted to the xylosylated and fucosylated N-glycans, and to the variations of α-gal epitopes for all three areas. These happened to be the glycan groups on the array that were the focus of this study. Additionally, an area-associated trend in signal intensities was observed, with signal intensities for the R area being highest, followed with signal intensities for the UL and UH areas respectively (Figure 4.2).

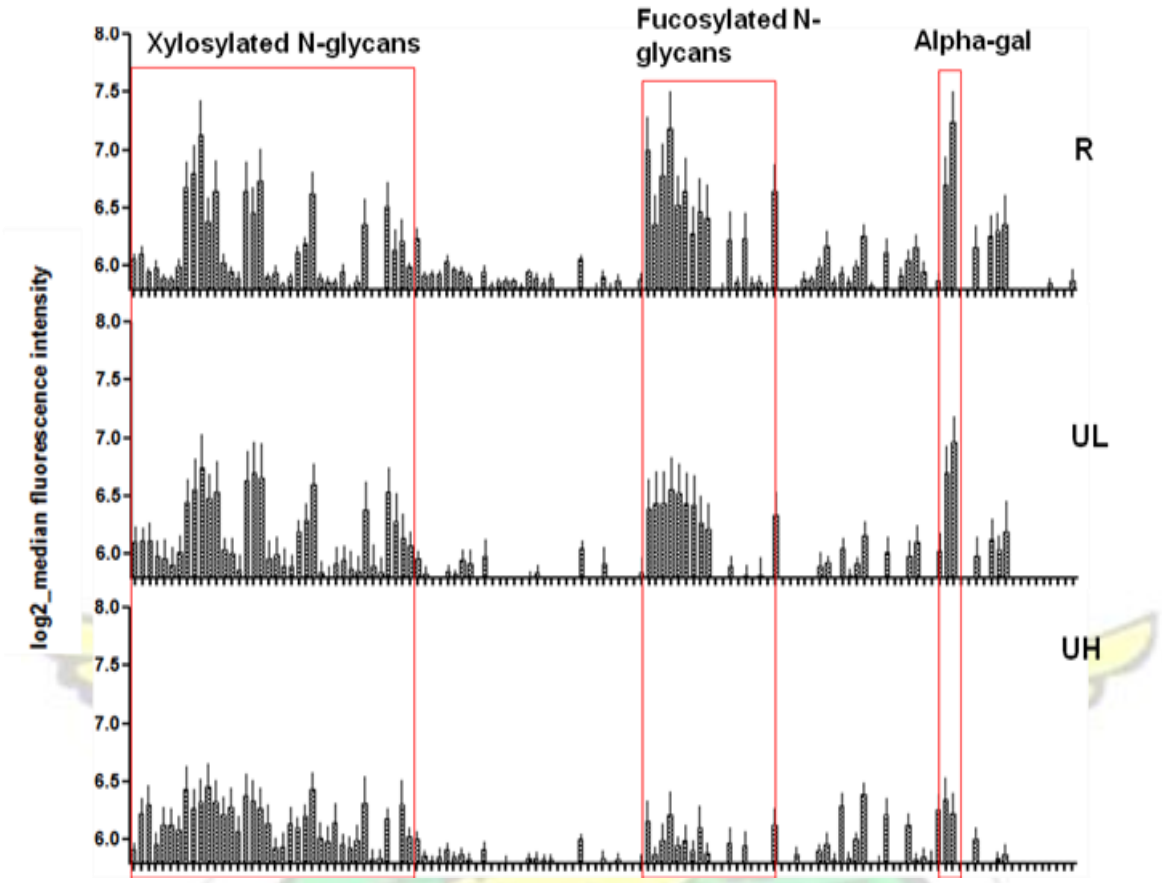


Figure 4.2: IgE fluorescence intensity graphs of the 128 glycans stratified by Area. Using the log2 median fluorescence intensity (log2 MFI) data, bar graphs showing the signal intensities of the 128 glycans at a glance, were constructed for each area. Graphs were arranged as shown for easy comparison. The red boundaries indicate glycan groups on the microarray chip that are the focus of this study. Each bar represents the mean of the log2 MFIs for 20 participants for a particular glycan, with the vertical lines on top of each bar representing the standard error of mean (SEM).

4.2.4 Identification of glycans with significant, area-associated IgE signal intensities

Further analysis of the IgE fluorescence intensity dataset presented with 5 glycans showing significant area-associated trends (Figure 4.3) namely, G73 ($p = 0.018$); G75 ($p = 0.036$); G99 ($p = 0.014$); G133 ($p = 0.002$); and G39 ($p = 0.030$). G73, G75, and G39 are N-glycans with various core modifications. For G73, a fucose is attached via an α1-3 linkage to the proximal N-Acetylglucosamine (GlcNAc) whilst for G75, two fucoses are attached via α1-3 and α1-6 linkages to the proximal GlcNAc. With regard to the third,

G39, not only is the proximal GlcNAc α3/α6 difucosylated, but a xylose is attached to the second mannose in the core structure via a β1-2 linkage (Figure 4.3e). For all three coremodified N-glycans, median intensities were highest in the R, followed by the UL, and UH Areas. This was similarly observed for α-gal-GlcNAc (70). For the Mannotriose (G133) however, the reverse trend was observed, with the highest median intensity recorded for the UH Area, followed by the UL, and R Areas (Figure 4.3).

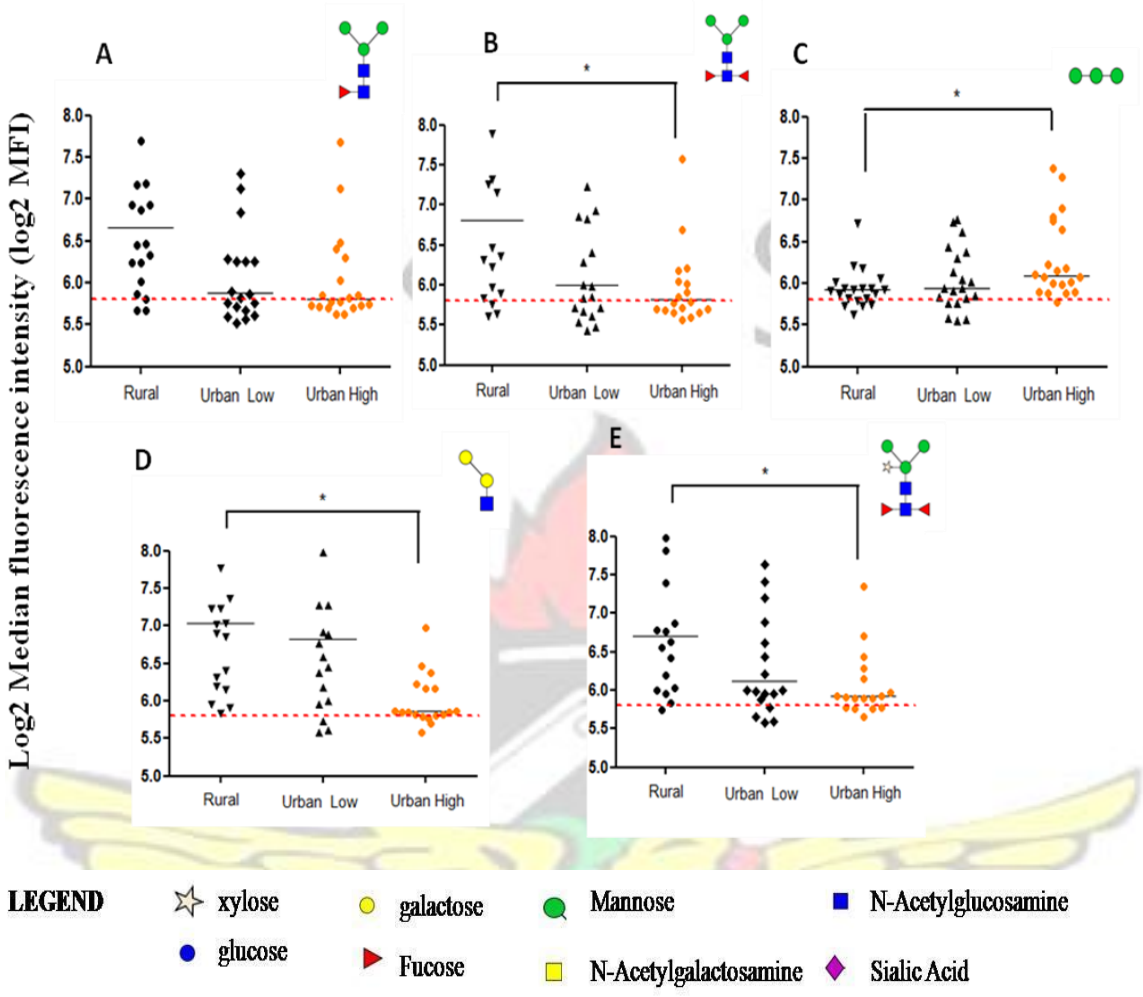


Figure 4.3: Glycans identified from the IgE fluorescence intensity dataset to exhibit an Area-associated trend in signal intensities. (a) G73; **(b)** G75; **(c)** G99; **(d)** G113 (70); and **(e)** G39. Area comparisons for each glycan were conducted using the Mann Whitney U test to compare two areas at a time. An asterisk (*) represents p values less than 0.05. Horizontal lines represent median fluorescence intensities for the various groups. The dashed red line is set at the threshold value for IgE (i.e. 5.8).

4.2.5 Associations between potential influencing factors and identified glycans from IgE fluorescence datasets

The associations between IgE response intensities to the identified glycans and such factors as gender, age, helminth infection status, and malaria parasitaemia, were assessed in an attempt to identify those factors potentially playing a role in cross-reactive IgE induction for this selection of participants (Table 4.2a). Additionally, the associations between IgE response intensities to the identified glycans and ImmunoCAP-determined IgE positivity to cockroach, house dust mite, peanut, and bromelain, were determined (Table 4.2a).

Firstly, gender was found to be significantly associated with G73 ($p = 0.007$), G75 ($p = 0.008$), and G39 ($p = 0.008$), an observation more likely due to inadvertent selection bias in the R area. Apart from Mannotriose (G99; $p = 0.007$), no significant association was observed between Age and IgE signal intensities to any other glycan. *N. americanus* infection status was observed to be strongly associated only with IgE signal intensities to G73 ($p = 0.026$). Put together however, infection status for any helminth was observed to be significantly associated with G73 ($p = 0.001$), G75 ($p < 0.001$), and G39 ($p = 0.001$), thus implying the possible expression of one or more of these glycan structures at some stage by one or more of these helminths (i.e. *Schistosoma sp*., *N. americanus*, *Ascaris lumbricoides*, and *T. trichiura*). Malaria infection status was only significantly associated with IgE intensities to the G113 epitope ($p = 0.029$), hence suggesting either mosquitoes or the *Plasmodium* parasites as potentially expressing this moiety.

Additionally, ImmunoCAP-measured IgE to cockroach was found to be strongly associated with IgE signal intensities to G73 ($p < 0.001$), G75 ($p < 0.001$), G39 ($p <$

0.001), and G113 ($p = 0.035$). Significant associations were also observed between ImmunoCAP-measured IgE to peanut and IgE signal intensities to G73 ($p < 0.001$), G75 ($p < 0.001$), and G39 ($p < 0.001$). This was similarly realized for the house dust mite allergen, and for bromelain, a well-known marker for cross-reactive carbohydrate determinants (CCD) (Table 4.2b). ImmunoCAP-measured IgE to α-gal was observed to be significantly associated with IgE signal intensities to all five glycans, including Mannotriose (G99; $p = 0.009$), hence implying strong cross-reactive IgE activity among our selection of participants.

Table 4.2a: Associations between identified glycans for IgE response intensities and potential influencing factors

	G73 (N=60)	G75 (N=60)	G39 (N=60)	G99 (N=60)	G113 (N=60)
Factor	**median (range)**	**median (range)**	**median (range)**	**median (range)**	**median (range)**
Gender					
Males	6.29 (5.67-10.17)	6.37 (5.60-10.36)	6.62 (5.74-10.42)	5.94 (5.72-7.38)	6.58 (5.61-10.25)
Females	5.78 (5.51-9.54)	5.79 (5.42-9.87)	5.96 (5.59-9.79)	6.00 (5.56-7.28)	6.22 (5.58-8.67)
p-value	**0.007**	**0.008**	**0.008**	0.9	0.126
Age					
< 11 yrs	5.85 (5.51-9.67)	5.90 (5.42-9.62)	6.10 (5.58-9.96)	5.87 (5.56-6.29)	7.12 (5.58-8.42)
≥ 11 years	6.25 (5.63-10.17)	6.19 (5.57-10.34)	6.29 (5.65-10.42)	6.03 (5.72-7.38)	6.27 (5.58-10.25)
p-value	0.16	0.197	0.42	**0.007**	0.214
Hookworm					
Positive	6.46 (6.01-10.17)	6.46 (5.76-10.04)	6.78 (5.83-10.42)	5.92 (5.74-6.18)	7.22 (5.91-10.25)
Negative	5.87 (5.51-9.67)	5.99 (5.42-9.87)	6.17 (5.59-9.96)	5.98 (5.56-7.38)	6.44 (5.58-9.19)
p-value	**0.026**	0.073	0.131	0.269	0.217
Any Helminth**					
positive	6.65 (5.87-10.17)	7.12 (5.77-10.36)	7.14 (5.83-10.42)	5.92 (5.74-6.43)	7.07 (5.61-10.25)
Negative	5.82 (5.51-9.67)	5.85 (5.42-9.62)	5.98 (5.59-9.96)	6.01 (5.56-7.38)	6.27 (5.58-9.19)
p-value	**0.001**	**<0.001**	**0.001**	0.192	0.074
Malaria					
Positive	6.86 (5.56-10.17)	6.85 (5.48-10.36)	6.78 (5.59-10.42)	5.91 (5.57-6.76)	7.27 (5.83-10.25)
Negative	5.89 (5.51-9.67)	5.99 (5.42-9.87)	6.01 (5.59-9.96)	5.98 (5.56-7.38)	6.32 (5.58-9.19)
p-value	0.184	0.28	0.186	0.316	**0.029**

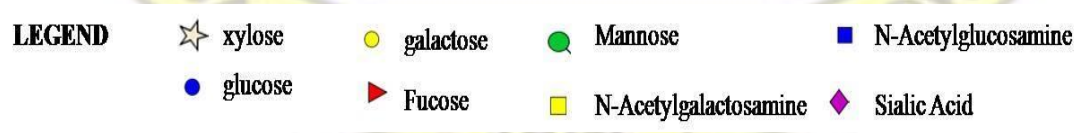
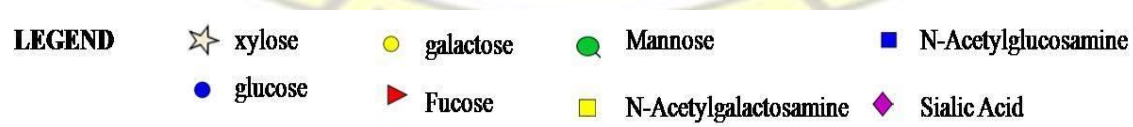


Table 4.2b: Associations between identified glycans for IgE response intensities and potential influencing factors

	G73 (N=60)	G75 (N=60)	G39 (N=60)	G99 (N=60)	G113 (N=60)
Factor (Allergens measured via ImmunoCAP)	**median (range)**	**median (range)**	**median (range)**	**median (range)**	**median (range)**
Cockroach (*Blatella germanica)*					
≥ 0.35kU/L	6.86 (5.76-10.17)	6.85 (5.71-10.36)	6.86 (5.96-10.42)	5.91 (5.57-5.56)	6.89 (5.61-10.25)
<0.35kU/L	5.82 (5.51-8.05)	5.79 (5.42-8.88)	5.95 (5.59-9.11)	6.02 (5.56-7.28)	6.17 (5.58-9.19)
p-value	**<0.001**	**<0.001**	**<0.001**	0.127	**0.035**
House dust mite (*Dermatophagoides pteronysinnus*) ≥ 0.35kU/L	7.03 (5.51-10.17)	7.28 (5.42-10.36)	7.03 (5.59-10.42)	5.89 (5.56-6.72)	6.96 (5.61-10.25)
<0.35kU/L	5.88 (5.56-9.67)	5.98 (5.48-9.62)	6.02 (5.59-9.96)	6.01 (5.57-7.38)	6.29 (5.58-9.19)
p-value	**0.019**	**0.021**	**0.035**	0.062	0.116
Peanut (*Arachis hypogaea*)					
≥ 0.35kU/L	8.67 (6.26-10.17)	8.75 (6.83-10.36)	8.77 (6.89-10.42)	5.93 (5.75-6.64)	6.86 (5.61-10.25)
<0.35kU/L	5.86 (5.51-8.05)	5.91 (5.42-8.88)	6.01 (5.58-9.11)	6.00 (5.56-7.38)	6.37 (5.58-9.19)
p-value	**<0.001**	**<0.001**	**<0.001**	0.488	0.748
α-gal					
≥ 0.35kU/L	6.46 (5.59-10.17)	6.41 (5.54-10.36)	6.71 (5.65-10.42)	5.89 (5.62-6.29)	7.22 (5.95-10.25)
<0.35kU/L	5.85 (5.51-9.54)	5.88 (5.42-9.87)	5.98 (5.59-9.79)	6.03 (5.56-7.38)	6.17 (5.58-8.69)
p-value	**0.023**	**0.045**	**0.013**	**0.009**	**0.002**
Bromelain ($MUXF^3$)					
≥ 0.35kU/L	7.92 (6.24-10.17)	8.63 (6.23-10.36)	8.08 (6.20-10.42)	5.91 (5.74-6.77)	6.81 (5.61-10.25)
<0.35kU/L	5.85 (5.51-8.05)	5.87 (5.42-8.88)	5.99 (5.59-5.99)	6.00 (5.56-7.38)	6.37 (5.58-9.19)
p-value	**<0.001**	**<0.001**	**<0.001**	0.355	0.671



Association between each glycan and factor was determined using the Mann Whitney U test. Effect of interaction was considered significant at $p < 0.05$. Significant associations

are in boldface. Any Helminth** comprises *S. haematobium*, *S. mansoni*, *A. lumbricoides*, *Trichuris trichiura*, and *Necator americanus*.

4.2.6 Area-stratified correlations between glycan-associated IgE signals and bromelain

Considering that bromelain is a well-known marker used in allergy-associated research and diagnostics to determine IgE crossreactivity to N-glycans with α3-fucose and β2xylose, graphs were generated which explored the strength of relationship between IgE signal intensities to identified glycans and ImmunCAP-measured IgE titres to bromelain (Figure 4.4). Stronger correlations were observed for G73/bromelain (Spearman's rho (ρ) = 0.736; $p < 0.001$); SS8_41/bromelain (ρ = 0.741; $p < 0.0001$); and G39/bromelain (ρ = 0.801; $p < 0.0001$). The α-gal-GlcNAc (70) epitope was found to correlate poorly with bromelain (Figure 4.4). Additionally, the strong correlations observed for G73/bromelain, SS8_41/bromelain, and G39/bromelain were found to be largely due to signal intensities realized from the R Area (red dots), implying a strong influence from the R area on the realized correlations (Figure 4.4). Although requiring further study, the poor correlation between α-gal and ImmunoCAP-measured bromelain (Spearman's rho = 0.36; $p < 0.0026$) could be due to differences between epitopes on complex N-glycans and α-gal.

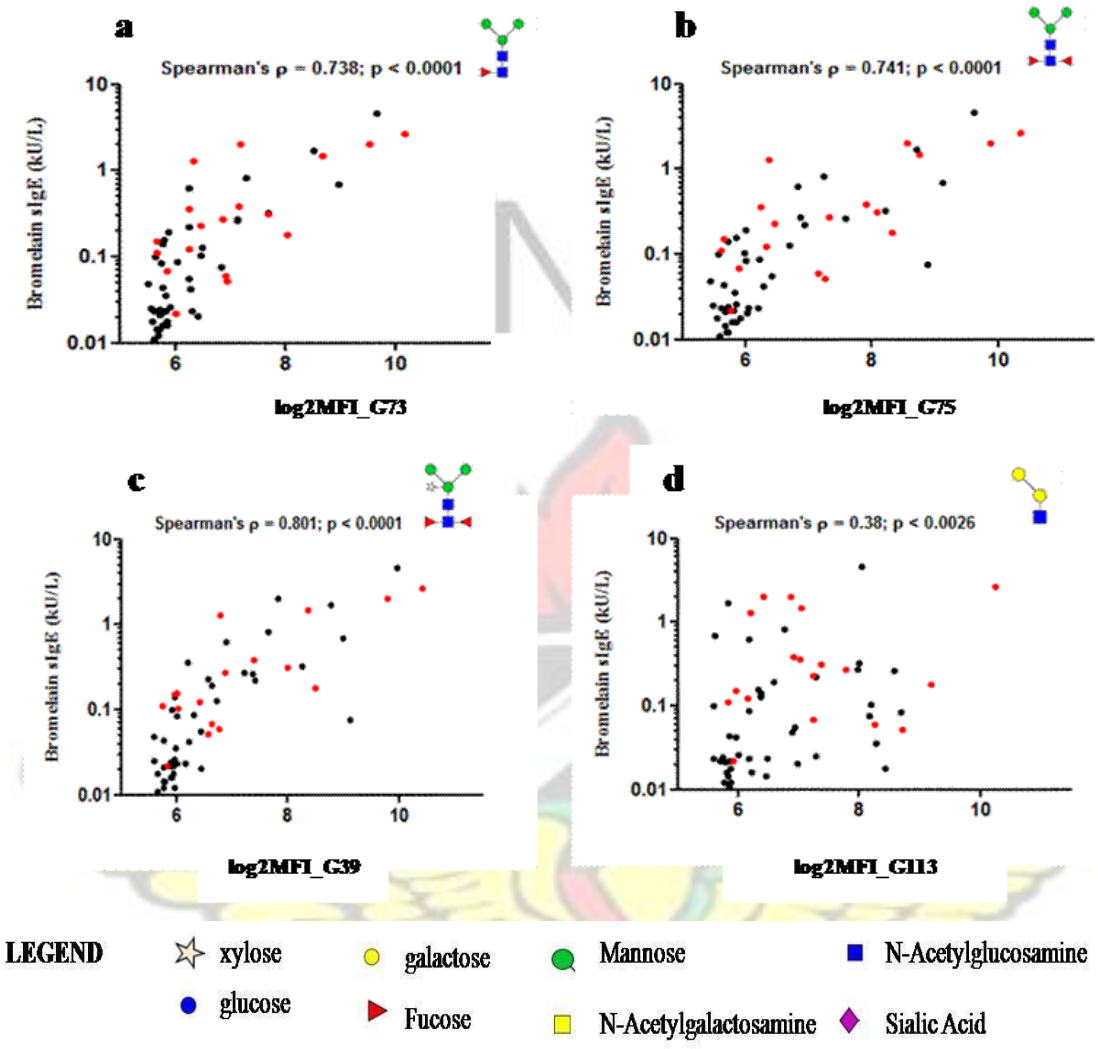
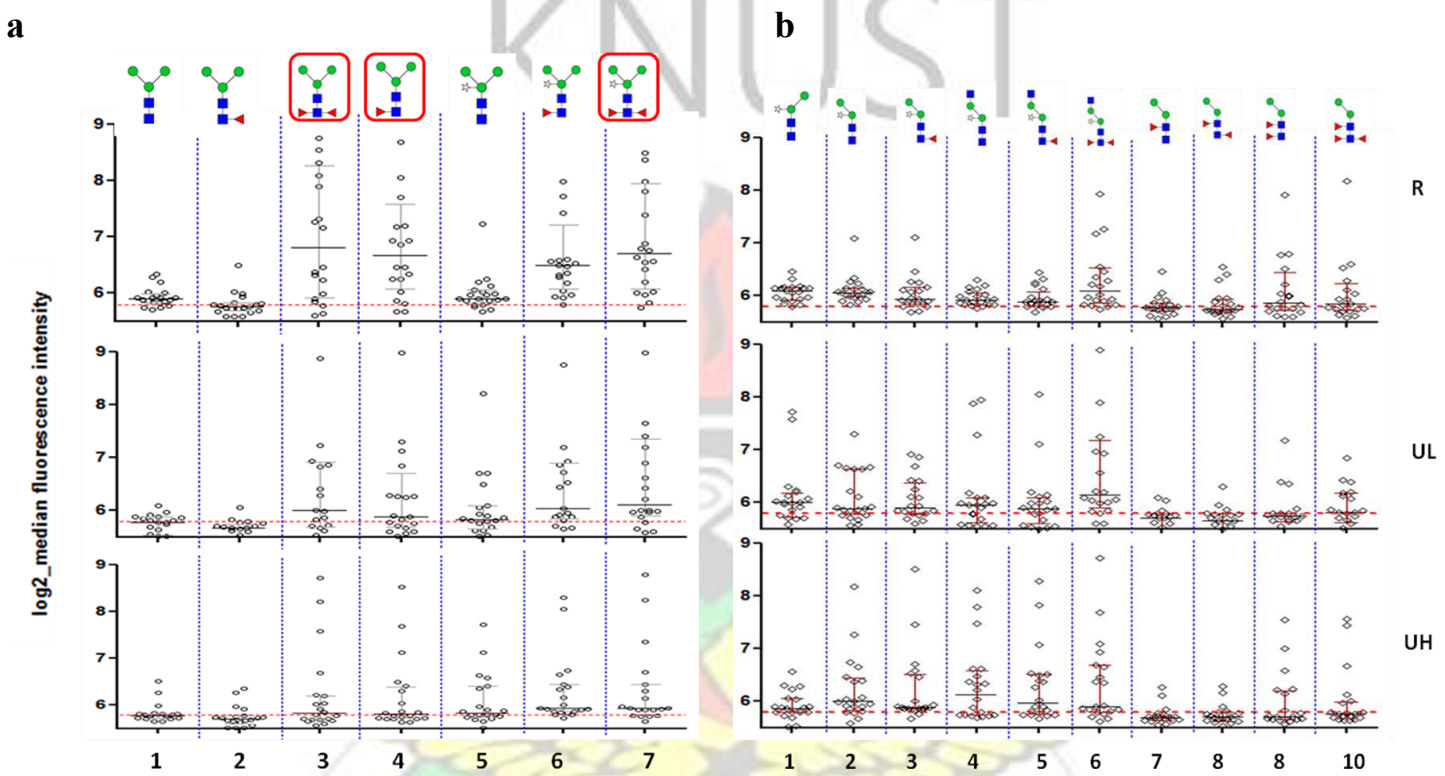


Figure 4.4: Correlations between IgE fluorescence intensities to selected glycans and ImmunoCAP-measured IgE to Bromelain (MUXF3): Correlations between ImmunoCAP-measured IgE to Bromelain and IgE signal intensities to **(a)** G73; **(b)** G75; **(c)** G39; and **(d)** G113 were measured using the Spearman's rho (ρ). Red dots represent subjects from the R area.

4.2.7 Urban-rural differences in IgE response patterns to selected glycans and their variations

Along with identifying factors associated with IgE responses to the selected oligosaccharides, area-stratified dot plots were constructed for each identified glycan and their variations, and studied for glycan components potentially influencing responses in each area. Variations of the synthetic glycans of interest (glycans of interest in red square boundaries) were selected from among the 128 glycans on the array.

For this selection of participants, it was apparent that IgE responses in the R Area were directed primarily at N-glycans with the α3 fucose core modification, irrespective of the presence or absence of other core modifications such as the α6-fucose or β2-xylose (Figure 4.5a, b). In the UL and UH Areas (Figure 4.5a, b) however, IgE response signals seemed directed against N-glycans with either an α3-fucose or a β2-xylose or both. Generally, signal intensities in the UH Area were much lower, as compared to signal intensities in the UL and R Areas (Figure 4.5a, b). Signal intensities directed at the α-galGlcNAc (70) epitope were higher in the R Area than in the UL and UH Areas respectively (Figure 4.5c1), a trend similarly observed with the G112epitope (Figure 4.5c2) and confirmed via ImmunoCAP (Figure 4.1).


a
b
log2_median fluorescence intensity
R
UL
UH
1
2
3
4
5
6
7
8
8
10

c

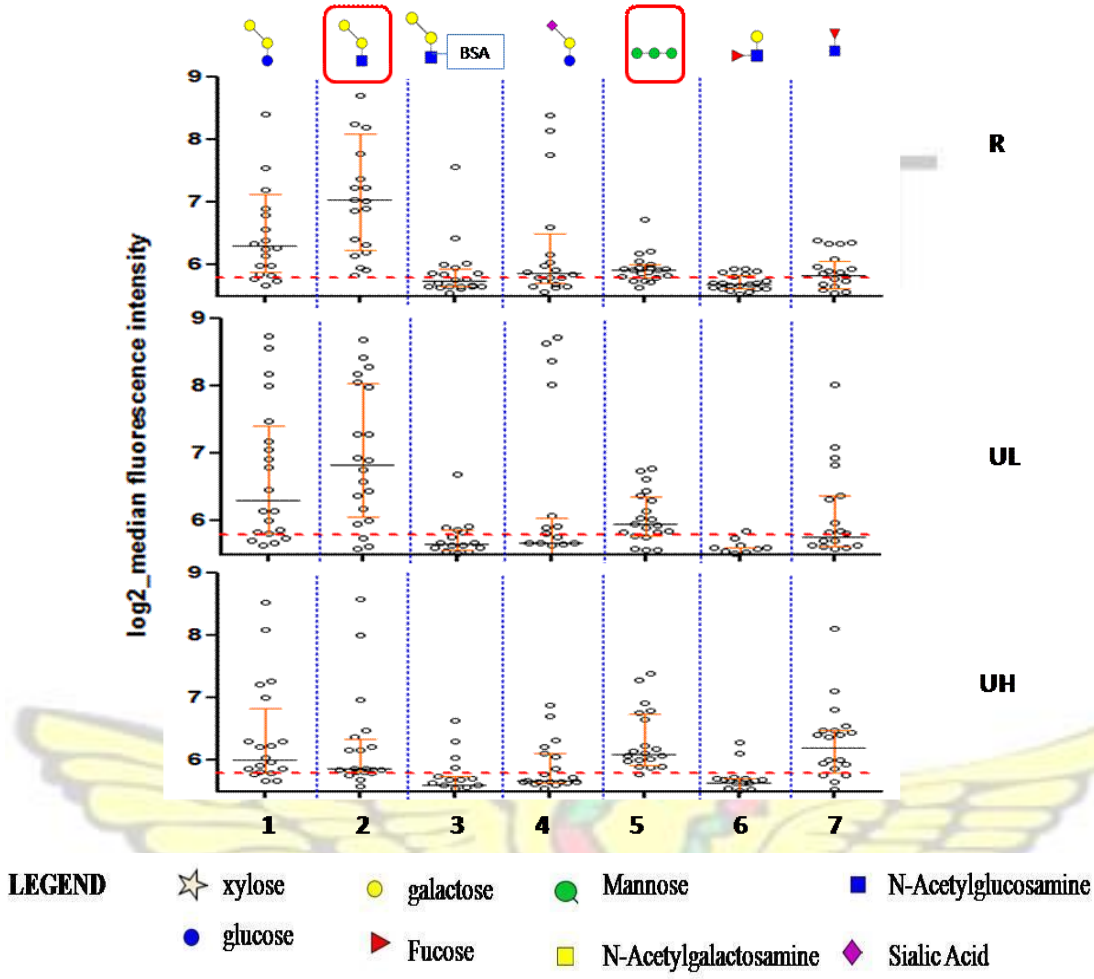


Figure 4.5: Determining urban-rural patterns for selected glycans and their variations: Urban-rural patterns in median IgE fluorescence signals to the selected glycans G73 (a4), G75 (a3), G39 (a7), G113 (c2) and G99 (c5) and their variations on the microarray were determined using dot plots. The reference glycans are in red square boundaries. Lines and error bars represent the median and inter-quartile range respectively. Red broken line is set at the threshold value (i.e. 5.8).

4.2.8 Association of measured IgE signal intensities to core-modified N-glycans with *S. haematobium* and *N. americanus* infection statuses

Analyses were carried out to determine, especially for participants with current helminth infections, whether IgE signal intensities were directed at particular epitopes on coremodified N-glycans (Figure 4.6). As was expected, participants with current *N. americanus* infection were observed to have higher median IgE fluorescence intensities to α3-fucosylated N-glycans than *N. americanus* negative participants. This was significantly higher for G73 ($p = 0.02$; Figure 4.6), an N-glycan with only an α3-fucose core modification. No differences in median IgE signal intensities were however observed between current *N. americanus* positive participants and *N. americanus* negative participants towards N-glycans with only xylose core-modifications. This was with the exception of G1 (Figure 4.6f), to which higher median IgE signal intensities were observed for *N. americanus* positive participants than *N. americanus* negative participants, although this was statistically insignificant ($p > 0.05$; Figure 4.6f). This could be indicative of an existing association between *N. americanus* and the α3-fucose epitope on N-glycans, and may also explain the observed IgE fluorescence patterns in Figure 4.5 in which signal intensities were mainly directed at the α3-fucose epitope in the R area.

Higher median IgE signal intensities were likewise observed for participants with current *S. haematobium* infection, than for *S. haematobium*-negative participants towards Nglycans with α3-fucose. Significant differences were particularly observed for G75 (Figure 4.7b; $p = 0.023$) which has α3- and α6-difucosylations; and for G39 (Figure 4.7c; $p = 0.014$), which has core modifications of both α3- and α6-difucoses and a β2-xylose. Interestingly, significantly higher median IgE signal intensities were observed among *S. haematobium*-positive than among *S. haematobium*-negative participants for N-glycans

with only β2-xylose (Figure 4.7e-g), namely G34 ($p = 0.0005$), G1 ($p = 0.009$), and G2 ($p = 0.0003$). This is suggestive of the influential role of *S. haematobium* on the observed area-associated fluorescence patterns in Figure 4.5 whereby IgE signal intensities in the UL and UH Areas were directed at both xylose and fucose. Additionally, this trend suggests a strong association between *S. haematobium* positivity and the β2-xylose epitope.

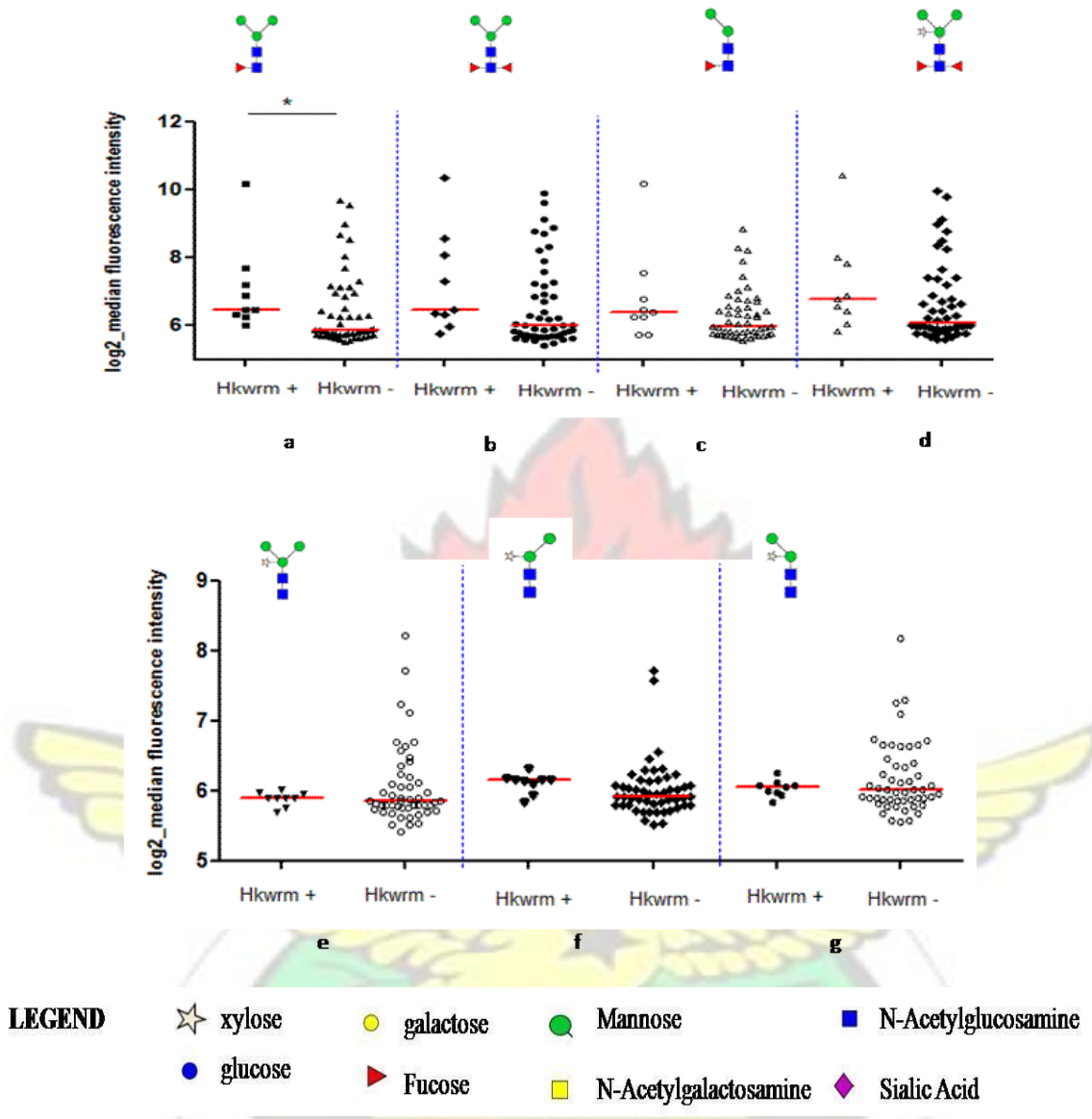


Figure 4.6: Comparing median IgE signal intensities between *N. americanus*-positive and negative participants for a selection of core-modified N-glycans. (a)G73 has an α3-fucose core modification, **(b)** Core modifications on G75 comprise α3- and α6fucoses, **(c)** G72 also possesses a single α3-fucose, **(d)** G39 has both α3- and α6difucosylations and a β2-xylose, while **(e)** G34, **(f)** G1, and **(g)** G2 possess only β2xylose. The Mann Whitney U test was used to compare between *N. americanus*-positive and negative participants for each glycan. The red lines are representative of the group medians. Asterisk (*) is indicative of comparisons with $p < 0.05$.

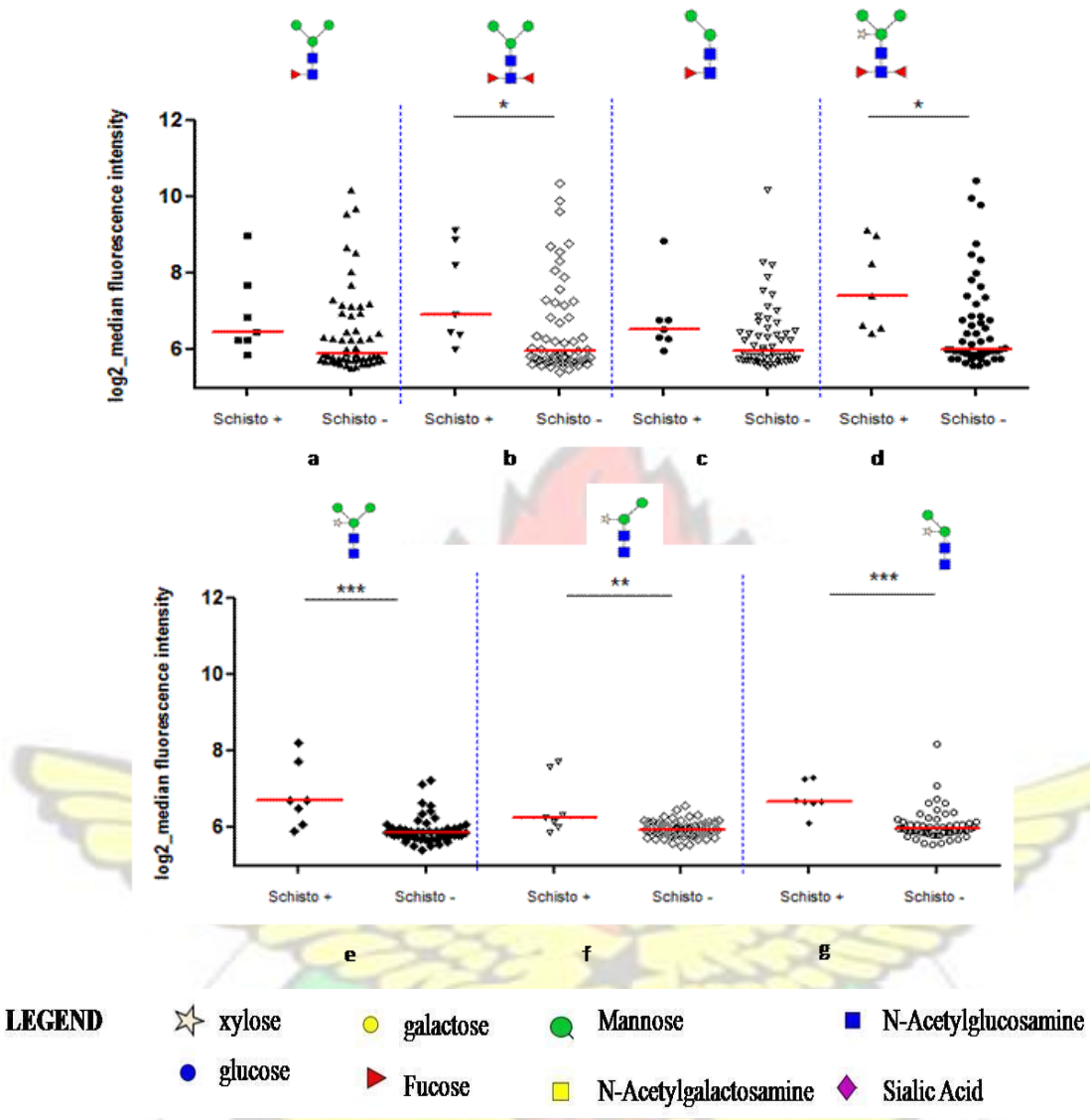


Figure 4.7: Comparing median IgE signal intensities between *S. haematobium*positive and negative participants for a selection of core-modified N-glycans. (a) G73 has an α3-fucose core modification, **(b)** Core modifications on G75 comprise α3- and α6fucoses, **(c)** G72 also possesses a single α3-fucose, **(d)** G39 has both α3- and α6difucosylations and a β2-xylose, while **(e)** G34, **(f)** G1, and **(g)** G2 possess only β2xylose. The Mann Whitney U test was used to compare between *S. haematobium*-positive and negative participants. The red lines are representative of the group medians. A single asterisk (*) is indicative of comparisons with $p < 0.05$; two asterisks (**) represent comparisons with $p < 0.01$; whiles three asterisks (***) represent comparisons with $p < 0.001$.

4.2.9 Differences in glycan-associated IgE, response patterns for European CCDpositive participants

For the selection of European CCD-positive participants, glycan-associated IgE signal intensities were studied in bar graph (Figure 4.8) and scatter dot plot formats (Figure 4.9). For the scatter dot plots, selections of glycans similar to those used for the Ghanaian participants (Figure 4.3) along with selected variations from the array, were utilized. From the generated plots, it was apparent that IgE response signals were directed against the α3-fucose and β2-xylose core modified N-glycans. Additionally, IgE, response signals to the respective variations of α-gal (Figure 4.8; Figure 4.9c1, c2, c7) and sialic acid epitopes were generally below threshold levels (Figure 4.9c5, c6).

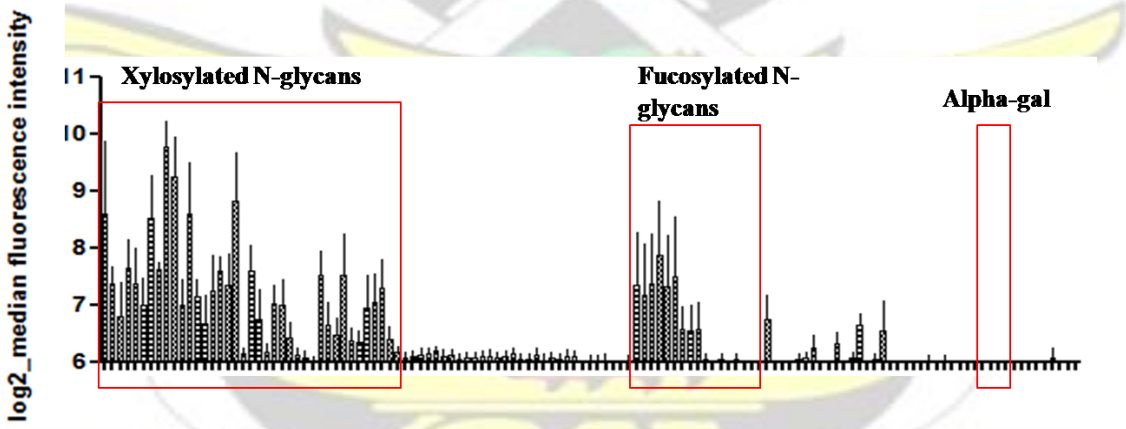


Figure 4.8: IgE fluorescence intensity graph of the 128 glycans for our European CCD-positives. This bar graph was constructed, using the log2 median fluorescence intensity (log2 MFI) data, and shows the signal intensities of the 128 glycans at a glance. The red boundaries indicate glycan groups on the microarray chip that are the focus of this study. Each bar represents the mean of the log2 MFIs for 5 participants for a particular glycan. The lines on top of each bar represent the standard error of mean (SEM).

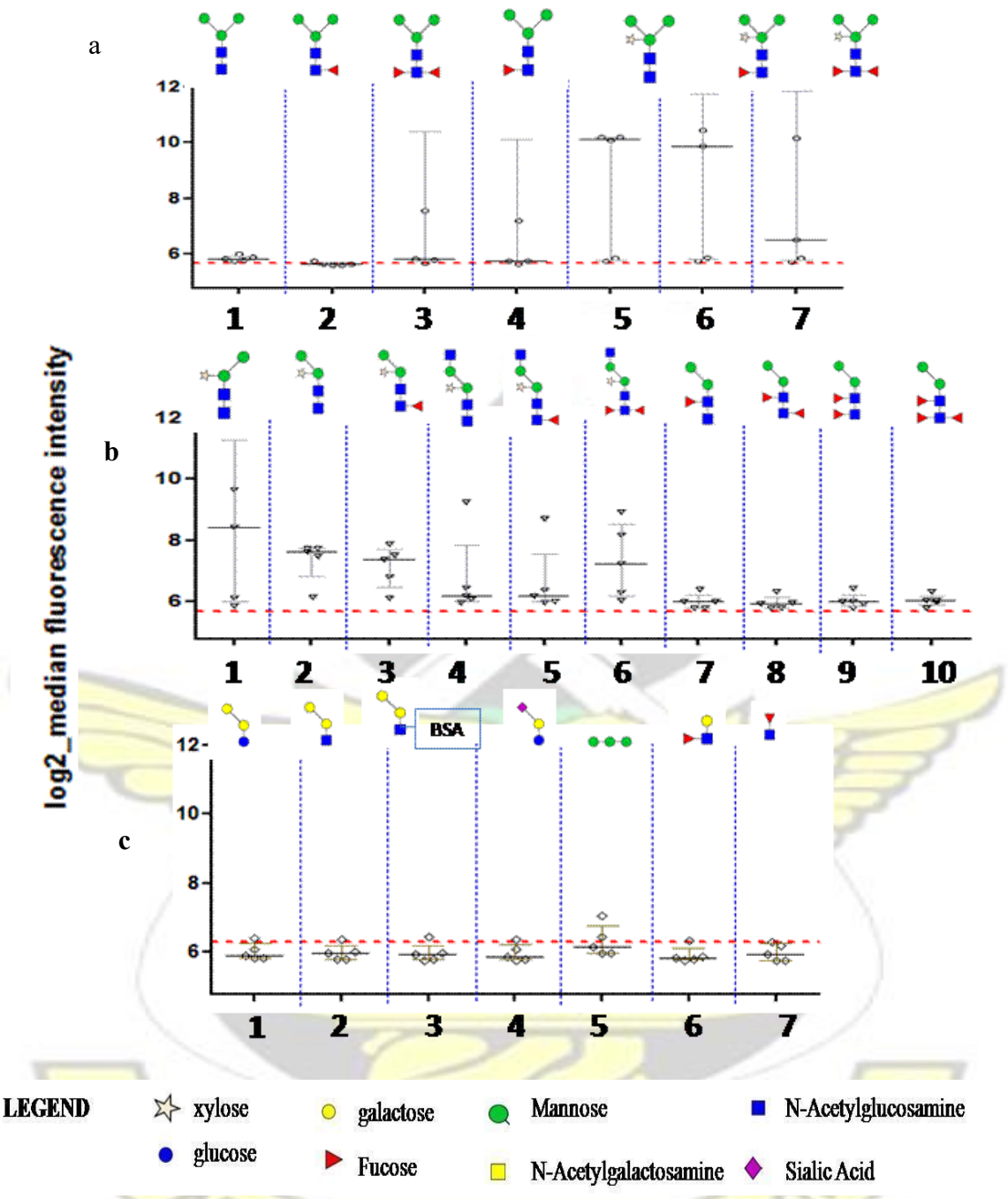


Figure 4.9: Determining patterns in anti-glycan IgE responses for EU CCD+ participants: Using the same glycans and their variations, dot plots were drawn to help determine the glycan moieties recognized by IgEs of selected European allergics. Lines and error bars represent the median and inter-quartile range respectively. Red broken line is set at the threshold value (i.e. 6.3 for IgE).

4.2.10 Comparing Ghanaian and European CCD-positive (EU CCD+) subjects in response patterns to selected glycans and their variations

Also with dot plots, comparisons involving glycan-associated IgE responses were made between both UH and R Ghanaian participants, and the EU CCD+ participants. The exclusion of the Ghanaian UL participants was because observed glycan response patterns for this group were similar to those for the UH group. It was observed from the generated plots, that the trends of glycan-associated IgE responses were similar for both the Ghanaian UH and EU CCD+ participants (Figure 4.10). This was inferred from the observation of response signals to such N-glycans as G34 (Figure 4.10a (5)), G1 (Figure 4.10b (1)), and G2 (Figure 4.10b (2)), which have only a β2-xylose core modification. IgE signal intensities to these glycans were found, compared to those for the EU CCDpositives and the Ghanaian UH participants, to be much lower in the R area (Figure 4.10). This was consistent with previous observations (Figure 4.5) whereby high signal intensities were realized in the R area only for N-glycans with the α3-fucose core modification.

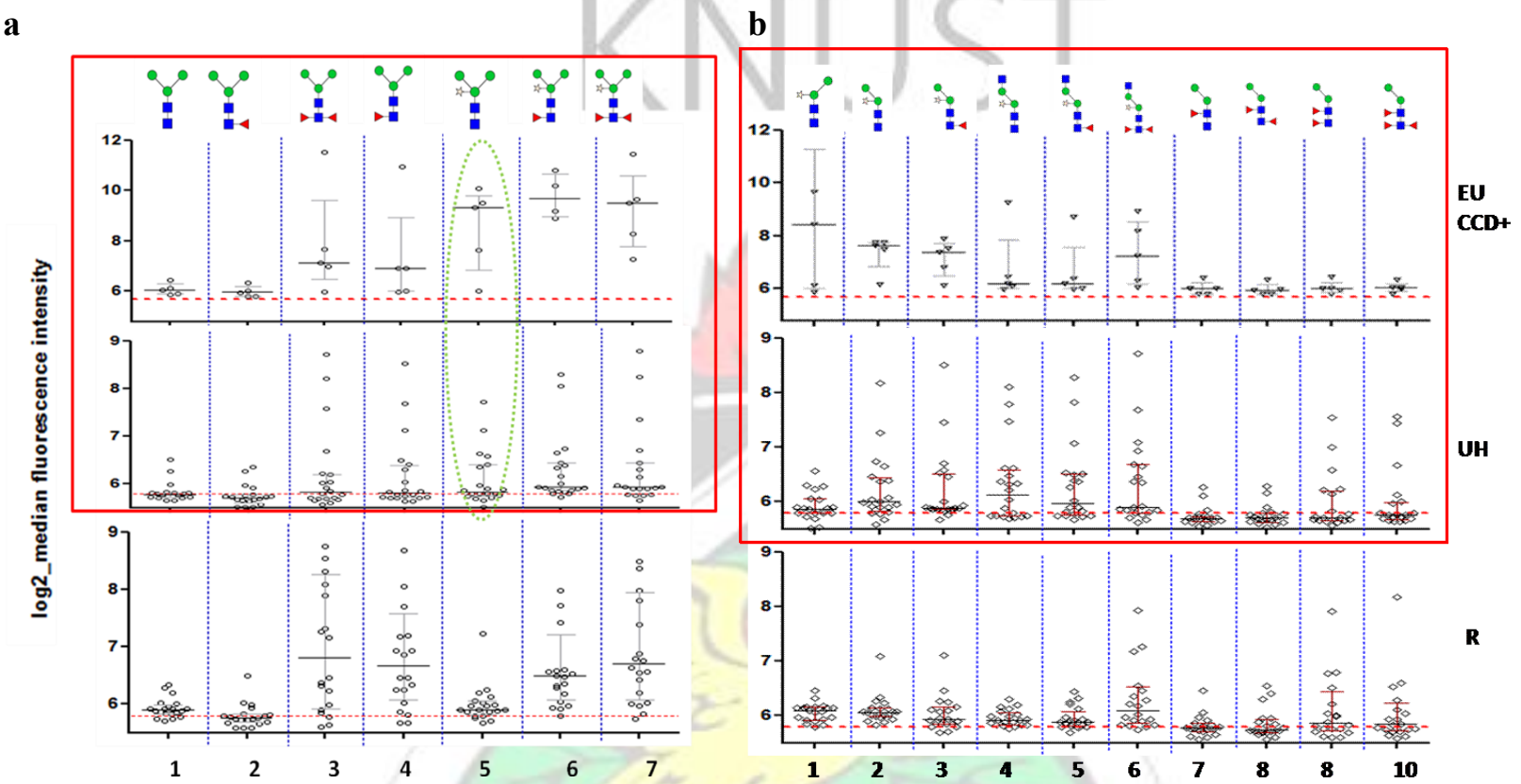


Figure 4.10: Comparing European CCD-positive (EU CCD+) glycan-associated IgE responses to IgE responses among Ghanaian UH and R participants for selected glycans and variations. Dot plots were constructed to ensure easy comparison. UL was excluded because response patterns were found to be closely similar to that of the UH area. Observations indicate that glycan-associated IgE response signals for the European and Ghanaian UH participants (red square boundaries) are directed at α3-fucose and β-2 xylose, while IgE response signals are directed against α3-fucose for the R Area. Lines and error bars represent the median and inter-quartile range respectively. Red broken lines are set at the respective threshold values (i.e. 5.8 for both Ghanaian and EU CCD+ participants).

4.3 Discussion

The objective for this part of the work was to compare glycan-associated IgE response patterns between the UH, UL, and R areas for this selection of Ghanaian participants as well as between the Ghanaian and European pollen allergics; and assess results for clues both into likely influencing glycan epitopes and environmental agents. Principal among the findings was the area-associated pattern of observed IgE fluorescence signals, which in terms of magnitude was found to be highest in the R area, followed with the UL and UH areas respectively. The measurement of seral titres of bromelain- and α-gal-specific IgE also yielded similar results, in which a higher prevalence of participants with α-galspecific IgE titres greater than 0.35kU/L was observed for the R Area, compared to the urban areas. These observations were in consonance with conclusions drawn from a previous related study, in which a significantly greater odds ratio was observed for peanut-specific IgE positivity/sensitization in the R Area as compared to the UH Area (Amoah *et al.*, 2013). Indeed, in a case-control study conducted in the Gambia involving three rural communities and the capital city Banjul, no case of asthma was observed in the rural communities while numerous asthmatic cases were noted in the capital city. Interestingly, the geometric mean of serum IgE titres measured for the rural pupils was nearly three times higher (962u/ml) than that of serum IgE titres measured for pupils in Banjul (368u/ml) (Godfrey, 1975).

Except for the peanut allergen, a higher prevalence of skin prick test positivity to cockroach and house dust mite was observed among rural than urban participants for this selection. Obeng *et al.*, (2014) made similar observations in a cross-sectional study involving a much larger number of subjects within the Greater Accra Region, whereby SPT positivity

prevalence was much higher in the rural than in the urban areas. On the contrary, Addo-Yobo *et al.* (2007) reported higher SPT prevalences among the urban affluent than among the urban poor and rural/suburban subjects in Kumasi. Similar trends were reported by Ng'ang'a *et al.* (1998) in Kenya, and by Nyembue *et al.* (2012) in Congo. Given the current knowledge on the pathophysiological mechanisms involved in skin prick testing (Janeway *et al.*, 2001), it was anticipated that SPT prevalence would be higher in the UH than in the UL and R areas respectively. However, the observed trend in atopic sensitization to cockroach and house dust mite SPTs (which were contrary to expectations) may mechanistically be due to the presence of low-affinity IgEs produced by B-lymphocytes which may have class-switched directly from IgM production (Davies *et al.*, 2013). Another possible cause could be the criteria employed in the categorization of urban areas into UH and UL, which upon considering the complexities associated with urbanization in our capital city, may have been over-simplified and inadequate in addressing the socio-economic and cultural differences important for atopy (Obeng *et al.*, 2014). Issues associated with the changing cultural and ethnic environments, as well as the limited access to amenities present different opportunities for diseases, and may also account for the observed discrepancies in SPT-associated trends. Further studies would certainly be necessary to ascertain observed SPT patterns.

Observations in this work indicate for this selection of Ghanaian participants that signal intensities were directed towards particular glycan epitopes, namely the xylosylated and fucosylated N-glycans, and α-gal. Furthermore, with area-stratified dot plots, the IgE signal intensities are seen to be directed primarily against the α3-fucose epitope of coremodified N-glycans for the R area (Figure 4.5) and against both the α3-fucose and β2xylose epitopes

in the UL and UH areas. Van Ree *et al.* (2000) in studying certain pollen and peanut allergens provided evidence demonstrating the involvement of the α3-fucose and β2-xylose epitopes in IgE binding. Faveeuw *et al.* (2003) also showed via murine models, the importance of these glycan moieties in generating a strong, Th2-based cellular response.

The lowest signal intensities were observed in the UH Area, suggesting comparatively less cross-reactive IgE activity in this group of subjects. This was in consonance with observations of lower prevalences of helminth and malarial infections in the UH area. Obeng *et al.*, (2014) in a related study also observed the near-absence of helminth infections among participants in the UH Area, with only 4 out of 239 (i.e. 1.7%) of them testing positive for any of the urinary or intestinal helminths. Interestingly, strong IgE reactivity to the α3-fucose and β2-xylose epitopes of core-modified N-glycans was observed for the selected European participants, but not to any of the α-gal epitopes on the microarray. This is important, considering that all our selected European subjects were previously known to have high titres of plant-induced cross-reactive IgE (Mari *et al.*, 2008).

Comparing between subjects with and without current *N. americanus* infections for selected N-glycans, the possible role of *N. americanus* in driving responses in the R area is indicated via the observed association between elevated IgE levels to α3-fucosylated N-glycans and *N. americanus* positivity. Structural studies on the glycoconjugates of *Haemonchus contortus*, a parasitic nematode to domestic ruminants and closely related to the human hookworms (*A. duodenale* and *N. americanus*) revealed among other structures in the adult stage, N-glycans with up to three fucoses attached to the core structure which included α3- and/or α6-fucoses attached to the proximal Nacetylglycosamine unit

(GlcNAc), and an α3-fucose attached to the distal GlcNAc unit of the core structure (Haslam *et al.*, 1996; as reviewed by Dell *et al.*, 1999). Mass spectrometric studies to determine the glycome of the human hookworms would therefore be critical in verifying these observations. Considering however the weak associations observed between current *N. americanus* infection and elevated IgE levels to almost all α3-fucosylated N-glycans, other factors may also be responsible in driving observed α3-fucose-associated IgE responses in the R area. Worth considering for later studies might be the role of insects in association with these observed responses.

More compelling however, was the comparison between subjects with and without *S. haematobium* infection for a selection of N-glycans. Significant associations between elevated IgE levels to xylosylated N-glycans and *S. haematobium* positivity were discovered, which might explain the observed elevated IgE reactivity to the β2-xylose epitopes in the UL and UH areas. It is worth noting here as well that for this selection of participants, *S. haematobium* prevalence was higher in the urban areas as compared to the rural area. Interestingly, significant associations between elevated IgE levels to some of the α3-fucosylated structures and *S. haematobium* positivity was observed, further buttressing observed IgE reactivity to this epitope in the UL and UH areas. Indeed, in a related study it was realized via inhibition assays that for almost 93% of participants, peanut-specific IgEs could also easily recognize the *S. haematobium* soluble egg antigen (SEA) (Amoah *et al.*, 2013). Additionally, *S. mansoni*-associated glycomics research has shown the egg stage of the Schistosome to express in their glycoproteins, N-glycans rich in β2-xylose, α3- and/or α6-fucose core modifications (Hokke *et al.*, 2007a; reviewed by van Diepen *et al.*, 2012).

It may be far-fetched, with evidence provided in this study to implicate any eukaryotic organism for cross-reactive IgE induction. However, it might be possible to predict the types of eukaryotes likely to influence observed IgE response patterns. Factors observed to be significantly associated with this selection of core-modified N-glycans include hookworm infection status; ImmunoCAP-measured IgE to cockroach (Bla g), house dust mite (Der p), peanut (Ara h), and bromelain. Considering however, that significantly higher prevalences of the above-mentioned factors were observed in the R area, than in the urban areas implies that helminth, plant, and insect sources may be potential inducing factors in the R and UL areas. In the UH area, IgE reactivity to glycan epitopes may be due to plants, considering the similarity in IgE response patterns between the UH and European CCD-positive participants, and the near absence of helminth and malarial infections among this selection of UH participants. Thus, further glycan-associated urban-rural studies should incorporate eukaryotes from the listed eukaryotic categories.

The reported high prevalence by Commins and Platts-Mills (2013) of IgE antibodies to galactose-α-1,3-galactose in a rural community 100 miles north of Nairobi, with no such reported associated symptoms as anaphylaxis or urticaria, was one of the strong indications of the presence of cross-reactive IgE in Africa, particularly in rural populations. This work indicates, for this selection of Ghanaian participants, a clear urban-rural trend both in prevalence and signal intensities of IgE to α-gal. Results from both the ImmunoCAP and microarray techniques yielded similar results in which significantly higher prevalence and signal intensities were observed for the R Area as compared to the urban areas. Interestingly, malaria positivity and cockroach-specific IgE were significantly associated with measured IgE signal intensities to the α-gal epitope (G113) on the microarray.

Considering that the inducing agent of IgEs to α-gal in the southeastern United States was discovered to be an insect (ticks: *Ambylomma americanum*), it certainly is not far-fetched to consider other biting insects typically associated with local Ghanaian communities as potential inducing sources. Indeed, evidence from a recent study in both mice and humans (Yilmaz *et al.*, 2014) has shown sporozoites of *Plasmodium spp*. to express the α-gal epitope. Although found by the same study to induce IgM but not IgG antibodies, the possibility of IgE induction to the epitope against sporozoites cannot be ruled out. Additionally, malaria positivity was observed to exhibit an urban-rural trend, with the highest prevalence realized in the R, followed by the UL, and UH areas respectively. It would be essential to determine via mass spectrometry, the glycomes of such insects as the mosquito, the cockroach, and the tick, as these would very well confirm or otherwise, current findings and postulations.

It is currently unclear however, whether or not glycan-associated cross-reactive IgEs are incapable of inducing mediator release from allergy-associated effector cells. This is because, although a number of studies have shown glycan-associated cross-reactive IgEs to be of low biological activity with no clinical symptoms exhibited by patients with high titres in their serum samples (Mari *et al.*, 2008; Amoah *et al.*, 2013), other studies seem to indicate otherwise (Fotisch *et al.*, 1999; Bublin *et al.*, 2003). Further studies are therefore needed to enhance our understanding of the capacity of these glycan-associated cross-reactive IgEs to induce the release of allergic mediators by effector cells, as well as the immune mechanisms involved in their production. This is essential in our quest for improved strategies in alleviating or eradicating clinical cases of type-1 hypersensitivity.

So far, this study shows firstly, that an Area-associated trend exists between R, UL, and UH Areas in terms of glycan-associated IgE responses, with the R Area exhibiting comparatively higher cross-reactive IgE activity than the UL and UH Areas respectively. Secondly, findings from this work indicate possible inducing agents for the observed cross-reactive IgE activity to include insects, helminths and plants in the Rural and Urban Low areas; and plants in the Urban High area.

CHAPTER 5:

ASSESSMENT OF GLYCAN-ASSOCIATED IGg1 RESPONSES

5.1 Introduction

It is well established that allergy-associated immune responses are largely Th2-driven, with IgEs bound by their F_c regions to $F_c\varepsilon$ receptors on inflammatory cells playing a key role in clinical outcomes (Shimoda *et al.*, 1996; Robinson, 2000). However, where allergy-associated immunity is concerned, other associated humoral activities occur, which most likely influence the biological activities of cell-bound IgEs, and the clinical symptoms exhibited.

The role of IgG1 in allergic reactions has been an area under intense research scrutiny in recent years. Findings from a recent study on IgE and associated IgG responses to α-gal indicated increases in IgG1 and IgG2 levels that characterized elevated levels of α-gal–specific IgE (Rispens *et al.*, 2013). In another study involving allergen-specific immunotherapy (SIT), IgG1 levels, along with IgA and IgG4, were found to increase significantly over a 70-day treatment period for the house dust mite allergen (Akdis, 2009). It was suggested that the observed increases in IgG1which characterized elevated IgE levels may have been due to the concurrent formation and production of IgG1 and IgE antibodies by immature B-cells in the germinal centres of spleens (Rispens *et al.*, 2013). Indeed, Stern *et al.*, (2006), who set out in a study to determine the extent to which exposure to a farming environment protected against IgE responses discovered for all allergens examined that IgG1 levels were concurrently detected where IgEs were measured; and that the failed expression of IgG1 was associated with a lowered prevalence of IgG4 or IgE

responses. It is generally agreed that IgG1 antibodies are capable of complexing with several allergens, thereby increasing the potency of cellbound IgE antibodies (Aalberse, 2011). However, regarding cross-reactive, or glycanspecific IgEs, little is known about the associated IgG1 response patterns.

Work was done in this part of the study, first to determine the existence of urban-rural patterns in anti-glycan IgG1 response signals; to secondly identify the glycans or glycan epitopes significantly associated with measured IgG1 responses from the selected participants in the urban high, urban low and rural areas, along with potential risk factors associated with the anti-glycan IgG1 responses recognized; to compare glycan-associated IgE responses with anti-glycan IgG1 responses both among this selection of Ghanaian and European participants; and to finally compare glycan-associated IgG1 responses between the Ghanaian and European CCD-positive participants. This work was conducted with the aid of a well-developed array of synthetic glycans.

5.2 Results

5.2.1 Urban-rural differences of measured IgG1response patterns to synthetic glycans by glycan microarray

Bar graphs depicting IgG1 fluorescence intensities for the 128 printed glycans stratified by area were constructed, with each bar representing the mean of IgG1 fluorescence intensities measured for 20 participants within a particular area for one glycan (Figure 5.1). For the IgG1 signal intensity graphs, signals were also largely restricted to the xylosylated and fucosylated N-glycans, as well as to the variations of α-gal. However, signal intensities appeared to be stronger for the UL area, followed with the R, and finally the UH Areas (Figure 5.1). Additionally, signal intensity patterns similar to those realized for IgE were observed for IgG1 across the array for all three areas (Figure 5.2).

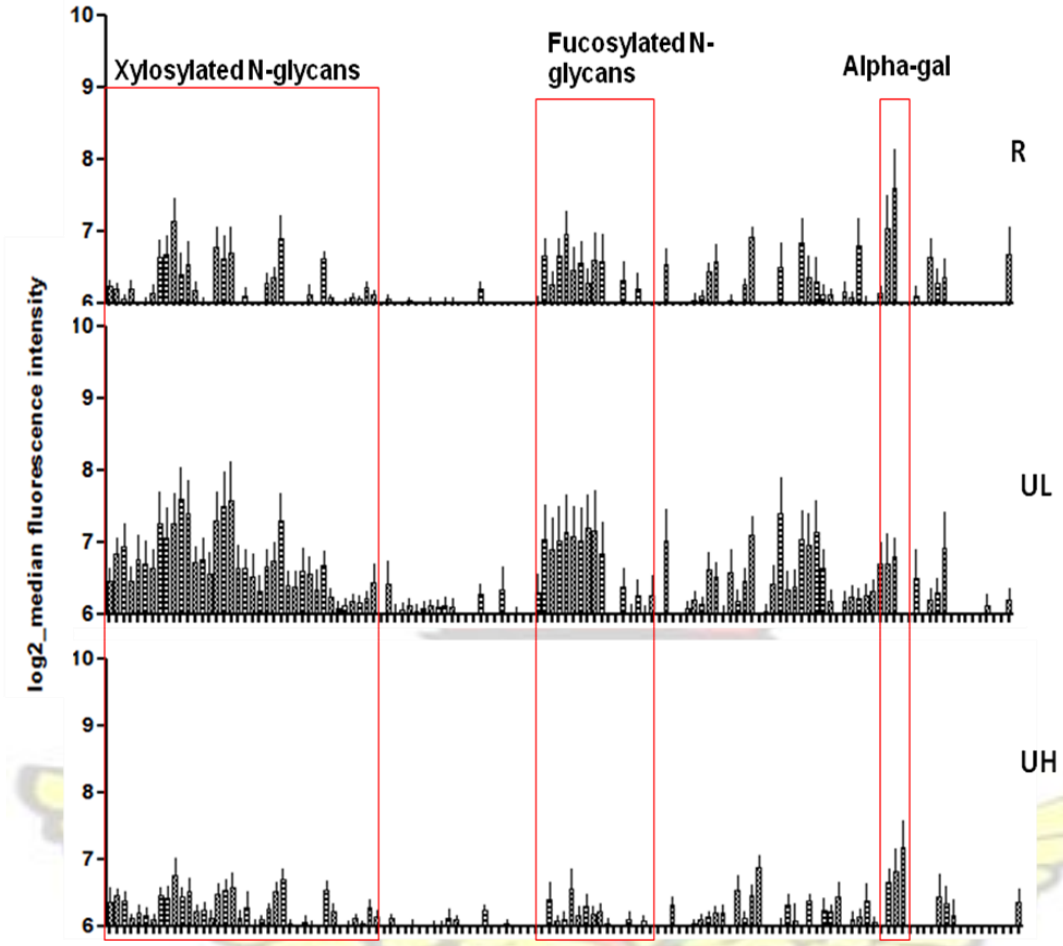


Figure 5.1: IgG1 Fluorescence intensity graphs of the 128 glycans stratified by Area. Using the log2 median fluorescence intensity (log2 MFI) data, bar graphs showing the signal intensities of the 128 glycans at a glance, were constructed for each area. Graphs were arranged as shown for easy comparison. The red boundaries indicate glycan groups on the microarray chip that are the focus of this study. Each bar represents the mean of the log2 MFIs for 20 participants for a particular glycan. The lines on top of each bar represent the standard error of mean (SEM).

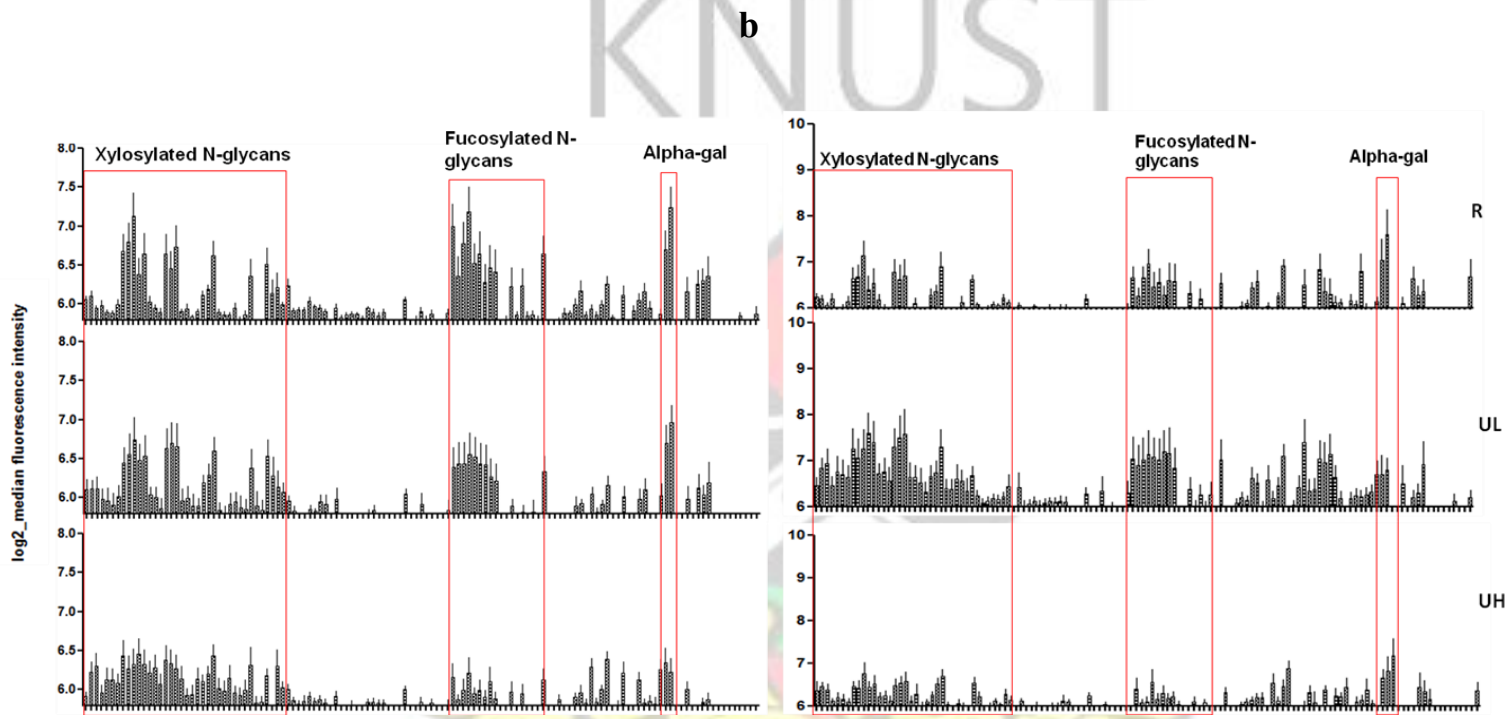


Figure 5.2: Comparing signal patterns on area-stratified fluorescence intensity graphs for both (a) IgE and (b) IgG1. Area-stratified graphs for (a) IgE and (b) IgG1 have been arranged as shown for easy comparison. The red boundaries indicate glycan groups on the microarray chip that are the focus of this study. Each bar represents the mean of the log2 MFIs for 20 participants for a particular glycan. The lines on top of each bar represent the standard error of mean (SEM).

5.2.2 Identification of glycans with significant, area-associated IgG1 signal intensities

Analysis of the IgG1 fluorescence intensity dataset using Mann Whitney U tests generated 5 glycans which showed significant difference between at least two Areas (Figure 5.3). These were G2 (UL/R; $p = 0.03$); G11 (UL/R; $p = 0.041$); G35 (UL/R; $p = 0.04$); G7 (UL/R; $p = 0.046$); and G111 (UH/R; $p = 0.024$). The first 4 are N-glycans, each with a β2-Xylose core-modification and a mannose absent from the core structure. Among the four however, only G2 is not fucosylated, while proximal GlcNAcs of both G35 and G7 are each attached to a fucose via an α-(1,6) linkage. For G11, the fucose is attached to the proximal GlcNAc via an α-(1,3) linkage. Additionally, for G11 and G7, an antennary GlcNAc is attached in each case to the first mannose via a β-(1,4) linkage (Figure 5.3). For the first four glycans, median signal intensity was highest in the UL (Figure 5.3), compared to the R and UH areas. Regarding G111 however, the median signal intensity was highest in the UH, followed by the UL and R areas respectively (Figure 5.3).

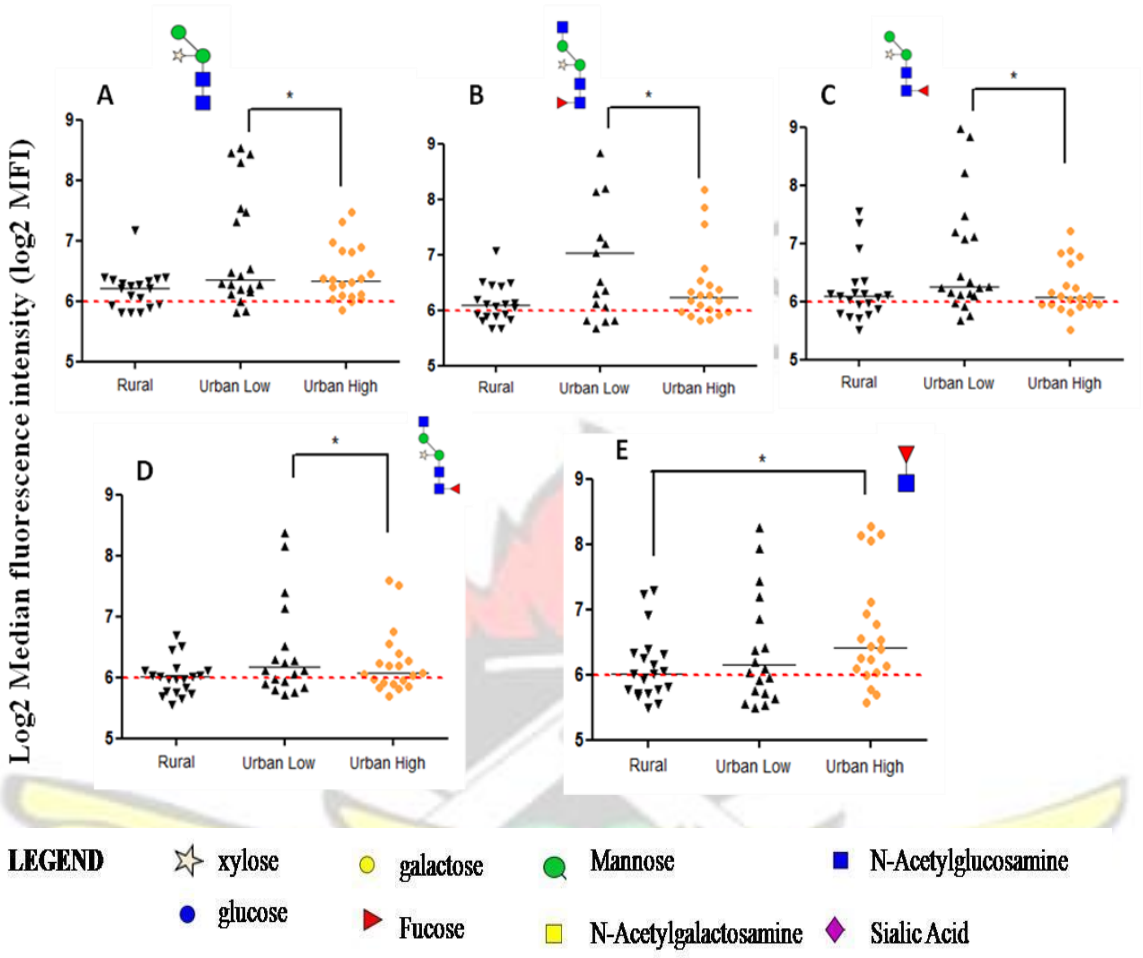


Figure 5.3: Glycans identified from the IgG1 fluorescence intensity dataset to exhibit significant differences in signal intensities in at least two Areas. Determining which glycans were significantly associated with Area was done using the Mann Whitney U test to compare between two areas at a time for all the 128 printed saccharides. The glycans are: **(a)** G2; **(b)** G11; **(c)** G35; **(d)** G7; and **(e)** G111. The single asterisk (*) represents p values less than 0.05. The red broken line is set at the threshold value (i.e. 6) which was determined by calculating average of the median fluorescence intensities for all the 128 glycans.

5.2.3 Area-stratified correlations between IgG1 and IgE

Given the striking similarity in signal intensity patterns realized between IgE and IgG1 (Figure 5.4), correlation graphs stratified by area were generated with the aim of exploring the strength of relationship between measured IgE and IgG1 responses (Figure 5.4). It was interesting to note that IgE/IgG1 correlation was strongest in the R Area (Pearson's r (r) = 0.63; $p < 0.001$), as compared to the UL (r= 0.216; $p < 0.001$) and the UH (r = 0.186; $p < 0.001$) areas, considering that higher IgG1 signal intensities were observed in the UL than in the UH and R areas (Figure 5.1).

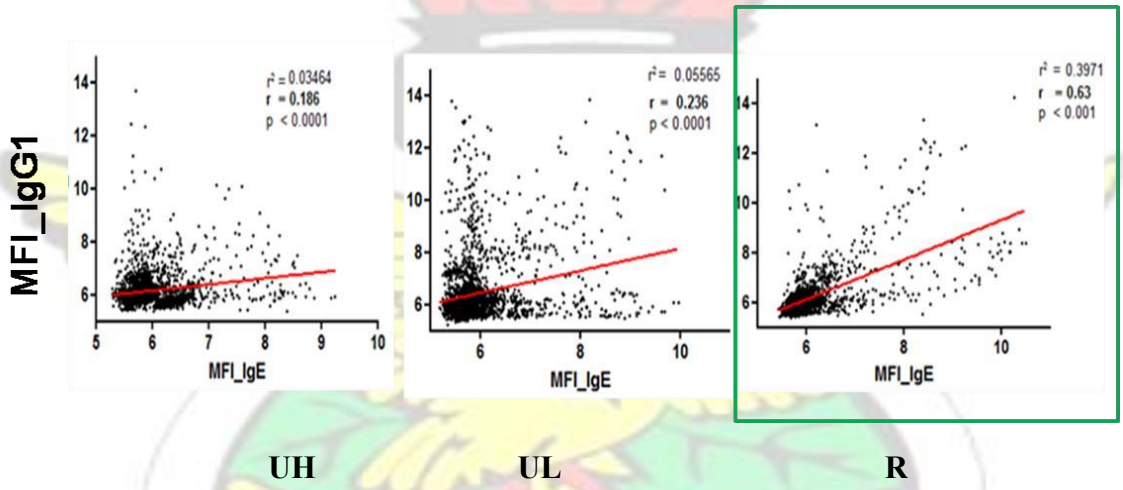


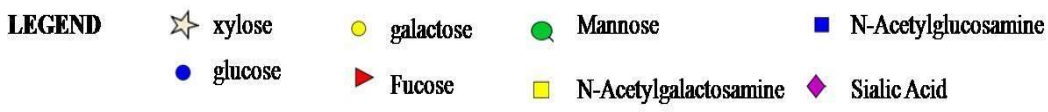
Figure 5.4: Area-associated correlations between IgG1 and IgE. Pearson's correlation coefficient (r) was used to determine the strength of correlation between IgG1 and IgE fluorescence intensities in each area (Red line = linear regression line/best fit line at total).Stronger IgG1/E correlations were observed for the R Area (Green square boundary) than for the UH and UL Areas.

5.2.4 Associations between potential influencing factors and identified glycans from IgG1fluorescence datasets

Strength of associations between IgG1 response intensities to identified glycans and such factors as gender, age, hookworm infection, infection with any helminth, and malaria positivity were assessed (Table 5.1). There was no significant association between gender and IgG1 signal intensities to any of the identified glycans. This was similarly observed for age. Hookworm infection status was found to be significantly associated with IgG1 signal intensities to G35 ($p = 0.026$), which is an N-glycan with a mannose absent from the core structure, and with α6-fucose and β2-xylose core-modifications. Also, the association between any helminth and G111, an N-Acetylglucosamine unit attached by an α-(1,3)-linkage to a fucose, was found to be of borderline significance ($p = 0.047$) (Table 5.1).

Table 5.1: Associations between identified glycans for IgG1 response intensities and potential influencing factors

	 G2	 G11	 G35	 G7	 G111
Factor	**median (range)**	**median (range)**	**median (range)**	**median (ramge)**	**median (range)**
Gender					
Males	6.29 (5.80-8.53)	6.28 (5.66-11.71)	6.09 (5.50-8.97)	6.03 (5.56-10.01)	6.09 (5.50-8.24)
Females	6.27 (5.80-8.46)	6.15 (5.67-10.21)	6.13 (5.67-8.83)	6.07 (5.65-9.58)	6.40 (5.49-10.28)
p-value	0.705	0.554	0.579	0.363	0.126
Age					
< 11 yrs	6.27 (5.80-7.32)	6.23 (5.67-11.56)	6.12 (5.67-7.55)	6.04 (5.73-9.58)	6.03 (5.49-10.28)
≥11 years	6.29 (5.80-8.35)	6.17 (5.66-11.71)	6.12 (5.50-8.97)	6.04 (5.56-10.01)	6.21 (5.50-9.28)
p-value	0.451	0.5	0.872	0.663	0.561
Hookworm					
positive	6.21 (5.80-6.38)	6.07 (5.66-7.08)	5.95 (5.50-6.90)	5.98 (5.56-6.69)	6.01 (5.50-6.90)
negative	6.29 (5.80-8.53)	6.28 (5.67-11.71)	6.14 (5.51-8.97)	6.11 (5.65-10.01)	6.19 (5.49-10.28)
p-value	0.054	0.137	**0.026**	0.066	0.153
Any Helminth**					
positive	6.34 (5.80-8.53)	6.34 (5.66-10.79)	6.11 (5.50-8.98)	6.02 (5.56-10.01)	5.91 (5.50-7.18)
negative	6.27 (5.80-8.29)	6.18 (5.67-11.71)	6.12 (5.51-8.83)	6.06 (5.68-9.58)	6.21 (5.49-10.28)
p-value	0.528	0.645	0.867	0.493	**0.047**
Malaria					
positive	6.20 (5.81-7.52)	6.20 (5.79-10.21)	6.08 (5.73-7.55)	6.03 (5.74-9.58)	6.02 (5.56-9.28)
negative	6.29 (5.80-8.53)	6.22 (5.66-11.71)	6.13 (5.50-8.98)	6.05 (5.56-10.01)	6.19 (5.49-10.28)
p-value	0.218	0.938	0.535	0.599	0.613

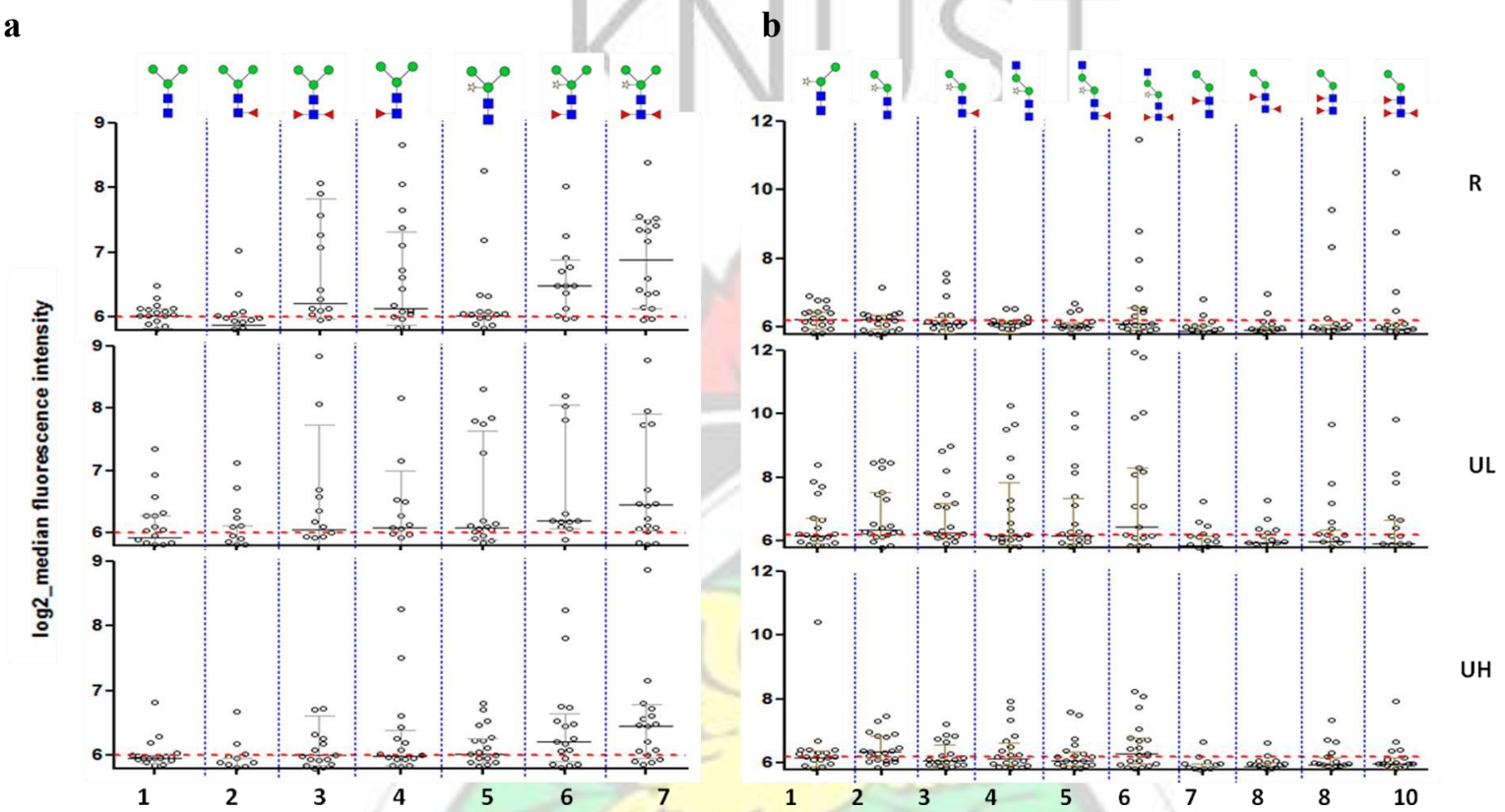


Association between each glycan and potential factor was determined using the Mann Whitney U test, and effect of interaction was considered significant at $p < 0.05$ (Significant associations are in boldface). Any Helminth** comprises *S. haematobium*, *S. mansoni*, *A. lumbricoides*, *Trichuris trichiura*, and *Necator americanus*. IQR = Interquartile range.

5.2.5 Urban-rural differences in IgG1 response patterns to selected glycans and their variations

Along with identifying factors associated with IgG1 responses to the selected oligosaccharides, area-stratified dot plots were constructed for the identified glycans and their variations, and studied for glycan components potentially influencing responses in each area (Figure 5.5). Variations of the synthetic glycans of interest were selected from among the 128 glycans on the array.

Principally, IgG1 response signals in the R area were observed to be directed against the α3-fucose core modification of N-glycans, irrespective of the presence or absence of α6fucose or β2-xylose (Figure 5.5a-b). In the UL and UH areas however, signal responses were observed to be directed against the β2- xylose and α3-fucose epitopes on coremodified N-glycans (Figure 5.5a-b). With respect to the G113 epitope, observed IgG1 signal intensities were higher in the R area than in the urban areas (Figure 5.5c (2)). An area-associated trend was also observed for signal intensities directed against the G111 epitope, where the highest responses were observed in the UH, followed by the UL, and R areas respectively (Figure 5.5c (7)).


a
b
log2_median fluorescence intensity
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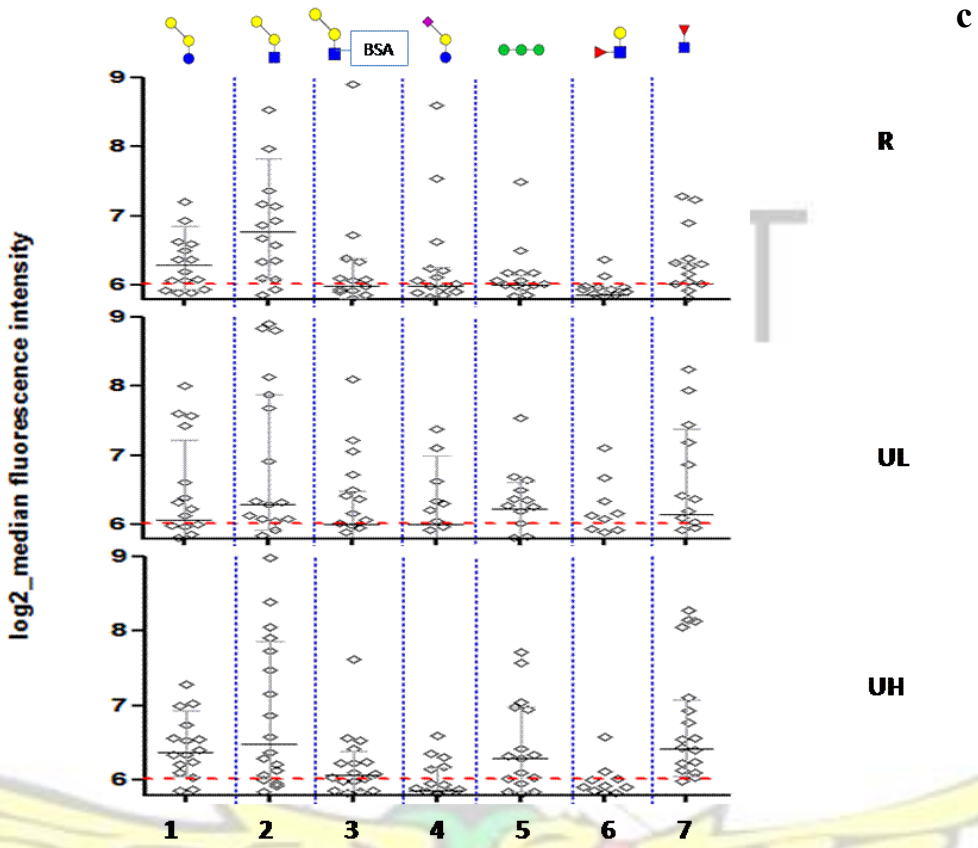

Figure 5.5: Identifying urban-rural patterns in anti-glycan IgG1 responses for selected glycans and their variations. Urban-rural patterns in median IgG1 fluorescence signals to the selected glycans G2(b2), G35 (b3), G7 (b5) and G111 (c7) and their variations on the microarray were determined using dot plots. Plots were stratified by area for easy comparison. Lines and error bars represent the median and inter-quartile range respectively. Red broken line is set at the threshold value (i.e. 6.0)

5.2.6 Association of measured IgG1 signal intensities to core-modified N-glycans with *S. haematobium* and *N. americanus* infection statuses

As was previously done with IgE signal intensities, analyses were conducted here to determine for participants with current helminth infections, whether IgG1 signal intensities were directed at particular epitopes on core-modified N-glycans (Figures 5.6 and 5.7). For participants with current *N. americanus* infection, no differences in median IgG1 fluorescence intensities were apparent between *N. americanus*-positive and negative participants towards the α3-fucosylated N-glycans (Figure 5.6a-d). Furthermore, comparison of median IgG1 signal intensities between current *N. americanus* positive participants and *N. americanus* negative participants towards N-glycans with only xylose core-modifications yielded no significant differences (Figure 5.6e-g). This was with the exception of G1 (Figure 5.6f), to which higher median IgG1 signal intensities were observed for *N. americanus* positive participants than *N. americanus* negative participants, although this was statistically insignificant.

Conversely, higher median IgG1 signal intensities were observed for participants with current *S. haematobium* infection, than for *S. haematobium*-negative participants towards N-glycans with α3-fucose (Figure 5.7a-d). This was similarly observed for median IgG1 signal intensities among current *S. haematobium* positive and negative participants towards N-glycans with only β2-xylose (Figure 5.7e-g), with the exception of G1 (Figure 5.7f). Significantly higher median signal intensities were observed among *S. haematobium* positive than negative participants, towards G2 ($p = 0.0024$). Hence, these observations are indicative of a potentially close association between *S. haematobium* positivity and the observed area-associated fluorescence patterns in Figure 5.5 whereby IgG1 signal intensities in the UL and UH areas were directed at both xylose and fucose.

Here again, a strong association between *S. haematobium* positivity and the β2-xylose epitope on N-glycans is suggested with the observed trends.

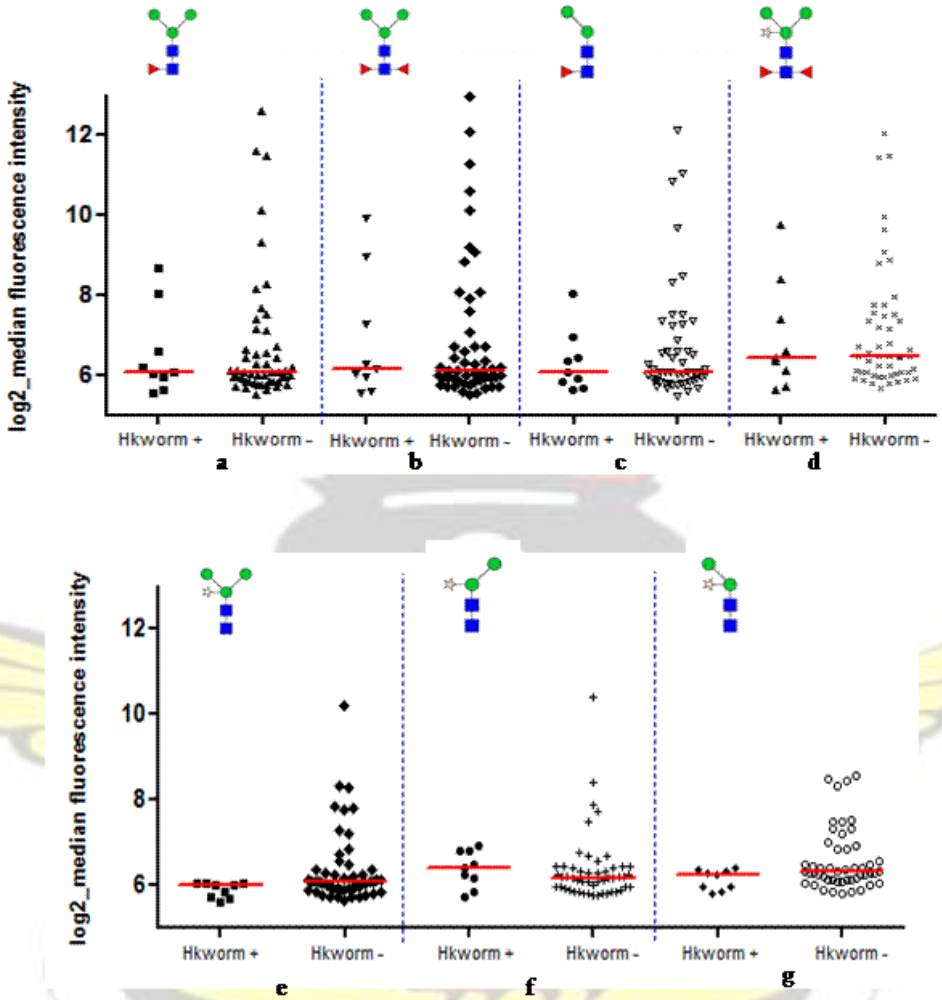

Figure 5.6: Comparing median IgG1 signal intensities between *N. americanus*positive and negative participants for a variety of core-modified N-glycans. The Mann Whitney U test was used to compare between *N. americanus*-positive and negative participants for each glycan. Glycans on the array representing the core modifications being studied are used. **(a)** G73 has an α3-fucose core modification, **(b)** core modifications on G75 comprise α3- and α6-fucoses, **(c)** G72 also possesses a single α3fucose, **(d)** G39 has both α3- and α6-difucosylations and a β2-xylose, while **(e)** G34, **(f)** G1, and **(g)** G2possess only β2-xylose. The red lines are representative of the group medians. Asterisk (*) is indicative of comparisons with $p < 0.05$.

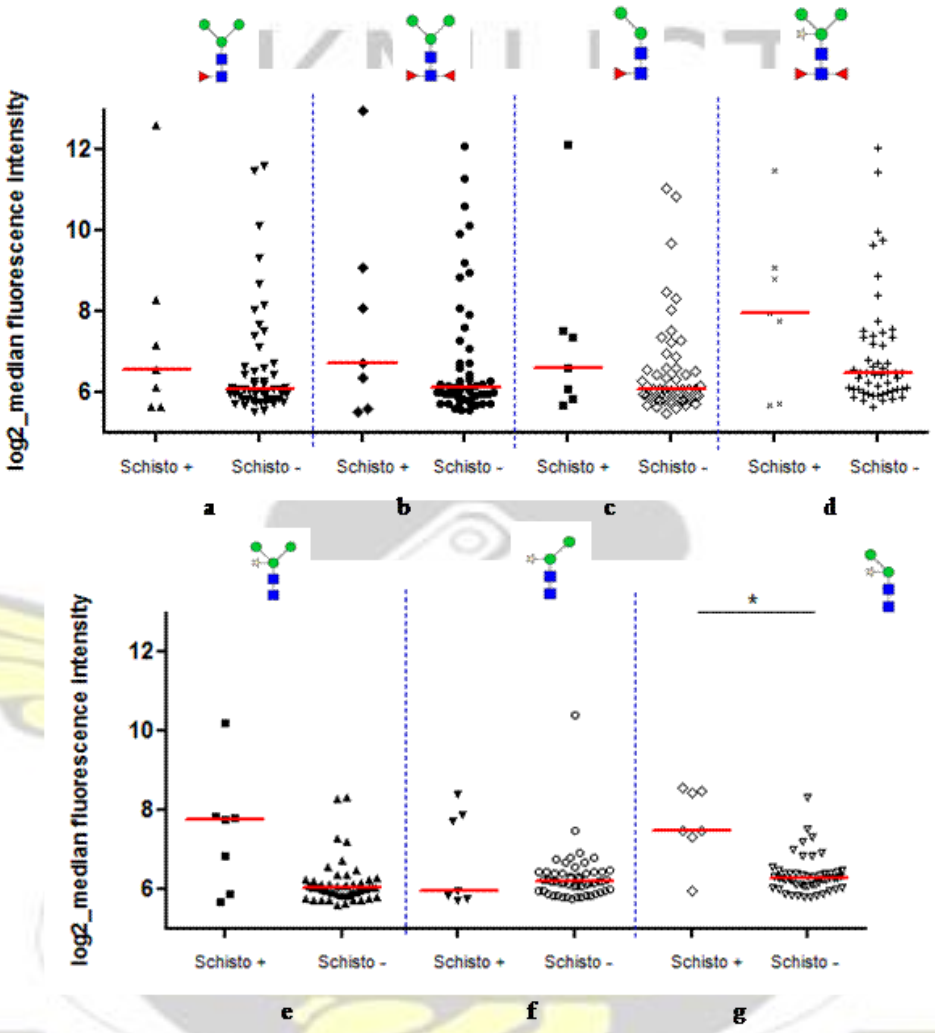

Figure 5.7: median IgG1 signal intensities between *S. haematobium*-positive and negative participants for a selection of core-modified N-glycans. The Mann Whitney U test was used to compare between *S. haematobium*-positive and negative participants for each glycan. Glycans on the array representing the core modifications being studied are used. **(a)** G73 has an α3-fucose core modification, **(b)** core modifications on G75comprise α3- and α6-fucoses, **(c)** G72 also possesses a single α3-fucose, **(d)** G39 has both α3- and α6-difucosylations and a β2-xylose, while **(e)** G34, **(f)** G1, and **(g)** G2possess only β2-xylose. The red lines are representative of the group medians.
Asterisk (*) is indicative of comparisons with $p < 0.05$.

5.2.7 Differences in glycan-associated IgG1 response patterns for European CCDpositive participants

For the selection of European CCD-positive participants, glycan-associated IgG1 signal intensities were studied in bar graph (Figure 5.8) and scatter dot plot format (Figure 5.9). With regard to the scatter dot plots, selections of glycans similar to those used for the Ghanaian participants (Figure 5.3), along with selected variations from the array, were utilized. From the generated plots, it was apparent that IgG1 response signals were directed against the α3-fucose and β2-xylose core modified N-glycans, as was similarly observed for IgE responses (Figure 4.8). Additionally, IgG1 response signals to the α-gal (Figure 5.9(1-3)) and sialic acid (Figure 5.9(4)) epitopes were generally below threshold levels for this selection of European participants (Figure 5.9).

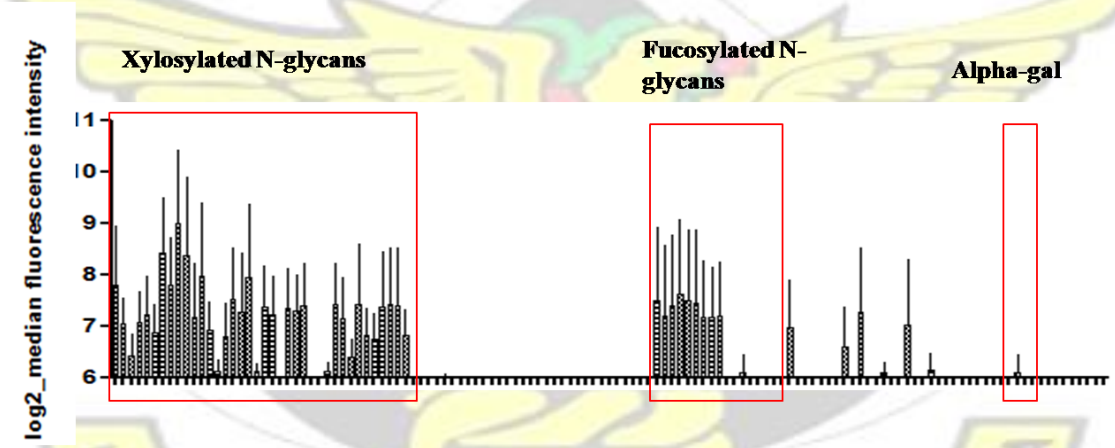

Figure 5.8: IgG1 fluorescence intensity graph of the 128 glycans for our European CCD-positives. The bar graph was constructed, using the log2 median fluorescence intensity (log2 MFI) data, and shows the signal intensities of the 128 glycans at a glance. The red boundaries indicate glycan groups on the microarray chip that are the focus of this study. Each bar represents the mean of the log2 MFIs for 5 participants for a particular glycan. The lines on top of each bar represent the standard error of mean (SEM).

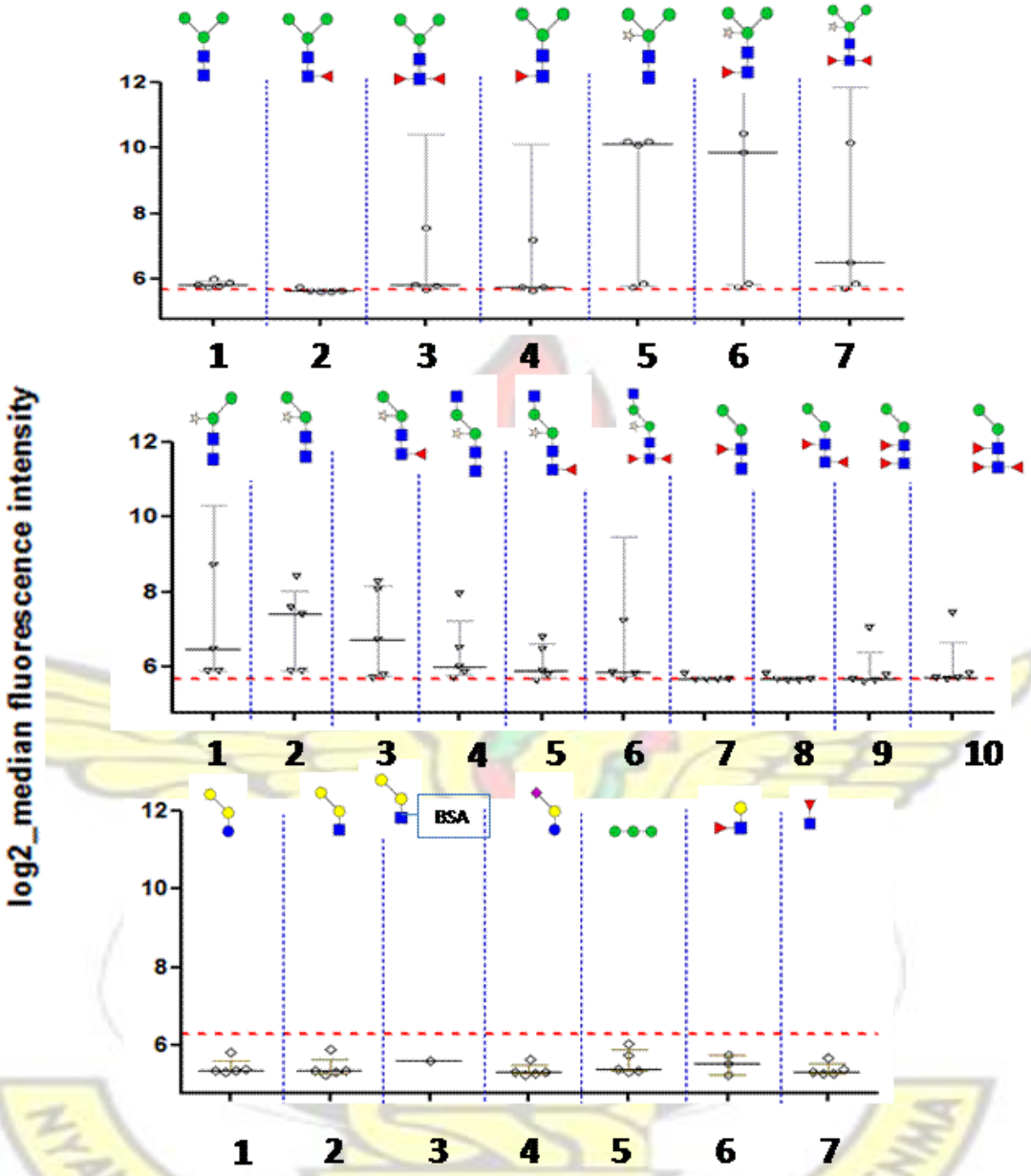

Figure 5.9: Identifying patterns in anti-glycan IgG1 responses for EU CCD+ participants. Using the same set of glycans and their variations as used for the Ghanaian selection, dot plots were drawn to determine epitopes being recognized by IgG1 for our selection of European allergics. Lines and error bars represent the median and interquartile range respectively. Red broken line is set at the threshold value (i.e. 6.0 for IgG1).

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5.2.8 Comparing Ghanaian and European CCD-positive (EU CCD+) subjects in response patterns to selected glycans and their variations

Also with dot plots, comparisons involving glycan-associated IgG1 responses were made between both UH and R Ghanaian participants, and the EU CCD-positive participants (Figure 5.10). The exclusion of the Ghanaian UL participants was due to similarities in observed glycan response patterns for both this group and the UH group. It was realized from the generated plots that IgG1 signal responses were directed at the α3-fucose and β2-xylose epitopes of core-modified N-glycans for both the UH and the European CCDpositive participants, whilst signal intensities were directed mainly against the α3-fucose for the R area. With particular reference to G34 (green dashed oval boundary; Figure 5.10a (5)), high median signal intensities were observed for both the UH and European CCD-positives than for participants from the R area. This was similarly observed for Nglycans either with only β2-xylose, or with both β2-xylose and α6-fucose core modifications (Figure 5.10b (1-4)).

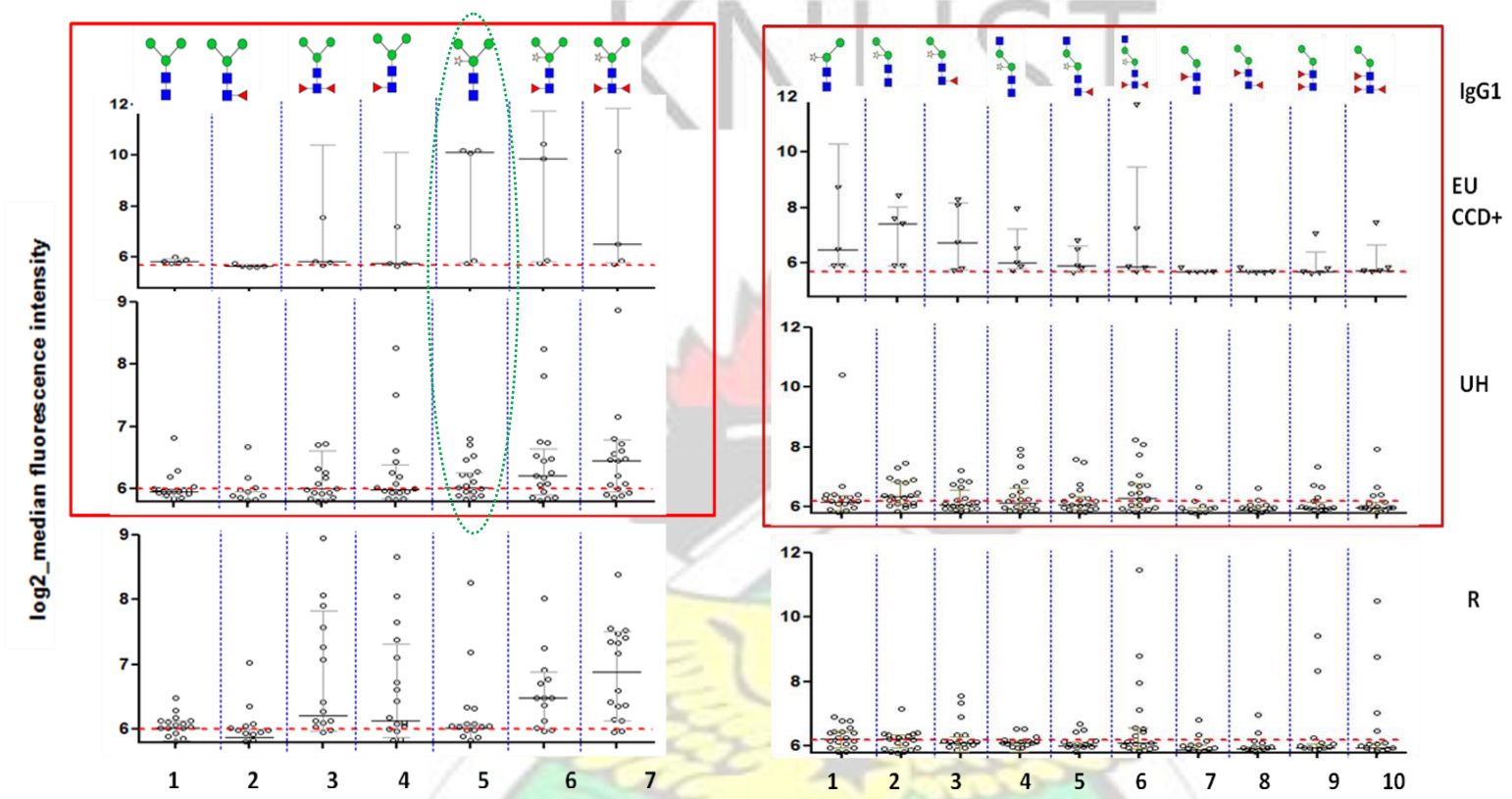


Figure 5.10: Comparing European CCD-positive (EU CCD+) anti-glycan IgG1 responses to Ghanaian urban-rural anti-glycan IgG1 responses for selected glycans and variations. Dot plots were constructed to ensure easy comparison. UL was excluded because response patterns were found to be closely similar to that of the UH area. Lines and error bars represent the median and inter-quartile range respectively. Red broken lines are set at the respective threshold values (i.e. 6.0 for both Ghanaian and EU CCD+ participants).

5.3 Discussion

Considering that IgG1 titres (5-12mg/ml) are higher in serum than IgE (0-0.002mg/ml) (Hamilton, 2001), coupled with the fact that no arm or component of the adaptive immune system functions in isolation, it would be erroneous to study glycan-associated cross-reactive IgE responses to glycans without determining signal response patterns of IgG1 in serum samples to these same glycans. Additionally, the advantage of using such a high through-put technique as the glycan microarray is the simultaneous assessment of many glycan-antibody interactions with minute volumes of serum (Reviewed by Park *et al.*, 2013).

Evidence provided in this work indicate a striking similarity in observed response patterns between IgG1 and IgE signals across the glycans in the area-stratified graphs. Even more interesting is the finding that like IgE, IgG1 reactivity is directed against the α3-fucose epitope in the R area, and against the α3-fucose and β2-xylose epitopes in the UL and UH areas. This trend is similarly observed for the selected European CCDpositives. Findings from recent studies suggest that IgE responses are characterized by increased levels of IgG1, among other antibodies (Akdis, 2009; Akdis and Akdis, 2011; Rispens *et al.,* 2013), although the mechanisms involved in the increase in these antibody levels, or their roles in allergy-associated immune responses remain unclear. Interestingly, higher IgG1/IgE correlation was observed for the R area than for the UL and UH areas, indicating comparatively stronger IgG1 to IgE activity in the R area. Taken together, it is clear that an association between IgE and IgG1 exists, and requires further scrutiny as far as type-1 hypersensitivity and cross-reactive IgE-associated immunity are concerned.

Apart from G111, all of the other glycans identified to be significantly associated with area are xylosylated N-glycans. Furthermore, findings in this work show higher IgG1 reactivity among *S. haematobium*-positive than *S. haematobium*-negative participants towards N-glycans with the β2-xylose core modification. Although evidence here indicate significantly higher responses among *S. haematobium*-positive participants towards only one of the N-glycans with a core β2-xylose, the likelihood of *S. haematobium* positivity driving observed responses particularly in the UL area remains strong. Using *S. mansoni*-infected murine models, Faveeuw *et al.* (2003) showed that egg-derived N-glycans with core α3-fucose and core β2-xylose elicited detectible titres of IgG1. Indeed, IgG1, according to immuno-epidemiological studies, has been associated with protective immunity to re-infection by *S. mansoni*, as has IgE (Khalife *et al.*, 1989). Furthermore, IgG1 reactivity patterns between our European CCD-positives and Ghanaian UH participants were found to be similar. This was anticipated, as the same glycan epitopes, namely α3-fucose and β2-xylose, were targets in IgG1 responses for both groups. This finding could be indicative of an inducing source that is present both among our European and Ghanaian urban participants, an area that would require further studies.

Essentially, the existence of an association between glycan-associated IgG1 and IgE antibodies is indicated here, which would require further research. Additionally, almost all glycans identified to be significantly associated with Area were xylosylated N-glycans to which higher responses were observed in the urban than in the rural areas.

Furthermore, observed similarities in response patterns between the Ghanaian UH and

European CCD-positive participants, is indicative of an inducing source that is common to both our European and Ghanaian urban participants. However, more extensive studies would be needed in order to draw firm conclusions.

CHAPTER 6:

ASSESSMENT OF GLYCAN-ASSOCIATED IgG4 RESPONSES

6.1 Introduction

Immunoglobulin 4 (IgG4) antibodies are well known to be beneficial during allergic or hypersensitivity reactions, although the mechanisms involved in their production by Bcells are yet to be elucidated (Davies *et al.*, 2013). For one, IgG4 has been found to be a non-precipitating antibody, so that even at high concentrations in double-diffusion assays, it doesn't precipitate antigens (Van der Zee *et al.*, 1986). Furthermore, evidence from experiments conducted in humans and mice involving grass and cat allergens have revealed IgG4 antibodies as capable of having the binding sites on their two Fab regions recognizing two different antigens or allergens, implying monovalency towards a single antigen (van der Neut Kolfschoten *et al.*, 2007). This in turn reduces the sizes of complexes formed between IgG4 and a particular allergen, hence inhibiting antigen presentation and inflammatory cell activation (Aalberse, 2011). Indeed, in a recent study aimed at investigating the role of IgG antibodies in the development of long-term tolerance to allergic rhinitis after therapy, it was discovered that close to half (38%) of the observed antibody inhibitory activity was due to IgG4, and this activity wttttttas maintained even when IgG4 antibody titres were reduced to near pre-treatment levels (James *et al.*, 2011).

Although our knowledge regarding the involvement of IgG4 in allergic responses has improved considerably in recent years, there still remain a number of issues to be resolved, one of which is how IgG4 antibodies respond to glycans against a backdrop of glycan-associated cross-reactive IgE responses. The major point of interest here would be whether

or not IgG4 responses are similar to, and associate well with observed glycanassociated IgE response patterns. This is because a good association would be suggestive of possible allergy-associated inhibitory activity involving IgG4 and other immunoregulatory factors.

In this part of the study, work was carried out to firstly, determine the existence of urbanrural patterns in anti-glycan IgG4 response signals; to secondly identify the glycans or glycan epitopes significantly associated with measured IgG4 responses from serum samples of selected participants in the urban high, urban low and rural areas, along with potential risk factors associated with the anti-glycan IgG4 responses recognized; to compare glycan-associated IgE responses with anti-glycan IgG4 responses both among this selection of Ghanaian and European participants; and to finally compare glycanassociated IgG4 responses between the Ghanaian participants and European CCDpositive participants. This work was conducted with the aid of a well-developed array of synthetic glycans.

6.2 Results

6.2.1 Urban-rural differences of measured IgG4 response patterns to synthetic glycans by glycan microarray

The mean of IgG4 fluorescence intensities for the 20 participants in each area was determined for each printed glycan on the array. A graphical representation of the measurements for all of the 128 glycans stratified by area is shown in Figure 6.1. Observation of the area-stratified graphs indicated generally lowered and non-specific signal intensity patterns across all three areas. Slight variations were however observed with the fucosylated N-glycans (Figure 6.1), where mean IgG4 response intensities were found to be higher in the R and UL areas, compared to the UH area. This was similarly observed for the α-gal epitopes on the array (Figure 6.1). Besides these, no clear areaassociated pattern could be discerned.

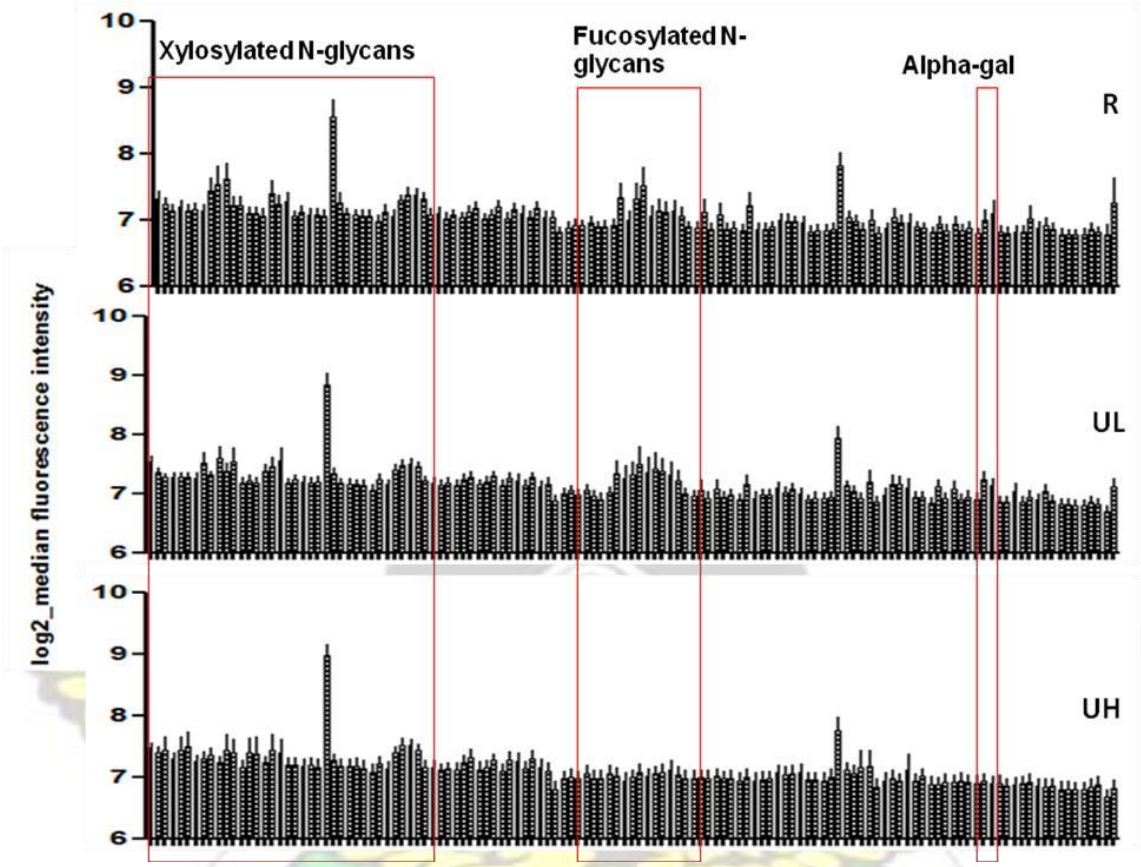


Figure 6.1: IgG4 Fluorescence intensity graphs of the 128 glycans stratified by Area. Using the log2 median fluorescence intensity (log2 MFI) data, bar graphs showing the signal intensities of the 128 glycans at a glance, were constructed for each area. Graphs were arranged as shown for easy comparison. The red boundaries indicate glycan groups on the microarray chip that are the focus of this study. Each bar represents the mean of the log2 MFIs for 20 participants for a particular glycan. The lines on top of each bar represent the standard error of mean (SEM).

6.2.2 Identification of glycans with significant, area-associated IgG4 signal intensities

For the IgG4 fluorescence intensity data three glycans showing significant difference between at least two areas were identified, following analyses with Mann Whitney U. These were α-gal-GlcNAc-BSA (UH/UL; $p = 0.041$), G112 (UH/UL; $p = 0.043$), and G120 (UH/R; $p = 0.04$). Both G112 and α-gal-GlcNAc-BSA are variations of the α-gal epitope present on the array (Figure 6.2). For all three glycans, median signal intensities were observed to be highest in the UL area, followed by the R and UH areas respectively (Figure 6.2).

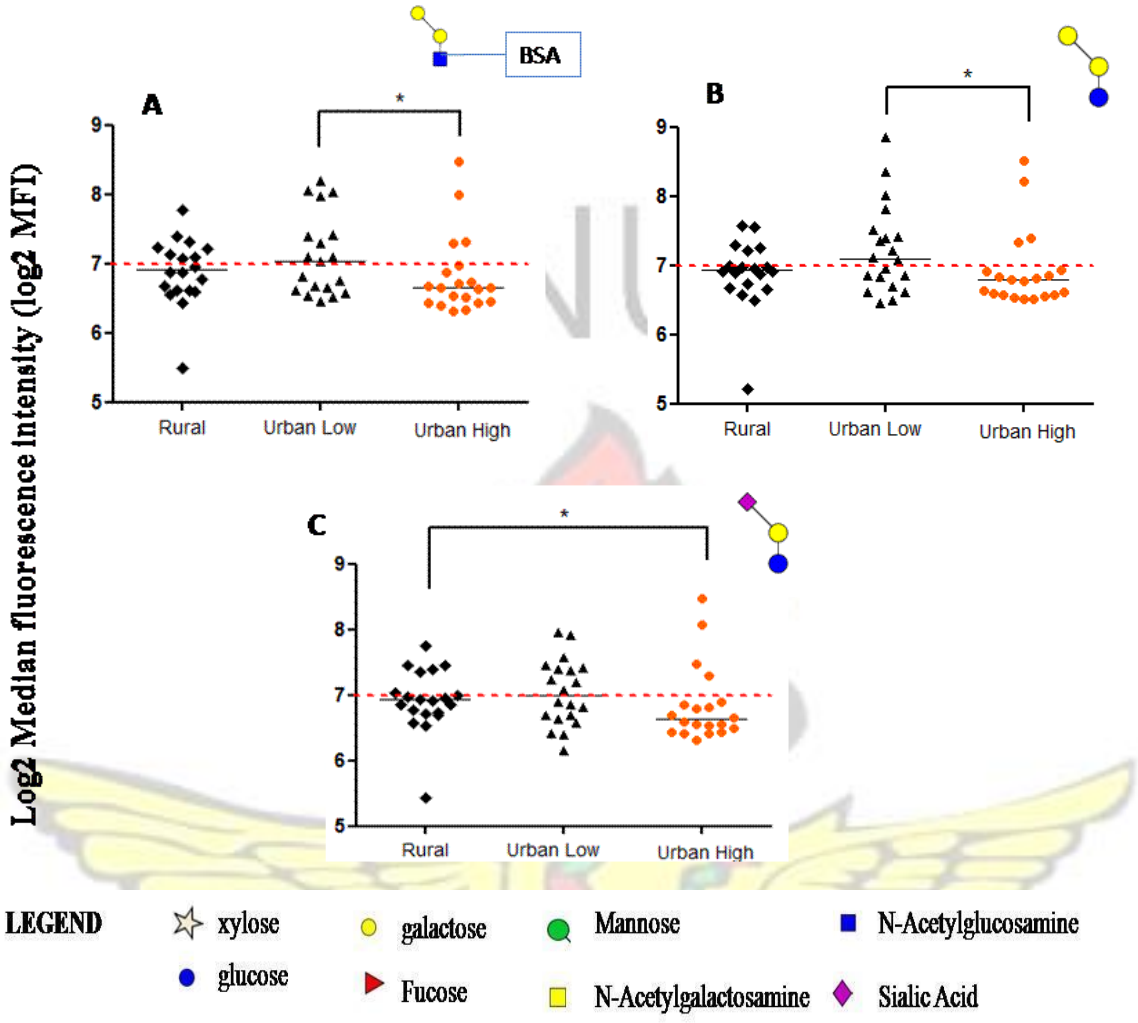


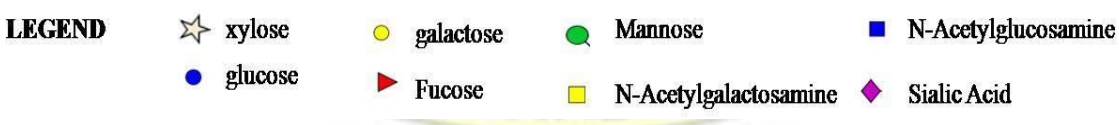
Figure 6.2: Glycans identified from the IgG4 fluorescence intensity dataset to exhibit significant differences in signal intensities in at least two Areas. Determining which glycans were significantly associated with Area was done using the Mann Whitney U test to compare between two areas at a time for all the 128 printed saccharides. The identified glycans are: **(a)** α-gal-GlcNAc-BSA; **(b)** G112; and **(c)** G120. The single asterisk (*) represents p values less than 0.05. The red broken line is set at the threshold value which was determined by calculating the average of the median intensities for all the 128 glycans (i.e. 7).

6.2.3 Associations between potential influencing factors and identified glycans from IgG4 fluorescence datasets

Analyses were conducted to identify factors which may potentially influence IgG4 responses to each identified glycan. Hence, IgG4 response intensities to identified glycans were assessed for strength of association with such factors as gender, age, *N. americanus* infection status, infection status for any helminth, and malaria positivity (Table 6.1). Gender was not significantly associated with any of the identified glycans, neither was age, *N. americanus* infection status, nor infection status for any helminth. Malaria positivity was however found to be significantly associated with G120($p = 0.013$), an oligosaccharide comprising a sialic acid unit attached via an α-(1,4) linkage to a galactose unit, which in turn is attached via a β-(1,4) link to a glucose unit.

Table 6.1: Associations between identified glycans for IgG4 response intensities and potential influencing factors

	G120	G112(10a)	α-gal-GlcNAc-BSA
			
Factor	**median (range)**	**median (range)**	**median (range)**
Gender			
Males	6.86 (5.43-8.47)	6.91 (5.22-9.22)	6.88 (5.49-8.47)
Females	6.82 (6.31-8.07)	6.86 (6.46-8.85)	6.74 (6.33-14.3)
p-value	0.717	0.819	0.976
Age			
< 11 yrs	6.90 (6.42-7.74)	6.94 (6.46-8.35)	6.93 (6.43-14.30)
≥11 years	6.72 (5.43-8.47)	6.85 (5.22-9.22)	6.68 (5.49-8.47)
p-value	0.093	0.302	0.073
Hookworm			
positive	6.91 (5.43-7.39)	6.98 (5.22-9.22)	6.95 (5.49-7.78)
negative	6.85 (6.16-8.47)	6.88 (6.46-8.85)	6.74 (6.33-14.25)
p-value	0.805	0.459	0.63
Any helminth			
positive	6.79 (5.43-7.39)	6.96 (5.22-9.22)	6.87 (5.49-8.04)
negative	6.85 (6.16-8.47)	6.87 (6.46-8.85)	6.74 (6.31-14.25)
p-value	0.341	0.763	0.862
Malaria			
positive	7.02 (6.72-7.91)	7.12 (6.56-9.22)	7.08 (6.43-7.97)
negative	6.79 (5.43-8.47)	6.86 (5.22-8.85)	6.73 (5.49-14.25)
p-value	**0.013**	0.084	0.381



Association between each glycan and potential factor was determined using the Mann Whitney U test, and effect of interaction was considered significant at $p < 0.05$ (Significant associations are in boldface). Any Helminth** comprises *S. haematobium*, *S. mansoni*, *A. lumbricoides*, *Trichuris trichiura*, and *Necator americanus*. IQR = Interquartile range.

6.2.4 Area-stratified correlations between IgG4 and IgE

Additionally, correlation graphs stratified by area were generated with the aim of exploring the strength of relationship between total IgE and IgG4 response signals (Figure 6.3). It was realized that correlation was generally weak in all three areas, indicating less IgG4 to IgE activity. This notwithstanding, correlation between IgG4 and IgE was observed to be strongest in the R Area ($r = 0.46$; $p < 0.001$), with the UH and UL Areas recording correlations of $r = 0.11$ ($p < 0.001$) and $r = 0.10$ ($p < 0.001$) respectively (Figure 6.3).

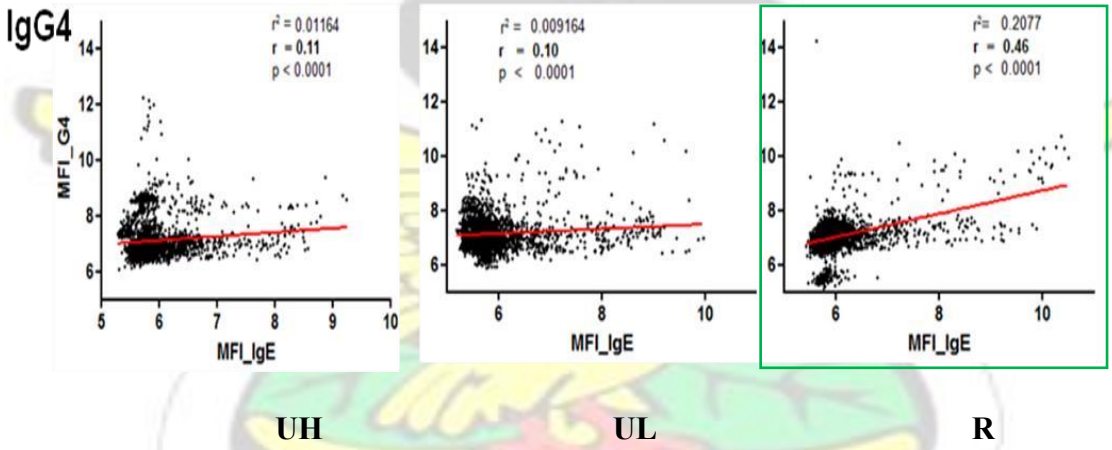
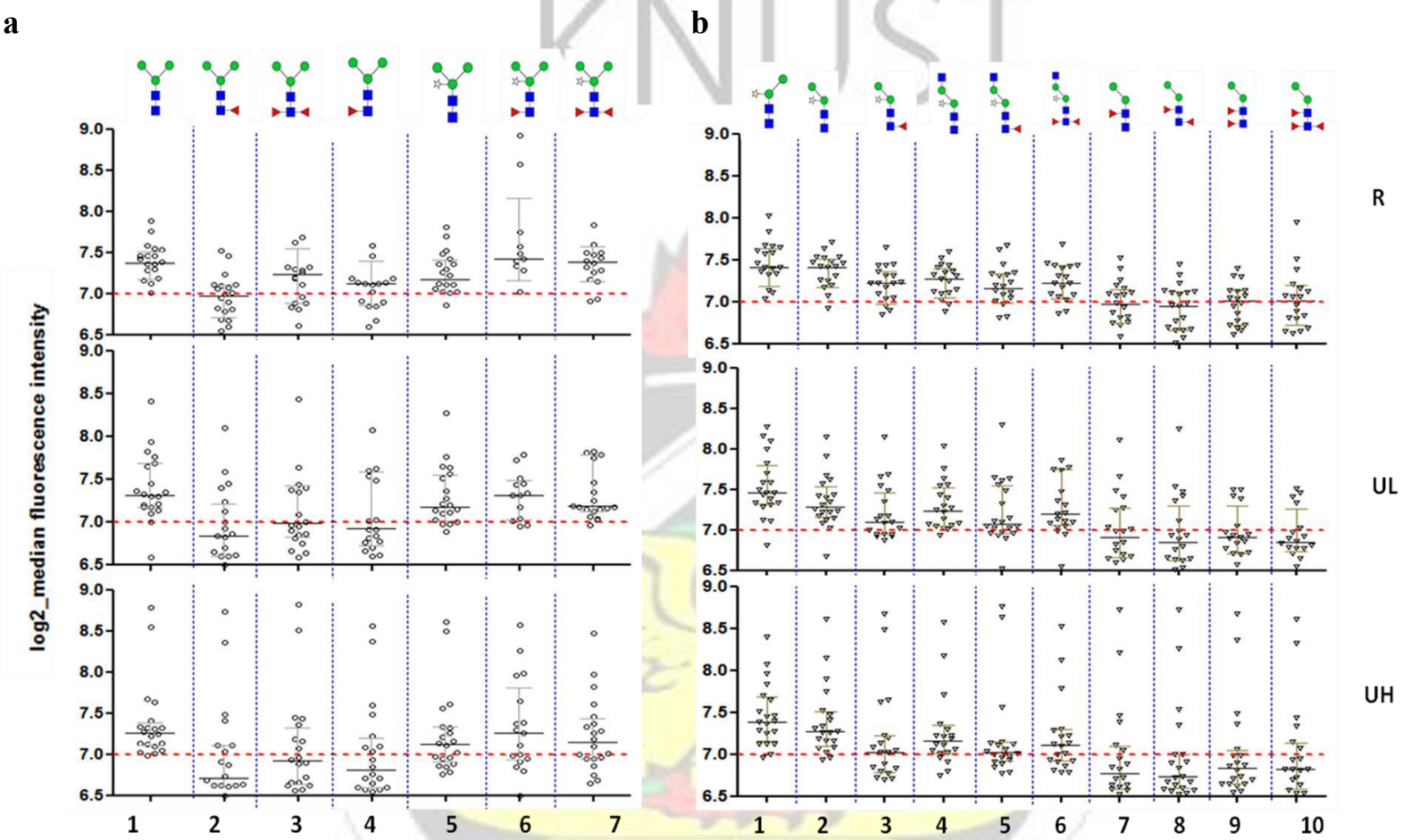


Figure 6.3: Area-associated correlations between IgG4 and IgE. Pearson's correlation coefficient (r) was used to determine the strength of correlation between IgG4 and IgE fluorescence intensities in each area (Red line = linear regression line/best fit line at total).Stronger IgG4/E correlations were observed for the R Area (Green square boundary) than for the UH and UL Areas.

6.2.5 Urban-rural differences in IgG4 response patterns to selected glycans and their variations

Area-stratified dot plots of IgG4-associated responses towards identified glycans and their variations were constructed and scrutinized for clues to glycan epitopes most likely influencing responses in each area (Figure 6.4). Variations of the glycans of interest were selected from among the printed glycans available. Interestingly, IgG4 responses were directed against both the α3-fucose and β2-xylose epitopes in the R area. This was similarly realized in the UL and UH areas, thus indicating a similarity in response patterns for all three areas (Figure 6.4a-b). For the variations of α-gal, namely G112, G113, and α-galGlcNAc-BSA (Figure 6.4c (1-3)), median signal intensities were observed to be higher in the UL area than in the R and UH areas. Median IgG4 signal intensities directed against the sialic acid (Sia-gal) (Figure 6.4c (4)), and G111 (Figure 6.4c (7)) were below threshold levels, although higher responses were observed again in the UL and UH areas.


a
b
log2_median fluorescence intensity
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UL
UH

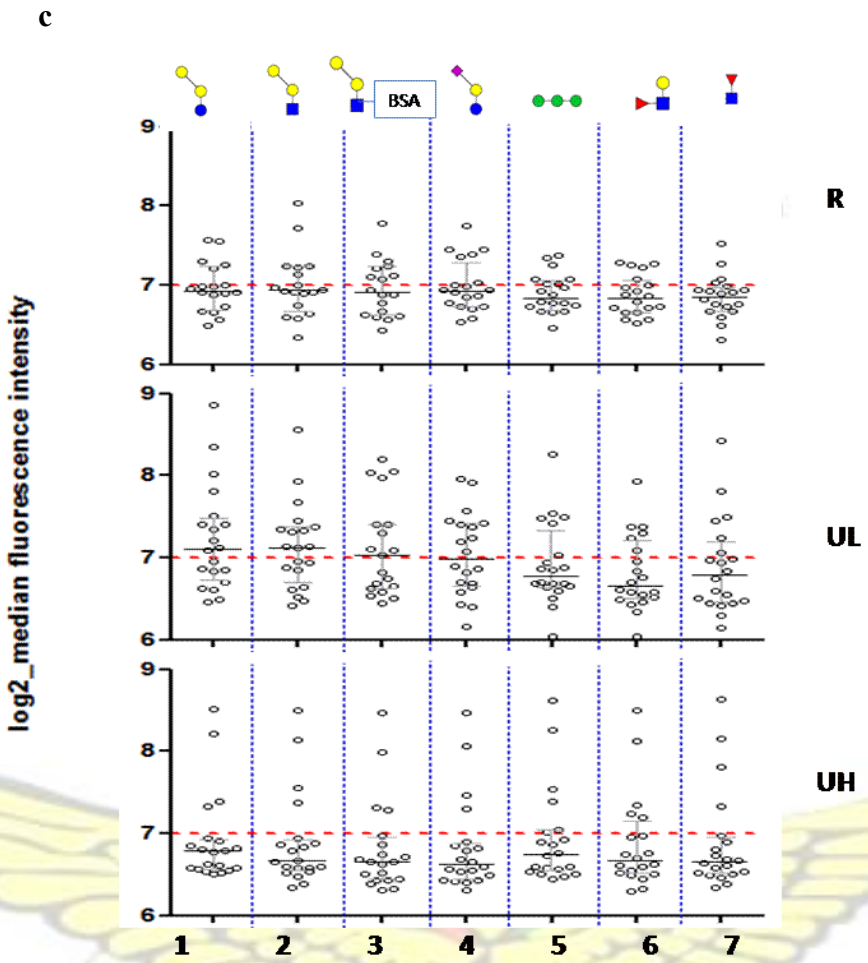

Figure 6.4: Identifying urban-rural patterns in anti-glycan IgG4 responses for selected glycans and their variations. Urban-rural patterns in median IgG4 fluorescence signals to the selected glycans G112(c1), α-gal-GlcNAc (70) (c2), and Sia-Gal (c4) with their variations on the microarray were determined using dot plots. Plots were stratified by area for easy comparison. Lines and error bars represent the median and inter-quartile range respectively. Red broken line is set at the threshold value (i.e. 7.0)

6.2.6 Association of measured IgG4 signal intensities to core-modified N-glycans with *S. haematobium* and *N. americanus* infection statuses

Further analyses were conducted to determine for participants with current helminth infections, whether IgG4 signal intensities were directed at particular epitopes on coremodified N-glycans (Figures 6.5 and 6.6). For participants with current *N. americanus* infection, no differences in median IgG4 fluorescence intensities were apparent between *N. americanus*-positive and negative participants towards the α3-fucosylated N-glycans (Figure 6.5a-d). Additionally, comparison of median IgG4 signal intensities between current *N. americanus* positive participants and *N. americanus* negative participants towards N-glycans with only xylose core-modifications yielded no significant differences (Figure 6.5e-g).

Similarly, no differences in median IgG4 signal intensities were observed for both *S. haematobium* positive and negative participants towards N-glycans with α3-fucose (Figure 6.6a-d). This was also observed for median IgG4 signal intensities among current *S. haematobium* positive and negative participants towards N-glycans with only β2xylose (Figure 6.6e-g). The absence of differences between helminth positive and negative subjects for the glycans studied could be reflective of the similar area-associated fluorescence patterns observed in all three areas (Figure 6.4).

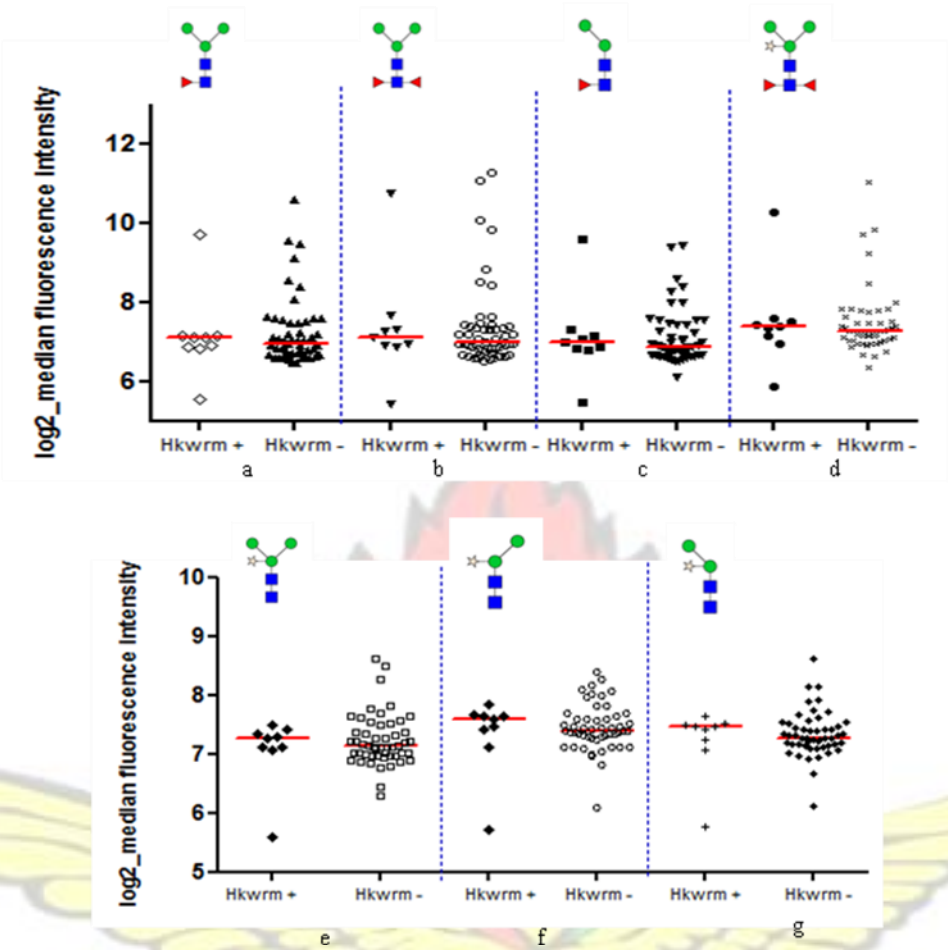

Figure 6.5: Comparing median IgG4 signal intensities between *N. americanus*positive and negative participants for a selection of core-modified N-glycans. The Mann Whitney U test was used to compare between *N. americanus*-positive and negative participants for each glycan. Glycans on the array representing the core modifications being studied are used. **(a)** G73 has an α3-fucose core modification, **(b)** core modifications on G75 comprise α3- and α6-fucoses, **(c)** G72 also possesses a single α3fucose, **(d)** G39 has both α3- and α6-difucosylations and a β2-xylose, while **(e)** G34, **(f)** G1, and **(g)** G2 possess only β2-xylose. The red lines are representative of the group medians. Asterisk (*) is indicative of comparisons with $p < 0.05$.

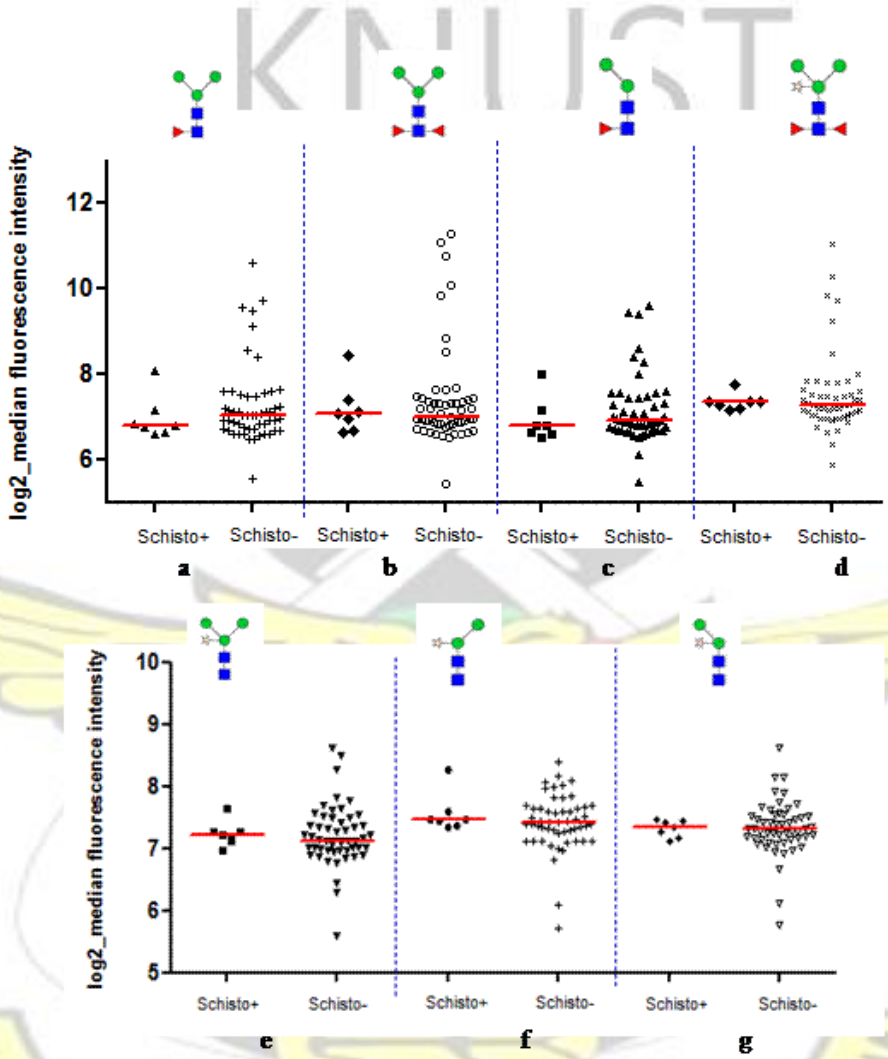

Figure 6.6: Comparing median IgG4 signal intensities between *S. haematobium*positive and negative participants for a selection of core-modified N-glycans. The Mann Whitney U test was used to compare between *S. haematobium*-positive and negative participants for each glycan. Glycans on the array representing the core modifications being studied are used. **(a)** G73 has an α3-fucose core modification, **(b)** core modifications on G75 comprise α3- and α6-fucoses, **(c)** G72 also possesses a single α3-fucose, **(d)** G39 has both α3- and α6-difucosylations and a β2-xylose, while **(e)** G34, **(f)** G1, and **(g)** G2 possess only β2-xylose. The red lines are representative of the group medians. Asterisk (*) is indicative of comparisons with $p < 0.05$.

6.2.7 Differences in glycan-associated IgG4 response patterns for European CCDpositive participants

Glycan-associated IgG4 signal intensities were also studied in bar graph (Figure 6.7) and scatter dot plot (Figure 6.8) formats for the five selected European CCD-positive participants. Regarding the scatter dot plots, selections of glycans similar to those picked for the Ghanaian participants (Figure 6.2), along with selected variations from the array, were used. Generally, median signal intensities were much lower for the European participants than for this selection of Ghanaian subjects, and bordered around the set threshold (Figure 6.7; Figure 6.8a-b). Nevertheless, slightly higher median intensities were observed towards N-glycans with both α3-fucose and β2-xylose core modifications. IgG4 signal intensities were even lower as far as the variations of α-gal (Figure 6.8c (13)); 3' G120 (Figure 6.8c (4)); G99 (Figure 6.8c (5)); G103 (Figure 6.8c (6)); and G111 (Figure 6.8c (7)) were concerned. Measured median responses were below the set threshold for most of the glycans, which may be indicative of probable IgG4 inactivity among the European CCD-positives towards the α3-fucose and/or β2-xylose coremodified N-glycans, and α-gal.

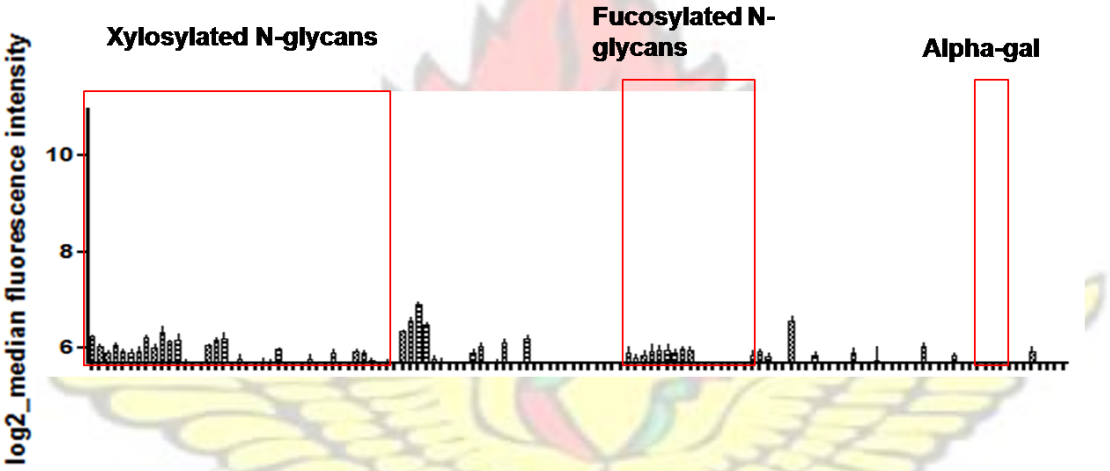

Figure 6.7: IgG4 fluorescence intensity graph of the 128 glycans for the European CCD-positives. The bar graph was constructed, using the log2 median fluorescence intensity (log2 MFI) data, and shows the signal intensities of the 128 glycans at a glance. The red boundaries indicate glycan groups on the microarray chip that are the focus of this study. Each bar represents the mean of the log2 MFIs for 5 participants for a particular glycan. The lines on top of each bar represent the standard error of mean (SEM).

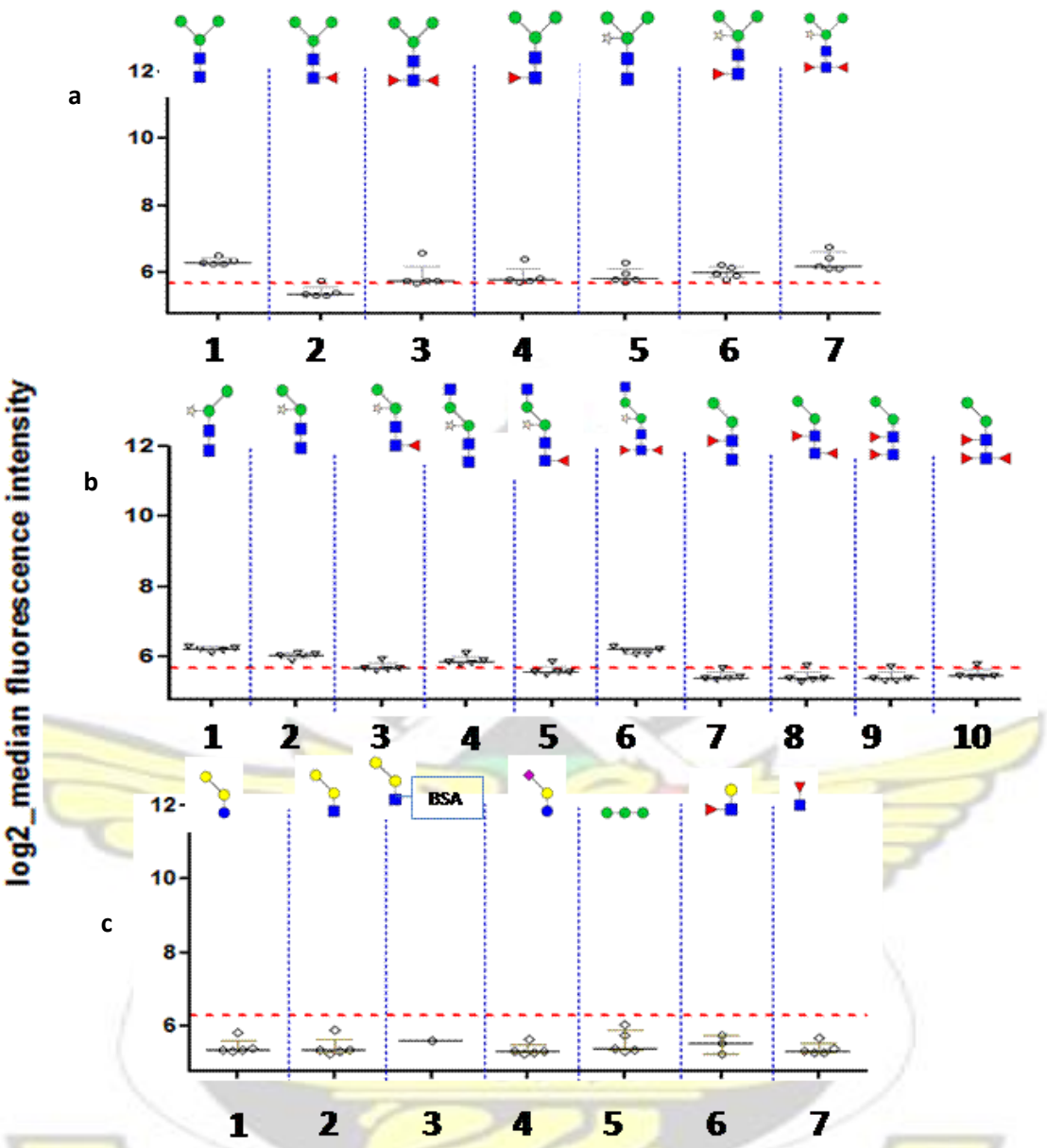

Figure 6.8: Identifying patterns in anti-glycan IgG4 responses for EU CCD+ participants. Using the same set of glycans and their variations as used for the Ghanaian selection, dot plots were drawn to determine epitopes being recognized by IgG4 for our selection of European allergics. Lines and error bars represent the median and interquartile range respectively. Red broken line is set at the threshold value (i.e. 5.7 for IgG4).

6.2.8 Comparing Ghanaian and European CCD-positive (EU CCD+) subjects in IgG4 response patterns to selected glycans and their variations

Also with dot plots, comparisons involving glycan-associated IgG4 responses were made between both UH and R Ghanaian participants, and those of EU CCD+ participants. Here as well, the exclusion of the Ghanaian UL participants was because observed glycan response patterns for this group were similar to those for the UH group. It was clear with the IgG4 responses however, that response patterns were similar for all three groups under scrutiny, and these were particularly lower for the EU CCD+ participants (Figure 6.9). Thus, whilst for the European CCD-positives, signal intensities slightly above threshold values could be observed for N-glycans with both α3-fucose and β2-xylose core modifications, much higher responses were realized among our Ghanaian UH and R participants towards N-glycans with either α3-fucose or β2-xylose, or both core modifications (Figure 6.9).

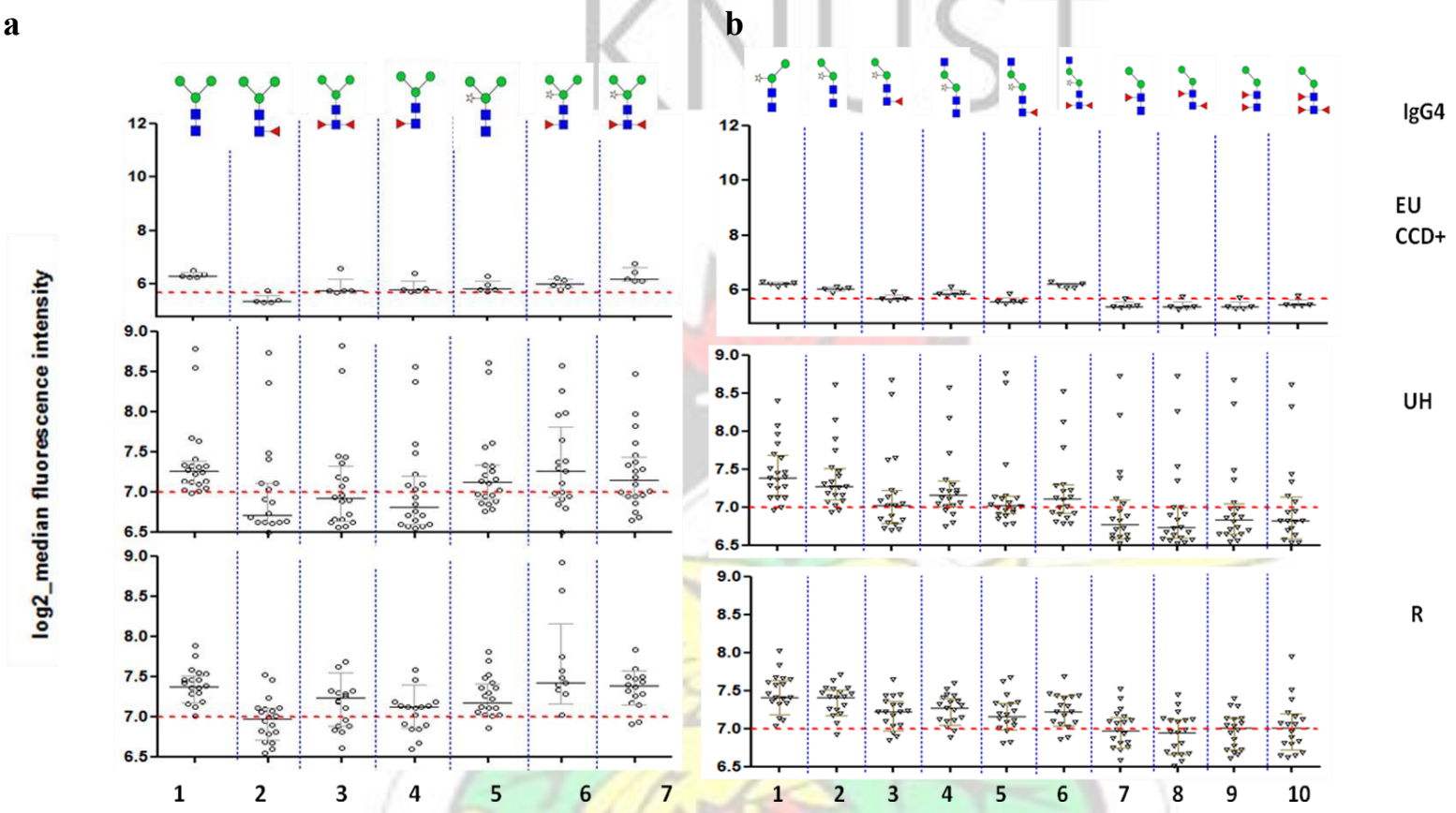

Figure 6.9: Comparing European CCD-positive (EU CCD+) anti-glycan IgG4 responses to Ghanaian urban-rural antiglycan IgG4 responses for selected glycans and variations. Dot plots were constructed to ensure easy comparison. UL was excluded because response patterns were found to be closely similar to that of the UH area. Lines and error bars represent the median and inter-quartile range respectively. Red broken lines are set at the respective threshold values (i.e. 7.0 and 5.8 for Ghanaian and EU CCD+ participants respectively).

6.3 Discussion

In this part of the study, area-associated patterns with regard to IgG4 responses to glycans were examined, and related to observations and findings with previouslyrealized glycan-associated IgG1 and IgE patterns. First of all, it was seen with regard to the Area-stratified fluorescence intensities and patterns for the 128 glycans that signal intensities for the three areas were similar. This was unexpected, as it was contrary to the speculation at the commencement of this work that, glycan-associated

IgG4 signal intensities would be stronger in the R Area, followed with the UL, and UH Areas respectively. This hypothesis was arrived at subsequent to indications from literature that, observed prevalences of helminth infections in endemic communities correlated positively with increased immuno-regulatory activity, characterized among others by increased levels of IgG4 (Garcia *et al.*, 1992; de Moira *et al.*, 2013b). Again, in contradiction to expectations, no difference in median IgG4 signal intensities was observed between either the *N. americanus*-positive/negative subjects or the *S. haematobium* positive/negative subjects towards the xylosylated and fucosylated structures. This study however did observe higher glycan-associated IgG4 to IgE correlation in the R area compared to the urban areas, indicating higher IgG4 activity in the R area. Indeed, findings from a number of studies have suggested the non-complex forming and non-precipitating capacities of IgG4 with antigen to be important in the development of immune tolerance, or the limiting of IgE-associated morbidity, both in allergic and helminth reactions (van den Biggelaar *et al.*, 2000; Akdis and Akdis, 2010; Aalberse, 2011; Fitzsimmons *et al.*, 2012; review by van

Diepen *et al.*, 2012; de Moria *et al.*, 2013b).

Secondly, evidence from dot plots indicated signal responses in the UH Area to be directed at the β2-xylose epitope, whilst responses in the UL and R Areas were directed at both the α3-fucose and β2-xylose epitopes of core-modified N-glycans. This was in contrast to urban-rural observations of IgE and IgG1 response patterns. Van Ree and Aalberse (1995) demonstrated, using subjects with high IgE titres to the glycosylated grass pollen allergen *Lol p* (*Lolium perenne*) X1, that IgG4 antibodies could recognize the glycan epitopes on the allergen, which comprised among other monosaccharides, fucose and xylose (van Ree *et al.*, 1995). Whether or not this recognition translates into blocking activity remains to be elucidated. In a separate study measuring antibody responses to various farm allergens (Stern *et al.*, 2006), IgG4 expression was discovered to be closely linked to the expression of IgG1, and was also associated with increased Th2-associated events. Evidence from findings elsewhere also suggests that IgG-associated blocking activity may not be restricted to
IgG4 alone, but may possibly involve other IgG subclasses (Ejrnaes *et al.*, 2004). Taken together, further studies are essential to facilitate our understanding on the mediatory mechanisms involving IgG4 in allergen- and glycan-associated immune responses.

For the European CCD-positive participants, IgG4 signal responses were at threshold levels for most of the glycans on the microarray, thus precluding the discernment of a response pattern for this group. This was surprising, considering that measurable IgG4 titres to complex N-glycan moieties were previously detected in a separate study in sera obtained from European patients undergoing grass pollen (*Lol p* 1) immunotherapy (van Ree and Aalberse, 1995). Indeed, differences in testing techniques, namely the glycan microarray and the radioallergosorbent test (RAST), along with the different European subject groups, and the nature of presentation of the glycan moieties on the microarray and RAST chips are likely to influence differences in observed results.

From the analysis carried out, two of the three glycans found in terms of IgG4 response signals to be significantly associated with area were the α-gal epitopes, namely G112 and α-gal-GlcNAc-BSA. In both cases, IgG4 signal intensities were higher in the R than in the urban areas (although at threshold levels), a trend similarly observed for α-gal-specific IgE. This could be indicative of the possible role of IgG4 in the development of α-gal-associated immune tolerance among this selection of participants. Indeed raised titres of IgG to α-gal in humans has been reported, the cause of which is perceived to be the continuous antigenic stimulation by the expression of these oligosaccharides by normal gastro-intestinal bacteria (Galili *et al.*, 1988; Commins, 2015); and elevated levels of α-gal-associated IgG4 responses have been found to characterize IgE anti-α-gal titres (Rispens *et al.*, 2013). However, while some studies have associated IgG4 with anti-inflammatory activity in allergic disease (Nouri-Aria *et al.*, 2004; Jin *et al.*, 2008; reviewed by Akdis and Akdis, 2011; James *et al.*, 2011), others have described IgG4 activity in allergic disease as unclear and complex at best (Stern *et al.*, 2006; reviewed by Mutius, 2007).

In summary, this aspect of the study indicates via correlation graphs, slightly higher IgG4 activity in the R area, as compared to the UL and UH areas, although from areastratified bar graphs, lowered responses with no discernible patterns are exhibited. From the dot plots, higher IgG4 reactivity to the β2-xylose and α3-fucose epitopes in the UH, UL and R areas were observed. Additionally, the diminished glycanassociated IgG4 responses observed for the European CCD-positives raises important questions as to the ontogeny of immune tolerance during allergen-specific immunotherapy (SIT).

CHAPTER 7:

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 General Discussion

This study so far, is the first of its kind that seeks firstly to characterize based on area, observed glycan-associated IgE, IgG1, and IgG4 responses among a selection of Ghanaian participants, using a synthetic glycan microarray; and to secondly identify potential influencing factors to the observed patterns. First, this study provides adequate evidence suggesting the presence of cross-reactive IgE in our Ghanaian population, particularly in the rural communities. For one, a clear area-associated trend with glycan-associated IgE responses were realized, in which higher response signals were observed for the R area than for the UL and UH areas respectively. Also, this work indicated strong correlation between IgE signal intensities to a selection of N-glycans with α3-fucose and β2-xylose and ImmunoCAP-measured IgE to bromelain. Furthermore, a clear urban-rural trend with respect to IgE responses to αgal whereby higher response signals were realized in the R area, followed by the UL and UH areas respectively was shown. Combined with significantly higher prevalences of helminth infections and malaria in the rural area, it is clear especially in the rural populations, that allergen-specific IgEs with low biological activity is present. Indeed, in a related larger study population, a tight correlation was observed between ImmunoCAP-measured peanut-specific IgE and bromelain-specific IgE in the serum samples of the study subjects (Amoah *et al.*, 2013). Also in the same study, 12 subjects were discovered to have ImmunoCAP-measured IgE titres equal to or greater than 15kU/L, a cut-off reported to have a 95% predictive value for clinical peanut allergy in a study population in Europe (Roberts and Lack, 2005; Amoah et al., 2013). These participants however did not exhibit any symptoms or clinical histories of allergy. In

two surveys conducted 10 years apart in Kumasi, the second largest metropolis in Ghana, Addo-Yobo *et al.* (2007) indicated a higher prevalence of exercise-induced bronchospasm (EIB) among urban affluent pupils as compared to urban poor and rural pupils respectively.

Contrary to expectations for this study, higher prevalences of SPT positivity to cockroach and house dust mite were observed in the rural than the urban areas. Obeng *et al.* (2014) made similar observations in a related study in urban and rural communities in the Greater Accra Region, although evidence from other studies (Ng'ang'a *et al.*, 1998; Addo-Yobo *et al.*, 2007; Stevens *et al.*, 2011) suggested otherwise. Observations made in this thesis certainly indicate that immunomechanisms associated with sensitization by skin prick tests may be more complex than initially realized. Indeed, it has been acknowledged elsewhere (Obeng *et al.*, 2014) that measured allergen-specific immunoglobulin levels are the strongest predictors of atopy.

Immunoglobulin (Ig) E, IgG1, and IgG4 responses to glycans with respect to area were also characterized for both our Ghanaian and European participants. For IgE and IgG1, similar area-associated trends were observed whereby the α3-fucose and β2xylose moieties of core-modified N-glycans induced elevated IgE and IgG1 reactivity in the UH and UL areas, and the α3-fucose primarily induced elevated reactivities of both antibodies in the R area. Conversely, elevated IgG4 reactivity was directed at the α3-fucose and β2-xylose epitopes in the R and UL areas, whilst directed at the β2xylose in the UH area. Similar IgE and IgG1 reactivity for our selection of European participants, which were directed against the α3-fucose and β2-xylose epitopes of Nglycan structures were also observed. Immunoglobulin (Ig) G4 responses were however not discernible, as median response signals were largely at or below set thresholds. One key observation

made was the striking similarity between IgE- and IgG1-associated responses in terms of the glycan moieties being recognized per area, and the similarities in signal intensity patterns across the 128 glycans for each area. There is accumulating evidence showing elevated IgE titres to be characterized by increased levels of IgG1 both in schistosomiasis (Faveeuw *et al.*, 2003; Khalife *et al.*, 1989; reviewed by van Diepen *et al.*, 2012) and in allergies (reviewed by Akdis, 2009; Rispens *et al.*, 2013). Indeed, a study involving 15 patients who had allergic rhinitis and/or asthma and were allergic to house dust mite, indicated after 70 days of treatment, a significant increase in IgG1 antibodies, which characterized modest increases in IgE titres (Jutel *et al.* (2003),). Observed IgG4 response intensities and patterns were contrary to anticipations. For the European CCD-positives as well, signal intensities were low, and barely exceeded set thresholds. It is unclear why this is the case especially for the Ghanaian participants involved in this study, considering that numerous immuno-epidemiological studies conducted in low- to middle-income countries associate chronic helminth infection with the development of immunoregulatory mechanisms characterized by elevated levels of IgG4 (Fitzsimmons *et al.*, 2012; De Moira *et al.*, 2013a, 2013b). Additionally, findings from various studies on allergic diseases have associated heightened serum titres of IgG4 with increased tolerance to the allergen in question (James *et al.*, 2011; Sommanus *et al.*, 2014; reviewed by Akdis and Akdis, 2011). In some studies however, this association was not clear (Stern *et al.*, 2006). Taken together, findings from this work show a clear need to research further to more fully understand the IgG1 and IgG4-associated mechanisms in allergic reactions, or in allergen-specific IgE sensitizations.

Furthermore, evidence presented in this study indicates significant associations between elevated IgE signal intensities and *S. haematobium* positivity with regard to β2-

xylosylated N-glycans. Some association, albeit not as compelling, was observed between elevated IgE signal intensities and *N. americanus* positivity towards α3fucosylated structures. Indeed, a higher prevalence of *S. haematobium* infection in the two urban areas than in the R area was observed, whilst a higher prevalence of *N. americanus* infection was realized in the R area than in the urban areas. Coupled with the targeted reactivity of IgE towards β2-xylose and α3-fucose in the UH and UL areas, and towards α3-fucose in the UH areas, it is certainly clear that an association worthy of further scrutiny exists. This is interesting as these evidences suggest potential factors driving observed urban-rural IgE response patterns. Indeed, it is established via glycomics-based research that the cercarium and egg stages of the

Schistosome is enriched with N-glycans core modified with β2-xylose and α3-fucose (Hokke *et al.*, 2007a; Meevissen *et al.*, 2012; reviewed by Meevissen *et al.*, 2012), and structural studies of the adult stage of *Haemonchus contortus* have revealed Nglycans saturated with α3-fucoses (Haslam *et al.*, 1996; reviewed by Dell *et al.*, 1999). Elevated IgG1 signal intensities were also associated with *S. haematobium* positivity towards β2-xylosylated N-glycans, while IgG4 signal intensities did not exhibit any association with either *S. haematobium* or *N. americanus* positivity towards either β2-xylose or α3-fucose. Considering that this study is the first to characterize IgG1 and IgG4 responses to printed synthetic glycans, it would be essential that these trends are confirmed in future studies.

A likely concern to be raised regarding this study is the fact that the glycan work was performed with printed synthetic oligosaccharides, hence raising questions concerning the similarity of these epitopes to their natural counterparts, as well as the validity of the results elicited. It is however possible to trust the validity of the microarray results obtained, as evidenced by the similarities in observed urban-rural data obtained from

the ImmunoCAP and glycan microarray techniques; and by the strong association observed between IgE signal intensities to G113 and IgE titres to ImmunoCAPmeasured α-gal. It must be noted that natural counterparts of these glycans are bigger structures with extensions on the branches, making IgE recognition of the epitope of interest even less likely. As this is a novel technique, further studies would be conducted to strengthen validity of findings.

7.2 Conclusions and Recommendations

Altogether, enough evidence is provided in this study validating the presence of crossreactive IgE in the Ghanaian population. Furthermore, this work has explained urbanrural patterns in IgE, IgG1, and IgG4 responses to printed glycans, and has enabled the prediction of likely factors associated with observed area-associated antibody response patterns to these glycans. Additionally, it can be seen from this study that observed IgE and IgG1 response patterns among the European and Ghanaian urban high participants are similar. It would however be interesting to see whether or not these findings are confirmed in urban-rural studies involving larger sample sizes. Additionally, determining the glycomes of such insects as the female *Anopheles* mosquito, and the cockroach (*Blatella germanica*/*Periplaneta americana*); as well as of such helminths as the hookworm (*Necator americanus*), could facilitate the search for sources of allergen-associated IgE and IgG cross-reactivity. Furthermore, more comprehensive immuno-epidemiological studies involving such techniques as the glycan microarray and ImmunoCAP assays, along with flow cytometry to study immune cell surface markers; and sandwich antibody ELISAs involving glycan antigens, would prove essential in understanding further, the immune environment associated with such cross-reactive IgEs.

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APPENDIX I: PREPARED MANUSCRIPT I FOR PUBLICATION

TITLE: Cross-Reactive IgE Responses Are Directed Mainly against the□-3 FucoseGlycan Epitope Among Ghanaian School children

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Abstract

Background: Population studies from helminth-endemic areas of the world have often observed elevated levels of allergen-specific serum IgE that does not translate into symptoms. Recent studies have shown that cross-reactive IgE recognizing Nglycan epitopes may largely account for these elevated levels.

Objective: To determine the N-glycan structures associated with IgE cross-reactivity in rural and urban children in Ghana using a synthetic glycan microarray

Methods: Stored sera samples from a subset ofchildren (n=60) enrolled in a larger cross-sectional survey conducted among Ghanaian schoolchildren were used to assessIgE responses against 128 synthetic glycan structures using a glycan microarray. Selected children attended a rural school (n=20), an urban low socioeconomic status (SES) school (n=20) and an urban high SES school. IgE against bromelain, house dust mite extract, cockroach extract, peanut extract and the oligosaccharide galactose-□-1,3-galactose (□-gal) were measured by ImmunoCAP.Data were also collected on demographic factors and parasitic infections.

Results:

Elevated levels of IgE to glycan structures were observed among rural children followed by urban low SES and urban high SES children. IgE responses were predominantly directed against structures with α-(1,3) fucose and not xylose. These responses were not

associated with current *S. haematobium* infection. Elevated levels of IgE to □-gal measured by ImmunoCAP as well as to two □-gal epitopes on the microarray were observed among rural children. IgE to the □-gal epitopes were not associated with helminth infection.

Conclusions: Elevated levels of anti-carbohydrate IgE may be directed at the α-(1,3) fucose epitope among rural and urban Ghanaian children.

Key Messages

Elevated levels of cross-reactive IgE may be directed mainly at glycan structures containing α-(1,3) fucose epitope and not core xylose among children living in helminth endemic countries

Glycan microarray technology presents a novel approach to characterize antibody responses to glycan structures in population studies

Capsule Summary: Synthetic glycan microarray technology presents a novel tool to characterize anti-glycan antibody responses and may have implications for clinical outcomes among allergic patients.

Key Words

Immunoglobulin E, Sub-Saharan Africa, IgE cross-reactivity, cross-reactive carbohydrate determinants, helminth infections, EuroPrevall, urban-rural, glycan microarray,

Abbreviations

CCD : Cross-reactive carbohydrate determinant

SEA: Soluble Egg Antigen

Introduction

Research indicates that in areas of the world where parasitic worm (helminth) infections are endemic, elevated levels of specific immunoglobulin (Ig) E to allergens are often observed that do not translate into allergic symptoms.[1]Cross-reactivity between helminth antigens and allergens is thought to explain these observations with IgE antibodies elicited to helminth antigen epitopes recognizing similar epitopes in other homologous molecules.[2]For example, individuals with elevated levels of IgE against *Ascaris lumbricoides* tropomyosin have been observed to have elevated levels of IgE to cockroach tropomyosin without symptoms of cockroach allergy.[3]

Studies have linked carbohydrates in glycoproteins known as cross-reactive carbohydrate determinants (CCDs) with IgE cross-reactivity. CCDs are the asparagine-linked carbohydrate components of plants and insect glycoproteins.[4] Currently, two distinctive non-mammalian substitutions to N-glycans of plant glycoproteins have been well-characterized. These are an α-1,3-linked fucose on the proximal N-acetyl glucosamine and a β-1,2-linked xylose on the core xylose.[5]N-glycans with □-fucose and □-2 xylose or with □-3 and □-6 fucose core modifications have also been identifiedin the egg stage of the helminth *Schistosoma mansoni*.[6, 7]An investigation we conducted in Ghana, West Africa demonstrated how IgE cross-reactivity between schistosome antigens and allergens may affect peanut-specific IgE sensitization. In that study, 90.9% of children with peanut-specific IgE sensitization (≥ 0.35 kU /L) were either peanut skin prick test negative or reported no symptoms.[8] In addition, infection with the helminth *S. haematobium* was strongly associated with peanut-specific IgE sensitization. Inhibition assays further demonstrated that both the CCD marker bromelain and *S. haematobium* soluble crude egg antigen were able to inhibitpeanut-specific IgE binding to peanut extract by more than 80%.[8]

In recent years, glycan microarray approaches have been used to investigate the antibody response profiles against helminth-derived glycan elements.[9] In addition, glycotechnology has allowed the synthesis of core N-glycan structural variants as well as non-xylosylated glycans on a microarray to further characterize IgE binding to glycan epitopes.[10]

Given that previous studies from Ghana have demonstrated that IgE cross-reactivity plays a key role in allergen-specific IgE sensitization, the aim of our current study was to investigate the specific N-glycan epitopes associated with IgE cross-reactivity among Ghanaian children using a synthetic glycan microarray. In addition, we also sought to characterize responses to other glycan epitopes including the oligosaccharide epitope galactose-□-1,3-galactose (□-gal) which has been linked to two forms of anaphylaxis in Western countries.[11]

Research from developing countries including Ghana have shown distinct urban-rural differences in allergy outcomes [12, 13] as well within-urban differences based on socioeconomic status.[14, 15] Therefore, we examined whether there were differences in IgE response profiles of urban versus rural children as well as the relationship between current parasitic infections and IgE reactivity to glycan epitopes.

We also examined IgG1 and IgG4 responses to glycan epitopes and correlations with IgE responses.

Materials and methods

Study subjects and sera

We conducted a glycan epitope characterization investigationamong a subset of subjects that had been recruited into a larger cross-sectional study on allergic sensitization and parasitic infections in schoolchildren in Southern Ghana. The cross-sectional survey was conducted between March 2006 and March 2008 and detailed methodology as well as population description have been reported elsewhere.[8]Briefly, the larger investigation was carried out within the framework of the European Commission–funded EuroPrevall [16] and GLOFAL [17]

projects and was conducted among children aged between 5 and 16 years attending rural and urban schools in the Greater Accra Region. The study was approved by the Noguchi Memorial Institute for Medical Research Institutional Review Board, Ghana (NMIMR-IRB CPN 012/04-05).

For the glycan epitope characterization investigation, 60 stored sera samples were randomly selected from three schools that reflected the dynamic environmental changes associated with urbanization in Ghana. The three schools were a rural school (TP) that was located in a rural community in the Dangme West district of the Greater Accra Region. The area is endemic for the waterborne helminth infection *S. haematobium,* other helminths and malaria. We targeted an urban low-socioeconomic status school (NB) which was a public school located in the city of Accra. An urban high-SES school (GR) was also selected. This was a private school also located in the city of Accra catering towards middle-to-high income individuals.

European CCD-positive samples

Stored sera samples from 5 European pollen allergic patients who had significantly detectable levels of specific IgE to bromelain and horseradish peroxidase were also included in our investigation for comparison purposes. These subjects have been characterized in detail elsewhere and had a median IgE to bromelain response of 13.2 kU/L.[18]

Glycan microarray binding assays

A microarray comprised of 128 synthetic glycan structures was used to assess IgE, IgG1 and IgG4 reactivity in sera samples. Glycan structures were prepared and immobilized on a solid phase (glass slide). Each glass slide consisted of seven arrays containing the 128 structures in quadruplicates. The synthesis of glycan structures as well as the slide printing procedures have been described in detail elsewhere.[10] Printed glass slides were then blocked with 50 mM, pH 9.0 for 1 hour, washed and then stored at -20□C. Figure E1 shows a selection of the 128 synthetic glycans that reflect the major xylose and α-1,3 fucose modifications, various core extensions as well as other epitopes of interest in the larger array.

Prior to the assay, microarray slides that had been stored at -20C were left at room temperature to thaw and dry. A7-well incubation chamber was placed on each glass slide to separate the printed arrays. Each array was incubated with serum diluted 1:30 in PBS, with 1% bovine serum albumin (BSA), 0.01% Tween 20 (T_{20}) and left to shake for one hour at room temperature. Each slide was washed first in PBS with 0.05% T_{20} and then in PBS alone. Washing was followed by a 30-minute incubation in the dark with PromoFluor 647-labelled anti-human IgE (Sanquin reagents, Amsterdam, the Netherlands) diluted1:400 in PBS and
0.01% T_{20} and also with Cy3-labelled anti-human IgG (Sigma Life Sciences, St. Louis, MO, USA) that had been diluted 1:1000 in PBS with 1% BSA and 0.01% T_{20}.The PromoFluor 647 labelling of the IgE antibody had been carried out using a Promokine kit (PromoCell GmbH, Heidelberg, Germany) according to manufacturer's instructions. Following incubation, the slide was washed first as previously described and then finally in Milli-Q water. The final washing step was followed by spinning to dry the slide, after which it was kept in the dark until scanning.

The measurements of IgG1 and IgG4 antibody responses to glycan epitopes were performed in a similar manner with a few modifications. Following the placement of the 7-well incubation chambers on each glass slide, the arrays were incubated with serum diluted 1:100 in PBS with 5% BSA and 0.01% T_{20} at room temperature with shaking for an hour. This was followed with a washing step and incubation for an hour in the dark with PromoFluor 647labelled anti-human IgG1 (Sanquin reagents, Amsterdam, the Netherlands) diluted 1:200 in PBS - 0.01% T_{20} and PromoFluor 555-labelled anti-human IgG4 (Sigma Life Sciences, St.
Louis, MO, USA) diluted 1:200 in PBS - 0.01% T_{20}.

Glycan microarray slide scanning and analysis

All microarray slides were scanned for fluorescence using a G2565BA scanner (Agilent Technologies, Santa Clara California, USA) at 10□m resolution using 2 lasers (532nm and 633nm). The GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, California) was used

to align the spots using circular features with a composite pixel intensity (CPI) threshold of 10. The resulting data was exported to Microsoft Office Excel 2007. Data was then processed as described by Oyelaran *et al.,* 2009. For IgE, IgG1 and IgG4 antibody responses, for each spot the fluorescence/background was calculated, then multiplied by the average background value (of all spots except BSA) averaged over 4 spots and then later log-2 transformed.

IgE antibody measurements to allergen extracts

For all participants, ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) measurements were carried out according to the manufacturer's instructions to determine serum-specific IgE levels to a panel of allergen extracts. The allergens were the CCD marker Bromelain which has well-characterized core α1,3-fucose and xylose modifications, house dust mite extract (*Dermatophagoides pteronyssinus*), cockroach extract (*Blattella germanica*), peanut extract (*Arachis hypogaea*) and the oligosaccharide galactose-□-1,3-galactose. The source material for galactose-□-1,3-galactose (□-gal) was bovine thyroglobulin.

Parasitological examinations

For each study participant, one stool sample was collected for the detection of intestinal helminth eggs by the Kato-Katz technique [19] using 25 mg of stool. Intestinal helminths detected were hookworm (*Necator americanus* and *Ancylostoma duodenale*), *A. lumbricoides*, *Trichuris trichiura* and *Schistosoma mansoni*. A urine sample was also collected to determine *S. haematobium* infection using the standard filtration method [20] in which 10ml of urine is filtered through a nylon nucleopore filter (pore size, 12 μm). A small quantity of blood was used to prepare a Giemsa-stained thick smear slide to detect malaria parasites.

Statistical Analysis

Analysis was carried out using IBM SPSS version 20.0 (SPSS, Chicago, IL, USA). Area differences in the distribution of subject characteristics were examined by Pearson's $\chi 2$ test (two degrees of freedom). A p-value less than .05 was taken as the level for statistical significance. Area differences in IgE, IgG1 and Ig4 responses to selected glycan were examined using Mann-

WhitneyU Tests. The associations between current parasitic infections and IgE responses to representative glycan structures were also examined using MannWhitney U Tests. Correlations between IgE to bromelain and N-glycan structures as well as correlations between IgE to □-gal and □-gal epitope structures on the microarray were examined using Spearman's rank correlation.

Results

Characteristics of study participants

Table 1 shows the characteristics of participants selected for this study stratified by area. There was no significant difference in age-group comparing the three areas although urban low SES children had a slightly higher median age. There were significantly more male subjects randomly selected from the rural school (15 out of 20) compared to the urban schools.

About 50% of rural subjects had at least one soil transmitted helminth (STH) infection while STH infections were below 5% for both urban schools. In this selection, 26.3% of urban low SES participants were positive for *S. haematobium* infection while 5% were infected with this helminth at the rural and urban high SES school. One of the *S. haematobium* positive individuals at the rural school had a concurrent *S. mansoni* infection. Malaria parasites were detected among 40% of rural children, 15% of urban low SES participants and none of the urban high SES children.

Regarding allergen-specific IgE sensitization ($\geq$ 0.35 kU/L), for all allergen extracts (bromelain, house dust mite, cockroach, peanut and □-gal), the proportion of individuals sensitized was greatest in the rural area followed by the urban low SES school and lastly the urban high SES school. This trend was statistically significant for house dust mite, cockroach and □-gal but not for IgE to bromelain and peanut. IgE sensitization to□-gal, was most marked with 70% of rural subjects being sensitized followed by 20% of urban low SES subjects and no urban high SES participants. Figure 1 shows the measurement of specific IgE to bromelain in each area with the median-specific IgE level indicated. We observed an area gradient with median levels being

highest among rural children followed by urban low SES and urban high SES children (Figure 1).

Figure E2 shows an overview of all the anti-glycan IgE responses stratified by area with the different glycan groups that are categorized in Figure E1 indicated. Overall, strong antiglycan IgE responses were observed in rural children followed by urban low SES and finally urban high SES children. Figure E2 also shows that relatively strong responses among rural children were elicited to structures with □ 1,3 fucose (Group V and VI). WeakIgE antibody reactivities were observed to structures with xylose alone (Group III) as well as with□-1,6 fucose alone (Group IV). Among rural and urban low SES children only, strong responses to structures containing the galactose-□1,3-galactose epitope were also seen (Group VII).

IgE responses to selectedN-glycan structures stratified by area

Figure 2 shows area differences in anti-glycan IgE responses to selected structures with or without the core xylose and core □-1,3 fucose epitopes. We observed that responses to structures with□-1,3 fucose alone(G73) were very similar to responses to structures with □1,3 fucose + □-1,6 fucose (SS8_41). In addition, these reactivities were very similar to the structure withxylose + □-1,3 fucose + □-1,6 fucose (G39). In both urban schools, a few children had elevated IgE reactivity to a structure that contained a core xylose with no fucose (G34).

Correlation between specific IgE to bromelain (measured by ImmunoCAP) and core xylose and □-1,3 fucose epitopes on the glycan microarray

Given that bromelain is a marker of cross-reactive carbohydrate determinants, we examined correlations between IgE reactivity to bromelain measured by ImmunoCAP and representative structures containing core xylose and □-1,3 fucose epitopes on the glycan microarray. These correlations are shown in Figure E3 (online repository). A relatively strong correlation was observed between bromelain-specific IgE and structure G73 which had □-1,3 fucose alone

(Spearman's rho = 0.74, p<0.0001). A similarly strong correlation was seen for bromelain-specific IgE and structure KB3_89 with core xylose and □-1,3 fucose (Spearman's rho = 0.70, p<0.0001).

IgE responses to other glycan structures stratified by area

Figure 3 shows responses to other glycan structures of interest. Elevated responses were observed to structures containing galactose-□-1,3-galactose (70 and 10a) especially among rural and urban low SES children. Other structures such as helminth-associated Lewis X moiety (44Lex) did not elicit much of a response.

Correlation between specific IgE to □-gal (measured by ImmunoCAP) and □-gal epitopes on the glycan microarray

The correlations between IgE to □-gal (measured by ImmunoCAP and IgE to two □-gal epitopes on the glycan microarray (70 and 10a) are show in Figure E4. We observed a moderate positive association between IgE to □-gal by ImmunoCAP and structure 70

(Spearman's rho = 0.52, p<0.0001) and a weak correlation with structure 10a (Spearman's rho = 0.28, p=0.023).

IgE responses to glycan structures among European pollen allergic patients

Figure E5 and E6 show IgE responses to selected glycan structures among the 5 European pollen allergic patients included in our analysis. Overall, responses to the glycan structures were greatly elevated among these allergic patients compared to our unselected population of Ghanaian children. Similar to the Ghanaian study participants, responses to structures without core xylose or □-1,3 fucose (SS5_67) among European pollen allergics were very low (Figure E5). In contrast to the Ghanaian responses, responses to structures with xylose alone (G34) appeared to the elevated among the European pollen allergics. Structures with core xylose and □-1,3 fucose such as KB03_89, produced responses similar to those with xylose alone suggesting that this response was xylose-driven.

Figure E6 shows the responses to the other glycan epitopes among European pollen allergics. Although responses were higher overall than for our unselected population of Ghanaian children, responses to epitopes that included □-gal structures were low.

Associations between demographic factors and anti-glycan IgE responses

Given the over-representation of males in the rural study group, we examined the role of gender on observed differences in glycan responses. This was done by examining associations between gender and glycan responses among urban high SES children only. We restricted this analysis to urban high SES children to minimize the influence of parasitic infections. For Nglycan structures that elicited robust responses overall (structures shown in Figure 2), there were no significant differences in median anti-glycan IgE responses in males compared to females. We also observed no differences in responses to the structures representing the □-gal epitope (structure 70 and 10a).

Associations between parasitic infections anti-glycan IgE responses

We examined whether current parasitic infection may account for elevated anti-glycan IgE responses. We restricted this analysis to rural and urban Low SES children since parasitic infections were prevalent in both these groups compared to the urban high SES children.

Figure 4 shows IgE responses to selected representatives N-glycan structures according to parasitic infection status. As shown in Figure 4A children infected with *S. haematobium* had significantly elevated levels of IgE to structure G34 which had xylose only (Mann Whitney U test statistic= 19.50, p=0.0008). For the structure with xylose and a □-1,3 fucose modification, elevated IgE levels were observed among *S. haematobium* positives but these were not significant (p=0.09).No significant associations were observed when it came to the two other structures that included a □-1,3 fucose modification.

We further examined the relationship between current *S. haematobium* and other glycan structures with core xylose but no fucose. (Figure E12). Among *S. haematobium*-infected compared to uninfected, we observed significantly elevated levels of IgE directed against xylosylated structures that did not contain fucose.

Among children positive for at least one soil transmitted helminth infection, elevated levels of IgE were observed to structure SS_29 which an □-1,3 fucose modification only. This association was of borderline significance (Mann-Whitney U test statistic = 94.50 , p=0.049). No differences in responses were observed between those who were malaria infection positive compared to negative.

We also examined associations between parasitic infections and IgE responses to the□-gal epitopes on the microarray (structures 70 and 10a). No significant associations were observed between any of the parasitic infections examined and these epitopes (Figure 5).

IgG1 responses to glycan structures and correlations with IgE

Area differences in anti-glycan IgG1 responses to selected N-glycan structures are shown in Figure E6. Overall, median IgG1 responses were much greater than for IgE for all the glycan structures examined. IgG1 responses followed a similar pattern to IgE responses for the Nglycans shown in Figure 2. Given the similarity in response patterns, correlations between IgE and IgG1 to N-glycan epitopes were also investigated (Figure E8A). We observed significant but weak correlations between IgE and IgG1directed against glycan epitopes with □-1,3 fucose. When it came to the other glycan structures including the α-gal epitopes, there were a few high outlier IgG1 responders in all three schools but no discernable pattern (Figure E9).

In addition, there was no correlation between IgE to α-gal epitopes and IgG1 (Figure E11A).

IgG4 responses to glycan structures and correlations with IgE

We also examined area differences in anti-glycan IgG4 responses to selected N-glycan structures (Figure E7). Median IgG4 responses were much greater than for IgE for all the glycan structures examined but did not follow the same pattern as IgE responses. In addition, althoughsignificant weak correlations were observed between IgE and IgG4 to the same Nglycans (Figure E8B), the pattern was hard to discern. For example, significant correlations were observed for the structure with only xylose (G34) as well as a structure with only □-1,3 fucose (G73) but not to the structure with both □-1,3 fucose and □-1,6 fucose (SS8_41). For the other glycan structures including the α-gal epitopes, responses were generally low for all the structures examined (Figure E10). Figure 11B shows that correlation between IgE and IgG4 was only observed for one of the twoα-gal epitopes (70).

Discussion

Our study is the first to use a synthetic glycan microarray to determine specific glycan epitopes associated with carbohydrate-related IgE cross-reactivity in children from a helminth-endemic part of the world. This investigation also allowed us to examine urban-rural differences in IgE reactivity to glycan epitopes as well as to examine within-urban differences based on socioeconomic status.

Overall, we observed greater IgE reactivity to both the glycan structures on the microarray and whole allergen extracts(measured by ImmunoCAP) among rural and urban low SES compared to urban high SES children. For the N-glycans structures, IgE reactivity was highest to structures with a fucose in the □-1,3 position. We also observed low IgE reactivity to structures with β-1,2-xylose alone or □-1,6-fucose alone. The□-1,3-fucose and β-1,2-xylose moieties have long been recognized as the main structural residues associated with carbohydrate-related IgE cross-reactivity.[4]Van Ree *et al* investigated the structural basis for

IgE recognition of plant N-glycans among European individuals who had elevated titers of IgE directed to pollen and/or vegetable foods and observed that both □-1,3)-fucose and β-1,2xylose were involved in IgE binding in this group of individuals.[5] In our study, we included samples from some European pollen allergic individuals and observed much elevated antiglycan IgE responses compared to our samples from Ghana. This observation can be explained by the fact that Europeans were a group selected on the basis of their elevated responses to allergens containing N-glycan structures. Our study participants on the other hand were unselected on the basis of their anti-carbohydrate IgE responses. However, we did observed elevated levels of IgE to the CCD marker bromelain. It also appeared that these elevated levels were to IgE directed mainly at the □-(1,3)-fucose epitope.

It is also known that plants contain both these epitopes while insects contain only the fucose.[4]Our observations seem to suggest that IgE associated with carbohydrate–related cross-reactivity was more specifically targeted towards the □-1,3-fucose rather than the β-1,2xylose.

With regards to whether helminth infection was associated with elevated levels of anti-glycan IgE, we observed that the systemic *S. haematobium* infection was significantly associated with elevated levels of IgE directed against structures with core xylose but not with fucose. In our published study from the larger study population from which our current subset were selected, peanut-specific IgE was strongly associated with CCD-specific IgE.[8] In addition, we observed a strong association between peanut-specific IgE sensitization (≥ 0.35 kU/L)and current infection with *S. haematobium* but not with soil transmitted helminths. This suggested a role for *S. haematobium* in inducing cross-reactive IgE directed against N-glycan structures. Our previously published findings from our study population that showed that Schistosome soluble egg antigen, which is rich in glycoproteins with both core xylose and core fucose structures, was able to block binding of IgE to peanut allergen using a serum pool from 17 individuals who had elevated levels of IgE to CCD (> 5.02 ku/L). [8]

Our current study findings seem to suggest that this cross-reactive IgE may be directed mainly against thecore xylose epitope as opposed to the □-1,3-fucose epitope. Although we did observe that soil transmitted helminth (STH) infection was significantly associated with elevated levels of IgE toone of □-(1,3)-fucose structures, this was not observed to all other structures containing □-(1,3)-fucose. Therefore, we did not find definite evidence of STH infection driving the elevated levels of IgE directed against □-(1,3)-fucose we found. Further investigations would have to be conducted to explore other factors aside from helminths that may be driving the elevated levels of specific IgE to □-(1,3)-fucose epitopes.

A novel aspect of our investigation was exploring IgE reactivity to the galactose-1,3-galactose epitope associated with clinically relevant reactions to the monoclonal antibody cetuximab [21] as well as delayed reactions to mammalian meat (Platts-Mills and Commins 2013). In our study, we observed 70% IgE sensitization to alpha-gal (≥0.35 kU/L) measured by ImmunoCAP among rural participants. Relatively high sensitization prevalences have also been observed in other helminth endemic areas such as rural Kenya (50%) and Esmeraldas Province, Ecuador

(35%).[22]In those studies, tick-bites rather than helminth infection were thought to explain the elevated levels of IgE to alpha-gal. The fact that we observed no association between current helminth infection and IgE to α-gal epitopes on the glycan microarray also suggests that factors other than helminths related to the rural environment may induce elevated levels of IgE to these epitopes such as tick-bites. Notably, a recent crosssectional study from rural Italy showed high prevalence of α-gal sensitization linked to exposure to the tick *Ixodes ricinus* and not associated with anaphylactic responses to mammalian meat.[23] Further studies are needed to better characterize asymptomatic α-gal sensitization.

In our study, we also investigated IgG1 and IgG4 responses to the glycan epitopes on the microarray. Our findings suggest correlations between IgE and IgG1 to some of the □-(1,3)fucose glycan epitopes that induced the most responses as well as correlations between IgE and IgG4. Jin *et al* investigated the affinity of IgE and IgG against cross-reactive carbohydrate determinants on plants as well as insect glycoproteins and observed that the affinity of IgG to CCD was high.[24] They concluded that IgG may function as a blocking antibody. The correlations we observed between IgE and both the IgG isotypes may be explained by the fact that IgG is acting as a blocking antibody.

The correlation between IgG and IgE may be related to the production and regulation of IgE by the immune system. Although the mechanisms by which IgE is produced are not clearly elucidated, mouse studies have demonstrated that there are two pathways of immunoglobulin class switching that lead to the production of IgE.[25] A direct pathway from IgM to IgE and an indirect/sequential pathway from IgM to IgG1 to IgE.[25]Therefore, it is possible that elevated levels of IgG are correlated with elevated responses to IgE due to the production and regulation of IgE antibodies.

Our study had some limitations such as the fact that there was an over-representation of males compared to females randomly selected from the rural school sera sampled. However,our analysis of the independent effect of gender showed there were no differences between the two genders when it came to IgE reactivity to the glycan structures.

Despite the limitations, our study provides insights into IgE responses directed against glycan epitopes using a novel technique.

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TABLES AND FIGURES

Table 1: Characteristics of Study Participants

Factor	AREA				#P value
	ALL, n/N (%)	Rural, n/N (%)	Urban low SES, n/N (%)	Urban High SES, n/N (%)	
Age §					
<11 years or less	21 (35.6)	8 (42.1)	8 (40.0)	5 (25.0)	0.47
≥more than 11years	38 (64.4)	11 (57.9)	12 (60.0)	15 (75.0)	
Gender					
Male	31 / 60 (51.7)	15 / 20 (75.0)	8 / 20 (40.0)	8 / 20 (40.0)	**.04**
Female	29 / 60 (48.3)	5 / 20 (25.0)	12 / 20 (60.0)	12 / 20 (60.0)	
Parasitic infections (positive)					
S. haematobium	7 / 59 (11.9)	1 / 20 (5.0)	5 / 19 (26.3)	1 / 20 (5.0)	.06
Soil Transmitted Helminth**	11 / 58 (19.0)	10 / 20 (50.0)	1 / 20 (5.0)	0 / 18 (0.0)	**<.001**
Plasmodium sp.	11 / 60 (18.3)	8 / 20 (40.0)	3 / 20 (15.0)	0 / 20 (0.0)	**.004**
Specific IgE levels (≥0.35kU/L					
Bromelain (MUXF3)	12 / 60 (20.0)	7 / 20 (35.0)	4 / 20 (20.0)	1 / 20 (5.0)	.06
House dust mite (Der p)	12 / 60 (20.0)	8 / 20 (40.0)	4 / 20 (20.0)	0 / 20 (0.0)	**.007**
Cockroach (Bla g)	23 / 60 (38.3)	13 / 20 (65.0)	6 / 20 (30.0)	4 / 20 (20.0)	**.009**
Peanut (Ara h)	9 / 60 (15.0)	15 / 20 (75.0)	3 / 20 (15.0)	1 / 20 (5.0)	.21
Galactose-□-1,3-galactose (□-gal)	18 / 60 (30.0)	14 / 20 (70.0)	4 / 20 (20.0)	0 / 20 (0.0)	**<.001**

P value calculated using Pearson's □2 test (two degrees of freedom)

§ Age of one rural subject was missing

Soil transmitted helminth = *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm **Significant associations are shown in bold.

Figure 1: Specific IgE to Bromelain (MUXF3)

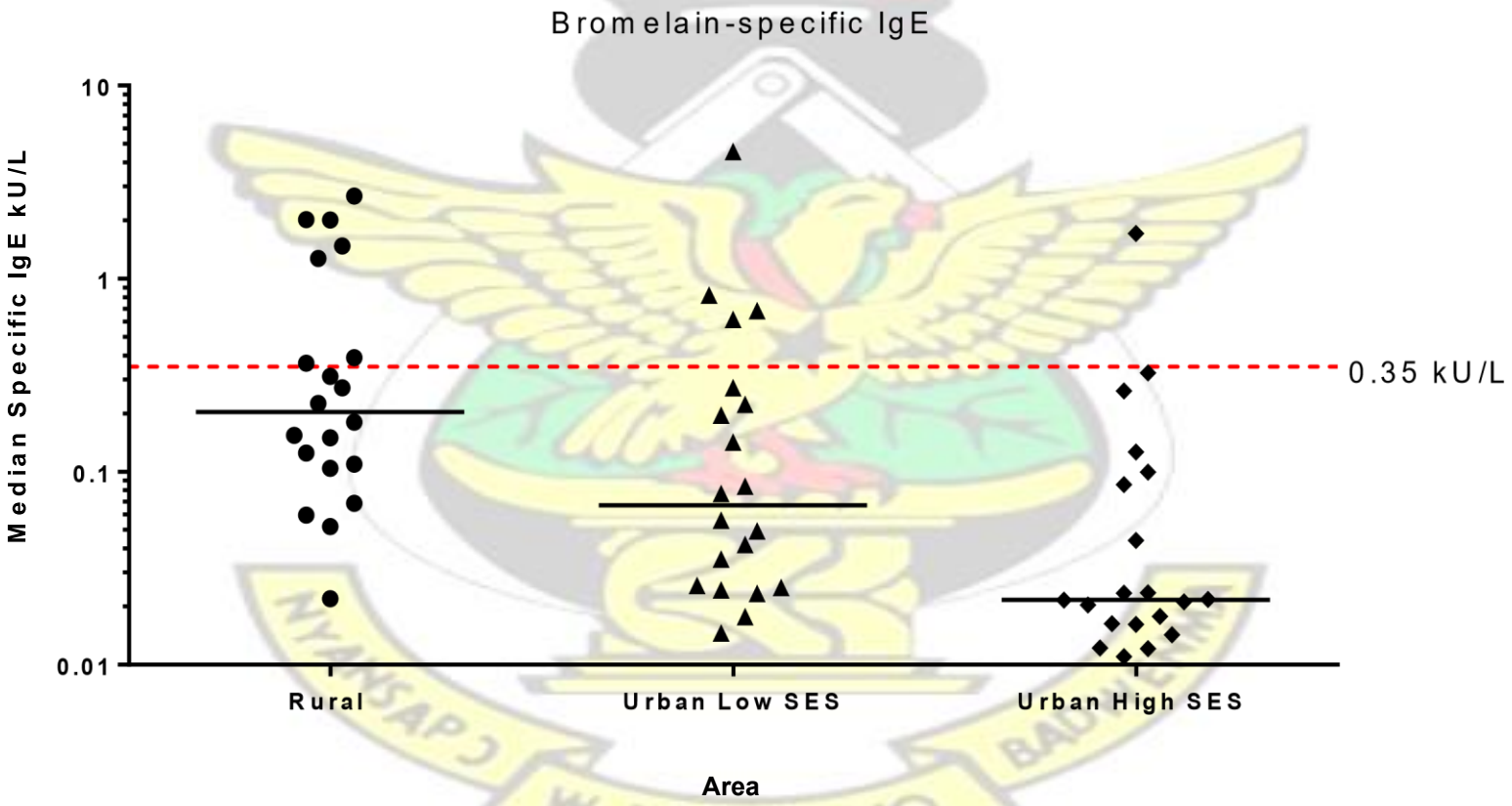


Figure 2: IgE responses to selected N-glycan structures

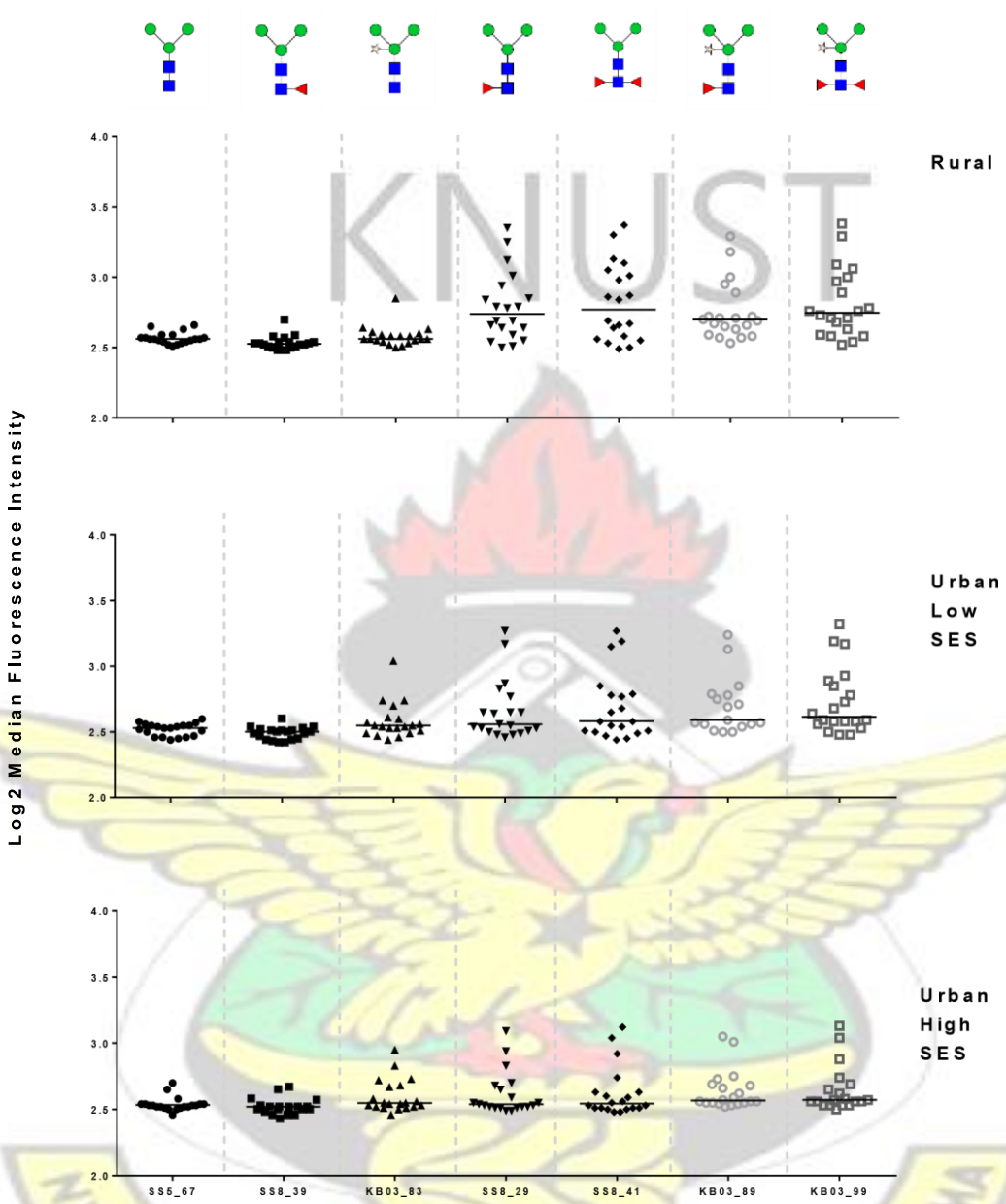


Figure 3: IgE responses to other selected glycan structures

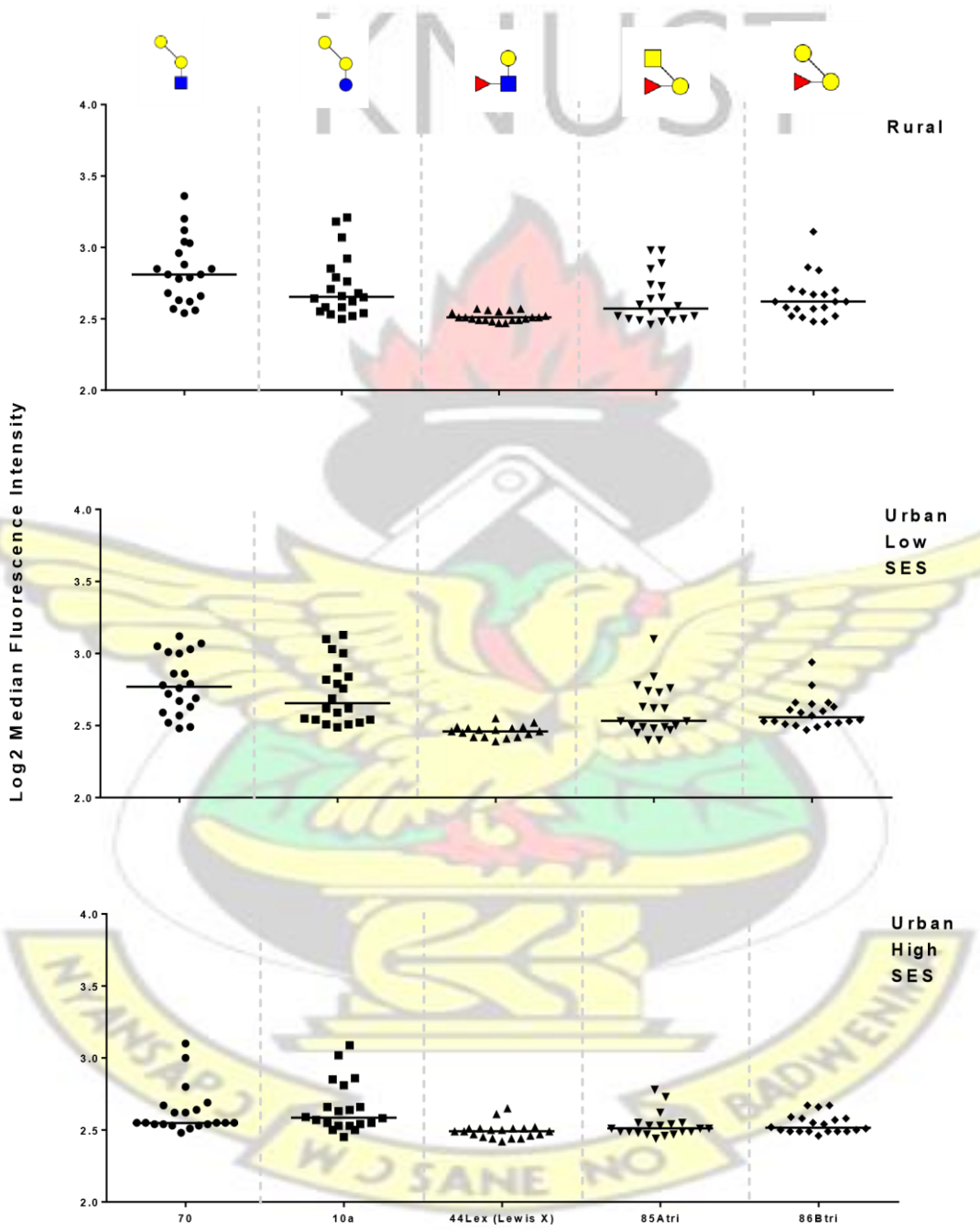
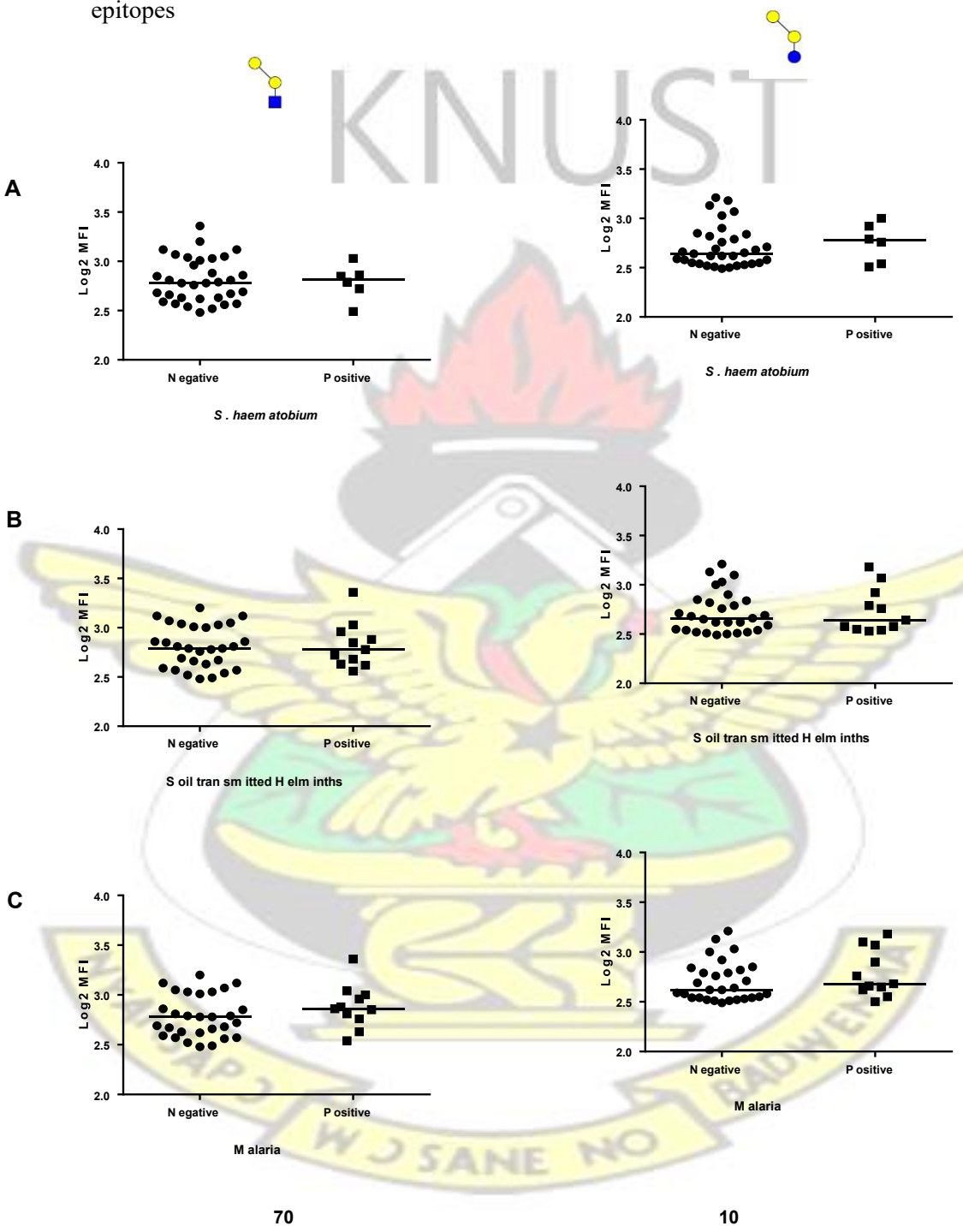


Figure 4:  Associations between   current parasitic infections and representative IgE responses to selected N-glycan structures

Figure 5: Associations between current parasitic infections and representative IgE responses to □-gal epitopes



APPENDIX II: PREPARED MANUSCRIPT II FOR PUBLICATION

TITLE: Urban-rural differences in pro- and anti-inflammatory cytokine production: a case for IgE cross-reactivity in a selection of Ghanaian pupils

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ABSTRACT

In low-to middle-income countries (LMICs), allergic diseases are becoming a Public Health concern, as increased urbanization is driving up observed incidences and prevalences. However, the striking urban-rural differences in allergic disease prevalence in LMICs provide opportunities for research studies attempting to explain the role of the environment in allergic disease outcomes while presenting with insights and findings essential for the development of novel therapeutic strategies against allergy. Previously, we showed that there was a higher prevalence of glycan-associated cross-reactive IgE in the rural (R) than the urban areas. In this study therefore, we characterized within an urban-rural context, proand anti-inflammatory cytokine responses following stimulation with innate and adaptive agonists. Additionally, we sought to determine the innate and adaptive immune responses associated with peanut-specific IgE (pIgE) positivity, as well as hookworm and *S. haematobium* infections.

Our results showed clear urban-rural differences with regard to TNF-α and IL-10 production to innate stimuli. Trends were not as clear following TNF-α, IL-10, and IL-13 production to adaptive stimuli, although generally low pro-inflammatory indices were observed. Also, higher titres of TNF-α were observed among pIgE-negative participants, whilst the converse was realized with regard to IL-10 levels after stimulation with innate agonists. Here as well, TNF-α/IL-10, and TNF/IL-13 indices were much lower than threshold values, indicating a generally dampened adaptive immune environment. For both helminths (hookworm and *S. haematobium*) scrutinized, we observed higher pro-inflammatory indices among both hookworm- and *S. haematobium*-negative participants following innate stimulation.

However, the opposite was observed for both helminths following stimulation with adaptive agonists. Our findings confirm suggestions of a dampened pro-inflammatory immune environment being associated with cross-reactive IgE.

Key Messages

Clear urban-rural differences exist following innate immune stimulations among children living in low-to middle-income countries (LMICs).

Observed lower pro-/anti-inflammatory indices following adaptive stimulation may be suggestive of a less hostile immune environment for subjects in rural or helminth-endemic areas.

Capsule Summary

Glycan-associated cross-reactive immunoglobulin Es (IgEs) are characterized by dampened pro-inflammatory innate responses, leading eventually to a generally more tolerant adaptive immune environment.

Key words:

Glycan-associated cross-reactive IgEs, peanut-specific IgE, hookworm, *S. haematobium*, pro-inflammatory index, immune environment

Abbreviations

LMICs:	Low- to middle-income counties
pIgE:	peanut-specific IgE
CCD:	cross-reactive carbohydrate determinant
PPD:	Purified protein derivative
PHA:	Phytohaemagglutinin
AWA:	Adult worm antigen
TNF-α:	Tumor Necrosis Factor- α
IL-10:	Interleukin 10
IL-13:	Interleukin-13
LPS:	Lipopolysaccharide
Poly(I:C):	Polyinosinic-polycytidylic acid

INTRODUCTION

Allergic disease within the last three decades has been on the rise globally. Current estimates for asthma, rhinitis, and food allergies indicate respectively 300, 400, and 250 million sufferers annually (Masoli *et al.,* 2004; Pawankar*,* 2014). These rates are further driven up by the increasing urbanization in low- to middle-income countries (LMICs), characterized by the adoption of a more 'Western' lifestyle; and by significant environmental changes which determine disease patterns (Haahtela *et al.*, 2013; Amoah *et al.,* 2013, 2014; WAO, 2013; Hamid *et al.,* 2013, 2015). This notwithstanding, it is accepted that the prevalence of allergies remain higher in industrialized countries than in LMICs (ISAAC, 1998; van den Biggelaar *et al.,* 2001; van der Vlugt *et al.,* 2012; Hamid *et al.,* 2015). Indeed, findings from numerous studies indicate a poor geographical overlap between infectious and allergic diseases (van den Biggelaar *et al.,* 2000, 2001; .Croese *et al.,* 2006; Fleming, 2011). Nevertheless, the striking differences in allergic disease prevalence between urban and rural populations, attributable to increased urbanization in LMICs (Cooper *et al.,* 2004; Davey *et al.,* 2005; Hamid *et al.,* 2013), provide opportunities for studies that would improve our understanding regarding the aetiology, factors, and mechanisms associated with allergies.

It is well established that, immune responses to allergies are T-helper 2 (Th2)-mediated, with immunoglobulin Es (IgEs), mast cells, eosinophils, and basophils playing key roles in clinical manifestations (van den Biggelaar *et al.,* 2000; Gloudemans *et al.,* 2009; Stone *et al.,* 2010; Hamid *et al.,*2011; Obeng *et al.,* 2014). Hence, among wealthier urban dwellers who have greater access to clean food, potable water, and adequate sanitation, higher prevalences of allergic and auto-immune diseases have been reported, as compared to inhabitants in urban

environments characterized by poor sanitation, limited access to potable water, and high population density and overcrowding (Cooper *et al.,* 2004; Davey *et al.,* 2005; Amoah *et al.,* 2014; Isunju *et al.,* 2011). Among inhabitants in rural areas, clinical symptoms of allergy are rarely reported because of their more agrarian lifestyles characterized by traditional diets, limited access to quality health care, and perpetual exposure to helminths (Alirol *et al.,* 2011; Amoah *et al.,* 2014).

Evidence from recent urban-rural studies in southern Ghana suggest that the IgEs produced, particularly among rural participants, most likely cross-react with glycan epitopes on allergenic and helminthic glycoproteins, thus contributing to its characteristically poor biological activity (Amoah *et al.,* 2013, 2014). Indeed, although studies have characterized innate and adaptive immune profiles associated with allergic reactions (Lambrecht *et al.,* 2000; Holt, 2002; Murphy *et al.,* 2008; Akdis and Akdis, 2011), such immune characterization is yet to be done in an urban-rural context in a LMIC where cross-reactive IgE has been identified within the population. Therefore, involving subjects previously recruited in a larger survey on allergic sensitization and parasitic infections in southern Ghana, we sought to determine the urban-rural differences in the pro-and anti-inflammatory profiles of our participants following 24- and 72-hour stimulations of whole blood samples with innate and adaptive agonists. Our hypothesis was that higher innate and adaptive proinflammatory activity would be associated with the urban than the rural areas. Additionally, we characterized and compared the innate and adaptive immune profiles of peanut-specific IgE-positive and peanut-specific IgE-negative participants. This was because evidence in a previous related study showed peanut-specific IgE to be tightly correlated with bromelain, a cross-reactive

carbohydrate determinant (CCD) (Amoah *et al.,* 2013). Similarly, we compared innate and adaptive immune profiles between helminth-positive and negative participants.

MATERIALS AND METHODS

Study Design and participants

This immunological study was conducted on a subset of participants who had previously been recruited in a larger cross-sectional survey on allergic sensitization and parasitic infections in school children in Southern Ghana. Carried out within the framework of the European Commission-funded EuroPrevall(Kummeling *et al.,* 2009) and GLOFAL (GLOFAL, 2006) studies, the survey was conducted between March 2006 and March 2008, and detailed information on population and study description has been reported elsewhere (Amoah *et al.,* 2013; Obeng *et al.,* 2011, 2014). Briefly however, this survey involved children between the ages of 5 and 16 years recruited from thirteen randomly-selected urban and rural schools within the Greater Accra Region (GAR). Ethical approval was obtained from the Noguchi Memorial Institute for Medical research Institutional Review Board, Ghana (NMIMR-IRB CPN 012/04-05), while written or verbal parental consent in the form of a signature or thumbprint for each child was obtained prior to commencing the survey.

We sought, with this study, to investigate innate and adaptive immune profiles among urban and rural subjects; and also among peanut-specific IgE (pIgE)-sensitized and un-sensitized participants. We randomly selected individuals from 4 of the 13 urban and rural schools: 35

from an urban high (UH) school (Green Hill International School (GR)), 34 from two urban low (UL) schools (Immanuel Presbyterian School (IP) and Nii Okai Basic School (NB)), and 35 pupils from a rural (R) school (Toflokpo Presbyterian Primary School (TP)). The two urban schools are situated in different parts of the Accra metropolis, and reflect the socioeconomic and environmental diversity associated with urbanization. The R school, on the other hand, is situated in a community in the Dangme East district of the Greater Accra Region, and is endemic for urinary schistosomiasis (*S. haematobium*), soil-transmitted helminths, and malaria. Apart from blood, participants were required to provide urine and stool samples for parasitological studies. Blood samples were incubated with innate and adaptive stimuli for 24 and 72 hours respectively. Supernatants were harvested, and titres of cytokines of interest measured using requisite techniques and equipments.

Skin prick tests

With the aid of 1mm standardized lancets, skin prick tests were performed on the volar part of the lower left arm of participants. The panel of allergens tested included house dust mite (*Dermatophagoides pteronysinnus* (Der p)), cockroach (*Blattella germanica* (Bla g)), and peanut (*Arachis hypogaea* (Ara h)) (ALK-Abello, Denmark). Saline and histamine hypochloride (10mg/ml) served as negative and positive controls respectively (ALK-Abello, Madrid, Spain). A skin reaction, following a 15-minute waiting period, was considered positive on assessment for an allergen when the average of the longest wheal diameter (D1) and its perpendicular length (D2) was 3mm or more.

IgE antibody measurements

Specific immunoglobulin E (IgE) titres to peanut (Ara h), house dust mite (Der p), and cockroach (Bla g) were measured in serum samples via ImmunoCAP (Phadia AB, Uppsala, Sweden), with a serum-specific IgE titre value of 0.35kU/L serving as the sensitization cutoff.

Whole blood stimulation assay

This was conducted on heparinised blood samples, 4 to 6 hours after venipuncture. Briefly, each sample was diluted at a ratio of 1:1 in RPMI 1640 medium (Invitrogen) supplemented with 100U/ml penicillin, 100µg/ml streptomycin, and 2mM glutamate. Next, 100µL of the mixture was transferred to each well of a round-bottomed, 96-well culture plate (Nunc, VWR International) containing 100µL of either medium or stimulus co-cultured with medium. For our innate immune studies, we co-cultured with Pam3CSK4 (a TLR 2/1 agonist), polyinosinic: polycytidylic (Poly(I:C); a TLR 3 agonist), lipopolysaccharide (LPS; a TLR 4 ligand), and the *S. haematobium*-specific adult worm antigen (AWA) for 24hours (Table 1).In studying the adaptive immune responses in our participants, we co-cultured for

72 hours with purified protein derivative (PPD), an adaptive immune agonist against the Bacillus Calmette-Guerin (BCG) vaccine received at birth in Ghana (Amoah *et al.,* 2014; Djuardi *et al.,* 2010); phytohaemagglutinin (PHA), which is a polyclonal T-cell stimulus; and AWA, which is capable of inducing both innate and adaptive responses (Labuda *et al.,* 2014) (Table 1). Supernatants were harvested following respective incubation periods, stored at -20^{o}C and later transferred on dry ice to a central laboratory where cytokine measurements were conducted.

Cytokine measurements

We measured the levels of Tumor Necrosis Factor α (TNF-α) and interleukin-10 (IL-10) in supernatants harvested from 24-hour cultures; whilst TNF-α, IL-10, and IL-13 titres were measured in supernatants harvested from 72-hour cultures. Measurements were conducted using a Luminex 100 cytometer (Luminex Corp., Austin Texas, USA) and Luminex cytokine kits (BioSource, Camarillo, California, USA) according to the manufacturer's instructions. The lower detection limit for IL-10 was 5pg/ml, and 10pg/ml for TNF- α and IL-13. Samples with concentrations below the detection limit were assigned one-half the respective threshold values.

Parasitological examinations

We collected a single stool sample from each individual, which we examined for the presence of eggs of such soil-transmitted helminths (STHs) as hookworm (*Necator americanus*, *Ancylostoma duodenale*), round worm (*Ascaris lumbricoides*), whip worm (*Trichuris trichiura*), and *Schistosoma mansoni*. Samples were prepared, using the kato-katz technique. Single urine samples (≥10ml) were collected per subject, prepared via the filtration technique, and examined microscopically for the presence of *S. haematobium* eggs. Additionally, a Giemsa-stained thick smear slide for the detection of malaria parasites was prepared per participant from a small quantity of blood.

Statistical analyses

Cytokine concentrations per stimulant were corrected for by subtracting median responses in the following equation: (TNF-$\alpha_{stimulus}$ - TNF-α_{medium}). Zeros and negative numbers were replaced with one-half the respective threshold values (Hence, 5.0pg/ml for TNF and Il-13; and 2.50pg/ml for IL-10). The pro/anti-inflammatory index of a stimulant was thence calculated as follows: (TNF-$\alpha_{stimulated}$-TNF-α_{medium})/(IL10$_{stimulated}$-IL10$_{medium}$) (Labuda *et al.,* 2014). The cytokine data was subsequently log_{10}-transformed to ensure normalized distribution. Urban-rural differences in participant characteristics were assessed, using the Kruskal-Wallis test, whilst the Dunn's post test was used to identify groups causing significant differences. Assessment of urban-rural differences in pro-and anti-inflammatory cytokine responses to innate and adaptive stimuli were also done using the Kruskal-Wallis and Dunn's post tests. Differences in cytokine production between pIgE-positive and negative, as well as helminth-positive and negative participants were determined using the Mann-Whitney U test. A p-value less than 0.05 was considered to be statistically significant. Statistical analyses were carried out using the IBM SPSS version 16.0 software (IBM Corp., Armonk, New York, USA), and graphs were drawn using the Graph Pad Prism 5.04 software (La Jolla, California, USA)

RESULTS

General characteristics of participants

For this study, a subset of 104 pupils was randomly selected comprising 35 from the UH area, and 34 and 35 participants from the UL and R areas respectively. No significant differences were observed with regard to gender ($p = 0.098$) and age ($p = 0.320$) in all three areas for this selection of pupils (Table 2). With respect to ImmunoCAP-measured IgE titres to our panel of allergens, area-associated trends were observed with the highest titres realized in the R, followed by the urban areas. Trends were particularly significant for the ImmunoCAP-measured cockroach (*Blatella germanica*; $p < 0.001$) and peanut (*Arachis hypogaea*; $p = 0.035$) allergens (Table 2). Area-associated trends were however less clear with respect to skin prick tests (SPT) involving the same allergens (Table 2). Nevertheless, the highest number of SPT-positive subjects to the cockroach allergen was observed in the R area, followed by the urban areas ($p = 0.002$; Table 2).

For our subset of study participants, *Schistosoma haematobium* infection was observed to be highest in the UL area, followed by the R and UH areas respectively ($p = 0.210$; Table 2). Also, a significantly higher number of pupils were found in the R than in the urban areas to be *Necator americanus* (hookworm) positive ($p < 0.001$; Table 2). This was similarly observed with regard to malaria parasitaemia ($p < 0.001$; Table 2). None of our study subjects were however found to be positive for *Schistosoma mansoni*, *Ascaris lumbricoides*, or *Trichuris sp.* (Table 2).

Factor-associated differences in cytokine responses following 24hr stimulation with selected agonists

Urban-rural differences were observed with regard to measured levels of pro- and antiinflammatory cytokines in whole blood stimulated with toll-like receptor (TLR) ligands and the *S. haematobium*-specific adult worm antigen (AWA) for 24 hours (Figure 2). Higher median titres of TNF-α were therefore realized in the UH, followed by the UL and R areas respectively upon stimulation with the TLR ligands Pam3CSK4 (Pam3) ($p < 0.001$), Poly(I:C) ($p < 0.001$), and LPS ($p < 0.001$), as well as with AWA ($p < 0.001$) (Figure 2a). This urban-rural trend was similarly observed for IL-10, with significant differences realized following stimulation with LPS ($p < 0.01$), and AWA ($p < 0.001$) (Figure 2b). Little difference in median IL-10 titres were observed between the areas following Poly(I:C) stimulation, whiles IL-10 titres were highest in the UH, followed by the R, and UL areas following Pam3 stimulation (Figure 2b). However, higher TNF-α/IL-10 indices were observed among the urban than the rural subjects with regard to Pam3, Poly(I:C) ($p < 0.001$), LPS, and AWA ($p < 0.05$) (Figure 2c). Interestingly, higher median titres of spontaneously-produced TNF-α ($p < 0.001$) and IL-10 ($p < 0.001$) in unstimulated whole blood were realized for the R than the urban areas (Figure 6a).

We also sought to compare the pro- (TNF-α) and anti- (IL-10) inflammatory cytokine profiles between subjects with peanut-specific IgE titres measuring 0.35kU/L or higher (pIgE-positive); and subjects with peanut-specific IgE titres below 0.35kU/L (pIgEnegative). Higher median titres of TNF-α were observed among pIgE-negative than among pIgE positive subjects following 24-hour stimulation with Pam3, Poly(I:C), LPS, and AWA (Figure 4a).

Conversely, higher IL-10 responses were observed among pIgE-positive than among pIgE-negative participants following 24-hour stimulation to the above-mentioned stimulants (Figure 4a). Essentially, a higher pro-inflammatory index was observed among pIgE-negative participants for the above-mentioned ligands and AWA (Figure 4b). On the other hand, higher TNF-α levels were observed for pIgE-positive than pIgE-negative participants following 24-hours of unstimulated incubation of whole blood. Median titres of IL-10 were however lower and comparable between the groups (Figure 6b)

Additionally, we made comparisons between hookworm-infected and uninfected pupils, and observed median TNF-α titres to be higher among our hookworm-uninfected subjects following 24-hour stimulation with Poly(I:C), LPS, AWA, and Pam3. Observed differences were especially significant for the TLR ligands Poly(I:C) ($p < 0.01$) and LPS ($p < 0.01$); and for AWA ($p < 0.001$) (Figure S1a). Similarly, IL-10 titres were observed to be higher among hookworm-negative than hookworm-positive participants for the above-mentioned stimulants, except Poly(I:C) (Figure S1b). Higher pro-inflammatory indices were also generally observed among hookworm-negative subjects to all the stimulants, with significant differences realized for Poly(I:C) ($p < 0.01$) and AWA ($p < 0.01$) (Figure S1c). Interestingly, higher titres of TNF-α ($p < 0.001$) and IL-10 ($p<0.001$) were observed among hookwormpositive than among hookworm-negative subjects in unstimulated whole blood (Figure 6c). We observed with regard to *S. haematobium* infection, higher titres of TNF-α among *S. haematobium*-positive participants following stimulation with Poly(I:C). Median TNF responses were however similar between the groups following stimulation with Pam3 and LPS (Figure S2a). Median IL-10 responses were much higher following stimulation with

Pam3 and LPS than with Poly(I:C). However, titres were similar between the groups for all the stimulants (Figure S2a). Also, higher pro-inflammatory indices were observed among *S. haematobium*-negative participants for Poly(I:C) and Pam3. Indices were similar between groups for LPS (Figure S2b). Again, higher TNF-α levels were observed among *S. haematobium*-positive subjects in unstimulated whole blood, whilst similar median titres were observed between the groups for IL-10 (Figure 6c).

Factor-associated differences in cytokine responses following 72hr stimulation with selected agonists

We further examined urban-rural differences in adaptive immune responses among our selected participants after incubating whole blood samples with PPD, PHA, and AWA for 72 hours. Higher titres of TNF-α were observed among pupils from the R than the urban areas for PPD ($p < 0.01$) and PHA (Figure 3a). The converse was however observed following stimulation with AWA ($p < 0.01$). With regard to IL-10 production, we observed median titres to be higher in the R than in the urban areas for PPD ($p < 0.05$), and higher among our urban than our rural subjects for AWA ($p < 0.001$; Figure 3b). IL-10 levels for PHA was higher in the UH than in the R area (Figure 3b). In addition, higher TNF-α/IL-10 indices were observed for all stimuli to be highest in the R, followed by the UL and UH areas respectively after incubation for 72 hours (Figure 3c). Titres of IL-13, a Th2 cytokine, were observed to be highest in the R area for PPD ($p < 0.05$) and similar in all three areas for AWA (Figure 3d). Interestingly, a higher TNF-α/IL-13 index was observed in the R than the urban areas for PPD ($p < 0.01$), whilst the opposite was observed for AWA ($p < 0.05$)

(Figure 3e). Also, spontaneous production of both TNF-α and IL-10 were found to be highest in the R than the urban areas, whilst levels were highest among our UL participants for IL-13 (Figure 6d).

For differences in cytokine production between pIgE-positive and pIgE-negative subjects, we observed higher TNF-α responses among pIgE-positives following incubations with PPD, PHA, and AWA for 72 hours (Figure 5a). IL-10 titres were also higher among pIgEpositive participants for PPD ($p < 0.01$) and PHA, whilst slightly higher among pIgEnegative participants for AWA (Figure 5a). Essentially, TNF-α/IL-10 indices were comparable between the groups for PHA and AWA, while slightly higher among pIgEnegative participants for PPD (Figure 5b). For IL-13, higher titres were observed among pIgE-positive participants for PPD, whilst similar median titres were observed for AWA (Figure 5c). This therefore translated into a higher TNF-α/IL-13 index among pIgE-positive subjects for PPD ($p < 0.05$) while similar indices were observed between the groups for AWA (Figure 5d). Spontaneous IL-10 and IL-13 production were observed to be similar between groups, whilst slightly higher among pIgE-positive participants for TNF-α (Figure 6e).

We also realized median titres of TNF-α to be slightly higher among hookworm-negative participants following stimulation with PHA and AWA for 72 hours. Median TNF-α titres were however significantly higher among hookworm-positive participants for PPD ($p < 0.05$) (Figure S2a). Similarly, higher IL-10 levels were observed among hookworm-negative subjects for PHA ($p < 0.05$) and AWA ($p < 0.01$), and among hookworm-positive participants for PPD (Figure S3a). Higher TNF-α/IL-10 indices were therefore realized among hookworm-positive participants for all the stimuli after 72 hours of incubation (Figure S3b). We only

observed higher Il-13 responses among hookworm-positive participants for PPD, and similar levels between groups for AWA (Figure S3c). Hence, a higher TNF-α/IL-13 index was realized among hookworm-positive subjects for PPD, whilst similar indices were observed for both groups following stimulation with AWA (Figure S3d). Following the incubation of unstimulated whole blood for 72 hours, spontaneous TNFα production was found to be higher among hookworm-positive participants ($p < 0.001$) (Figure 6f). IL-10 levels were low and similar between groups, whiles higher IL-13 titres were realized among hookworm-negative participants ($p < 0.05$) (Figure 6f). Following the stimulation of whole blood with PPD and PHA for 72 hours, we observed higher TNF-α levels among *S. haematobium*-positive than negative subjects (Figure S4a). Median IL-10 titres were however comparable between groups for both PPD and PHA (Figure S4a). Furthermore, higher TNF-α/IL-10 indices were observed among *S. haematobium*-positive participants for PPD and PHA (Figure S4b). Higher IL-13 responses were also observed among *S. haematobium*-positive subjects following incubation with PPD for 72 hours (Figure S4c). Interestingly, a higher TNF-α/IL-13 index was observed among *S. haematobium*-positives for PPD (Figure S4d). Higher spontaneous TNF-α and IL-13 production were realized among *S. haematobium*-positive participants, whilst IL-10 levels remained similar in both groups after incubation of unstimulated whole blood for 72 hours (Figure 6f).

DISCUSSION

We sought in this study to examine along a socio-economic gradient, the pro- and antiinflammatory cytokine profiles of subjects, following the 24- and 72-hour stimulation of whole blood samples with innate (i.e. Pam3, Poly(I:C), LPS, and AWA)and adaptive (i.e. PPD, PHA, and AWA) stimuli. The unique feature regarding this work was the recruitment of participants from a larger sample found in earlier studies to exhibit glycan-associated IgE cross-reactivity (Amoah *et al.,* 2013, 2014; Asuming-Brempong *et al.,* submitted manuscript). Indeed, except for the recruitment of pupils from an additional UL school (i.e. IP), all others were randomly selected from the same urban and rural schools involved in a recent glycan-associated IgE cross-reactivity study, comprising GR for the UH participants, NB for the UL subjects, and TP for the R participants (Asuming-Brempong *et al.,* submitted manuscript). It was therefore not surprising that urban-rural trends similar to those realized in the glycan study, with regard to hookworm and *S. haematobium* infections, ImmunoCAPmeasured allergen-specific IgE, and allergen-specific SPT results, were observed (Table 2; Asuming-Brempong *et al.,* submitted manuscript). This confirms that observations regarding innate and adaptive immune profiles in this subset of participants are applicable to related subsets, and the larger survey population.

Secondly, our findings indicated clear urban-rural trends in TNF-α and IL-10 production following the 24-hour stimulation of whole blood with TLR ligands Pam3, Poly(I:C), LPS, as well as AWA. The *S. haematobium*-specific adult worm antigen was studied both as an innate and adaptive immune stimulus, not only because of its capability in interacting with

TLRs and other innate immune receptors (van der Kleij *et al.,* 2002; Caldas *et al.,* 2008; Ritter *et al.,* 2010), but also because it has been found to contain antigens which can be processed and presented for interaction with T-cell receptors (Labuda *et al.,* 2011). Thus, for all stimuli, TNF- α production was highest in the UH, followed by the UL, and R areas respectively (Figure 2). This trend was similarly observed for IL-10, thus leading to higher pro-inflammatory indices in the urban, as compared to the R areas. This is in line with current evidence suggesting the involvement of such innate PRRs as TLR 1/2 (triggered by Pam3), TLR 3 (triggered by Poly(I:C)), and TLR 4 (triggered by LPS) in the promotion of Th1-cell and pro-inflammatory responses (as reviewed by Chang, 2010; Labuda *et al.,* 2011; Park and Lee, 2013). Growing evidence for the 'Trained Immunity' postulation, whereby an innate immune cell initially triggered by a ligand is 'imprinted' and likely to respond differently upon subsequent triggering by the same ligand, may explain the generally low innate response profiles among subjects in the R area (Netea *et al.,* 2011; Labuda *et al.,*2014). Therefore, the greater degree of exposure of R participants to helminthic and other pathogens as compared to participants in the urban areas (Table 2) may have over time, led to the lowered responses to these ligands (Roy *et al.,* 2007; Netea *et al.,* 2011). These results are interesting, as they indicate a prevailing lower pro-inflammatory innate environment in the R area, where glycan-associated cross-reactive IgE was more prevalent. However, as to what adaptive immune environment these responses would induce, remains to be elucidated. Also, these findings are in consonance with evidence presented by Smolen *et al.,* (2013), indicating higher and similar innate immune responses among European, North, and South American children, as compared to African children. Further studies are however required to confirm trends, as a

recent study has provided results indicating trends that are contradictory to those reported here or by Smolen *et al.,* 2013 (Labuda *et al.,* 2014).

Our observations, following the stimulation of whole blood samples for 72 hours were however to the contrary. Results generally indicated higher TNF- α and IL-10 production in the R than the urban areas, especially following stimulation with PPD and PHA (Figure 3).TNF-α/IL-10 indices indicated a clear urban-rural trend with the UH area reporting the lowest indices. Considering that calculated indices were below the threshold, it could be concluded that pro-inflammatory activity was generally low (Figure 3). In a related HDMassociated case-control study, PHA was found after 72 hours of stimulation in whole blood samples, to induce strong responses of both pro-and anti-inflammatory cytokines. In the same study as well, area stratification of adaptive cytokine responses revealed higher TNF-α production in the R than the urban area in association with HDM-positivity (Amoah *et al.,* 2014). This is in consonance with our observations, despite the different selection criteria of subjects in both studies. Indeed, for our subjects, high IL-10 production was observed in all three areas for PPD and PHA. Furthermore, van den Biggelaar *et al.,* (2000), from cellular experiments involving Gabonese *S. haematobium*-positive subjects, reported increased peripheral blood mononuclear cell (PBMC) proliferation for all study subjects following stimulation with PHA. This was similarly observed for PPD in related T-lymphocyte proliferation assays. For PHA, this is not surprising, as it is a well-known stimulus of polyclonal T-cell activity. However, the observation of higher TNF-α and IL-13 production in the R than the urban areas with regard to PPD is interesting, considering firstly, the higher prevalence of helminth infections in the R area; and secondly, that children in Ghana are administered the tuberculosis vaccine, Bacillus Calmette-Guerin (BCG), within the first week

of birth (Amoah *et al.,* 2014). In a longitudinal study in Indonesia involving children recruited at birth from 2 adjacent villages and followed up on at various time points for 24 months, whole blood stimulation assays indicated consistently lower TNF-α production to PPD at all time points for those born to helminth-infected mothers, as compared to those born to uninfected mothers (Djuardi *et al.,* 2010). Although this contradicts our observations, differences in results may be due to differences in study design and ages of participants recruited. Clearly, our findings necessitate further study to ascertain trends in our population.

Following a recent discovery in related studies of a tight correlation existing between ImmunoCAP-measured peanut-specific IgE (pIgE) and bromelain among our subjects (Amoah *et al.,* 2013), we sought to examine in our current subset, pro-, anti-inflammatory, and Th2-associated cytokine profiles following stimulation with innate and adaptive stimuli. Bromelain is a well-established cross-reactive carbohydrate determinant (Amoah *et al.,* 2013, 2014). It is also a glycoprotein derived from the stems of pineapples, and bears asparagine (N)-linked oligosaccharide side chains with α3-fucose and β2-xylose core modifications (van Ree and Aalberse, 1995; van Ree *et al.,* 1995; van Ree, 2002). Although IgEs recognizing these glycan epitopes are generally considered to be clinically irrelevant with regard to allergic disease diagnostics (Mari *et al.,* 2008), their possible relevance in ameliorating clinical symptoms of allergic reactions is being intensively explored. We found, after stimulating whole blood with Pam3, Poly(I:C), LPS, and AWA for 24 hours, higher TNF-α production among pIgE-negative than pIgE-positive participants for all innate agonists. The converse was observed for IL-10 production to all innate stimuli (Figure 4), hence the higher pro-inflammatory index among pIgE-negative participants. This is indicative of a lower pro-inflammatory environment among pIgE-positive participants, an expected outcome

considering that a larger number of our pIgE-positive subjects are from the R area, where a higher prevalence of parasitic infections was observed (Table 2). Indeed, we have unpublished data indicating higher median IgG4 levels in the R, as compared to the urban areas. Although higher TNF-α levels were observed among pIgE-positive subjects after stimulating with PPD and PHA for 72 hours, IL-10 production was even higher for these same stimuli, and pro-inflammatory indices generally very low (Figure 5a, b). This was similarly observed, regarding Th1/Th2 (TNF-α/IL-13) indices for PPD and AWA.

Regarding helminth infections, we observed among hookworm-positives lower TNF-α levels to all the innate stimuli than among hookworm-negative participants. This influenced the lower pro-inflammatory indices observed among hookworm-positive participants (Figure S1). Furthermore, our observations are in line with suggestions that the dampening of TLR function may be due to the continuous exposure of subjects to high microbial or parasitic stimuli (Savidge *et al.,* 2006; Braun-Fahrlander *et al.,* 2002; Netea *et al.,* 2011). However, observations following 72-hour stimulations with PPD, PHA, and AWA indicate higher proinflammatory cytokine production among hookworm-positives (Figure S3). Nevertheless, the generally low pro-inflammatory indices are indicative of the influence of innate immune responses on adaptive immune outcomes (Nookola *et al.,* 2004; Everts *et al.,* 2010; Labuda *et al.,* 2014). Similar to hookworm-positive participants, we realized among *S. haematobium*-positives lower TNF-α/IL-10 indices than among *S. haematobium*-negatives subjects after 24h stimulation with Poly(I:C), Pam3, and LPS (Figure S2). The opposite was however realized among *S. haematobium*-positives, following 72-hour stimulations with PHA and PPD. It is not clear why this is the case, considering that chronic schistosomiasis is generally attributed with a more regulatory immune environment (van den Biggelaar, 2000,

Labuda *et al.,* 2011). Indeed, in a study conducted by Labuda *et al.,* (2011), PBMCs from Gabonese *S. haematobium*-infected children were found to produce significantly higher IL10 titres, following stimulations for 72 hours with schistosomal egg antigen (SEA) and AWA. Observed differences between both observations may be due, among other factors, to the different stimuli and samples used. Further studies would be necessary to test observed trends.

Altogether, we have described urban-rural differences in innate and adaptive immune responses for our subset of participants, and have attempted to describe the innate and adaptive immune environments existing in the presence of glycan-associated cross-reactive IgE positivity, as well as hookworm and *S. haematobium* infections. As this is a pilot study, it would be essential to further scrutinize findings using a larger sample size.

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TITLE: Urban-rural differences in pro- and anti-inflammatory cytokine production: a case for IgE cross-reactivity in a selection of Ghanaian pupils

MAIN TABLES AND FIGURES

Table 1: Stimuli used in this study, and the cytokines measured

Stimulus	Classification	Final Concentration	Incubation Period	Cytokine(s) measured
Pam3CSK4	TLR1/2 stimulus	100µg/ml	24hrs	TNFα, IL10
poly(I:C)	TLR3 stimulus	50µg/ml	24hrs	TNFα, IL10
LPS	TLR4 stimulus	1ng/ml	24hrs	TNFα, IL10
AWA	*S. haematobium* adult worm antigen	-	24hrs & 72hrs	TNFα, IL10,IL13
PHA	Polyclonal T-cell stimulus	2µg/ml	72hrs	TNFα, IL10
PPD	Tuberculosis antigen	10µ/ml	72hrs	TNFα, IL10, IL13

.

Table 2: General Characteristics of participants involved in study

Factor	Urban High [n/N (%)]	Urban low [n/N (%)]	Rural [n/N (%)]	p-value
Gender				
Male	23/35(65.71)	18/34(52.94)	14/35(40.00)	0.098
Female	12/35(34.29)	16/34(47.06)	21/35(60.00)	
Age				
< 11 years	11/35(31.43)	15/34(44.12)	17/35(48.57)	0.320
≥ 11 years	24/35(68.57)	19/34(55.88)	18/35(51.42)	
Parasitic infections (infected)				
Schistosoma haematobium	0/35 (0.00)	3/34(8.82)	2/35(5.71)	0.210
Schistosoma mansoni	0/35 (0.00)	0/34(0.00)	0/35 (0.00)	-
Ascaris lumbricoides	0/35 (0.00)	0/34(0.00)	0/35 (0.00	-
Trichuris sp.	0/35 (0.00)	0/34(0.00)	0/35 (0.00)	-
Necator americanus	0/35 (0.00)	0/34(0.00)	15/35(44.12)	**<0.001**
Any Helminth	0/35 (0.00)	3/34(8.82)	17/35(48.57)	**<0.001**
Plasmodium sp.	2/35(5.71)	2/34(5.88)	13/35(37.14)	**<0.001**
Measured IgE levels by ImmunoCAP (≥0.35kU/L)				
Cockroach (Bla g.)	9/35(25.71)	10/34(29.41)	23/35(65.71)	**<0.001**
House Dust Mite (Der p.)	8/35(22.86)	8/34 (23.53)	14/35(40.00)	0.217
Peanut (Ara h.)	5/35(14.29)	5/34 (14.71)	13/35(37.14)	**0.035**
Skin Prick Test results (positives)				
Cockroach (Bla g.)	4/35(11.43)	2/34(5.88)	13/35(37.14)	**0.002**
House Dust Mite (Der p.)	5/35(14.29)	5/34(14.71)	5/35(14.29)	0.990
Peanut (Ara h.)	3/35(8.57)	0/34 (0.00)	1/35(2.86)	0.168

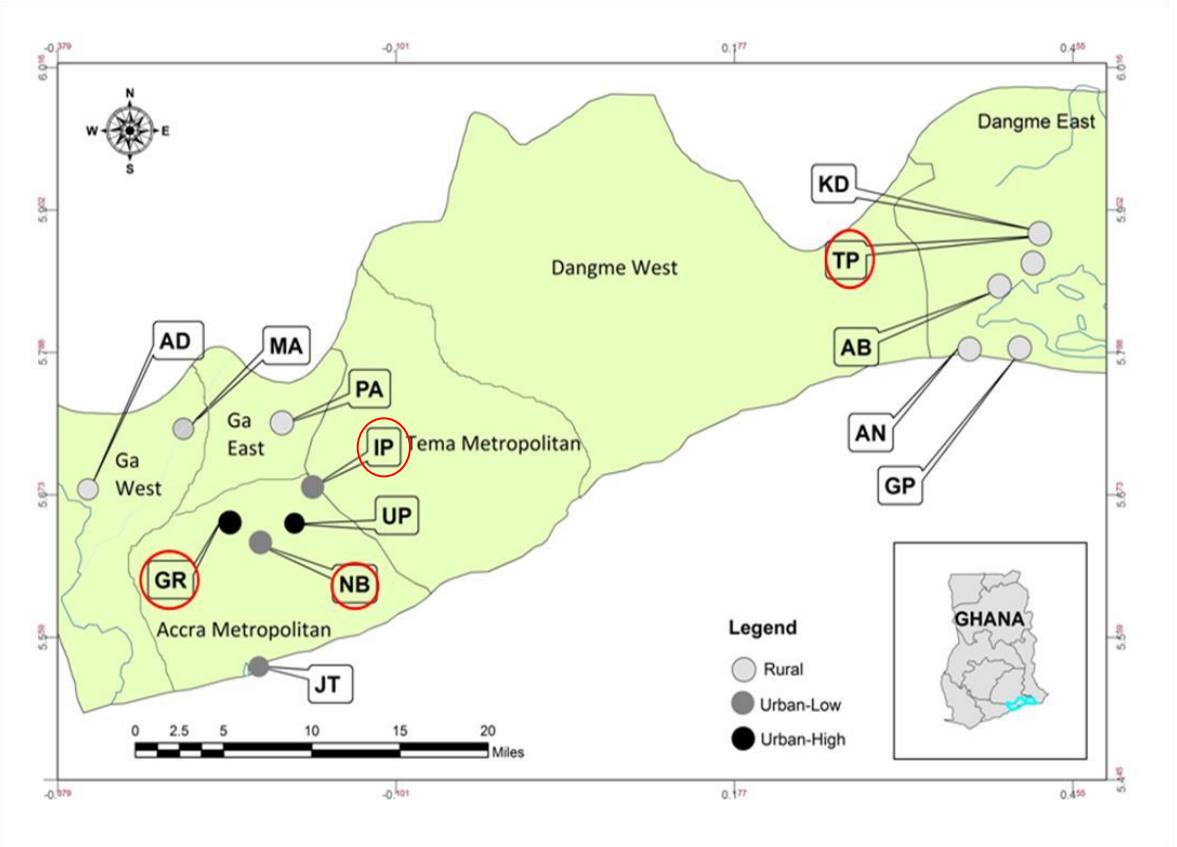

Figure 1: A map of the Greater Accra Region depicting the locations of the schools selected for this study. Selected schools are circled in red (Courtesy, Amoah *et al.*, 2013)

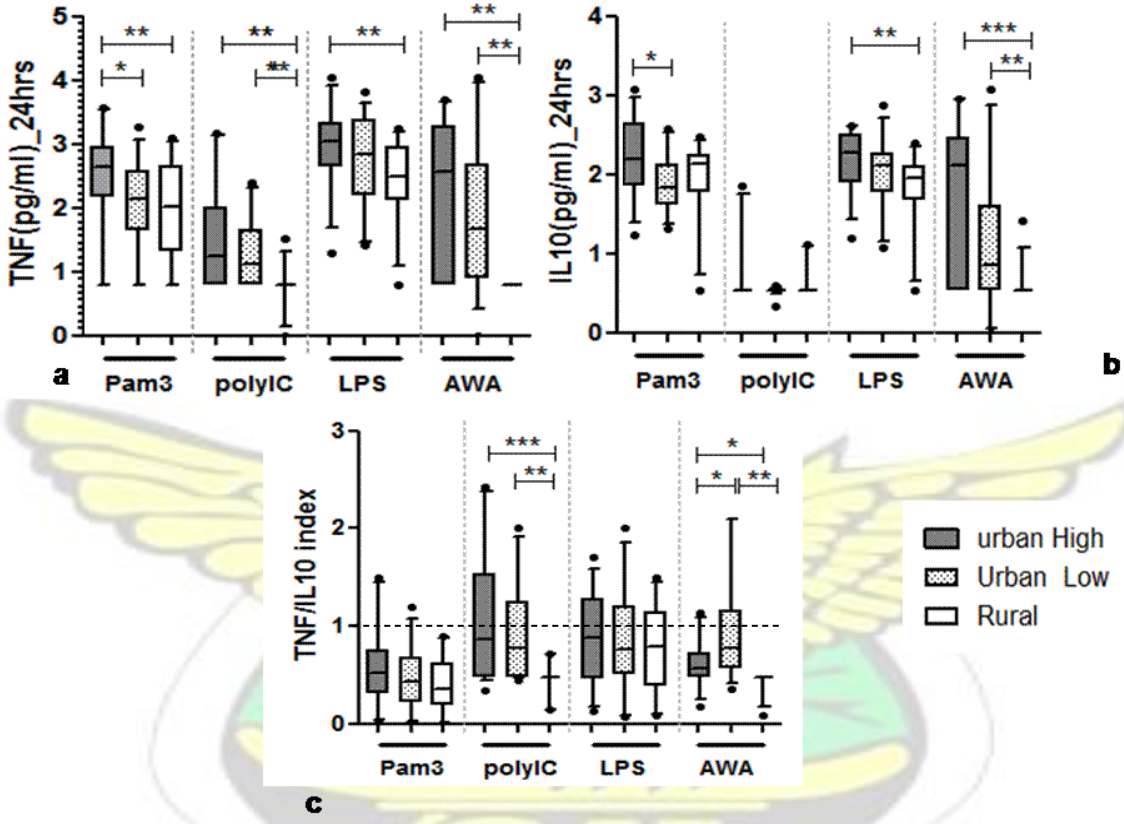

Figure 2: Urban-rural differences in the measurement of pro- and anti-inflammatory cytokine production in whole blood after 24hr stimulation. (a) Urban-rural comparisons of TNF-α production following 24h stimulation with Pam3, Poly(I:C), LPS, and AWA. **(b)** Urban-rural comparisons of IL-10 production following 24h stimulation with the same stimuli. **(c)** Urban-rural comparisons of TNF/IL-10 cytokine indices following 24h incubation with innate stimuli. The Kruskal-Wallis and Dunn's post tests were employed in analysis. An asterisk (*) indicates a p-value less than 0.05, two asterisks (**) represents a p-value less than 0.01, and three asterisks (***) denotes a p-value less than 0.001. Broken line is set at threshold value (i.e. 1).

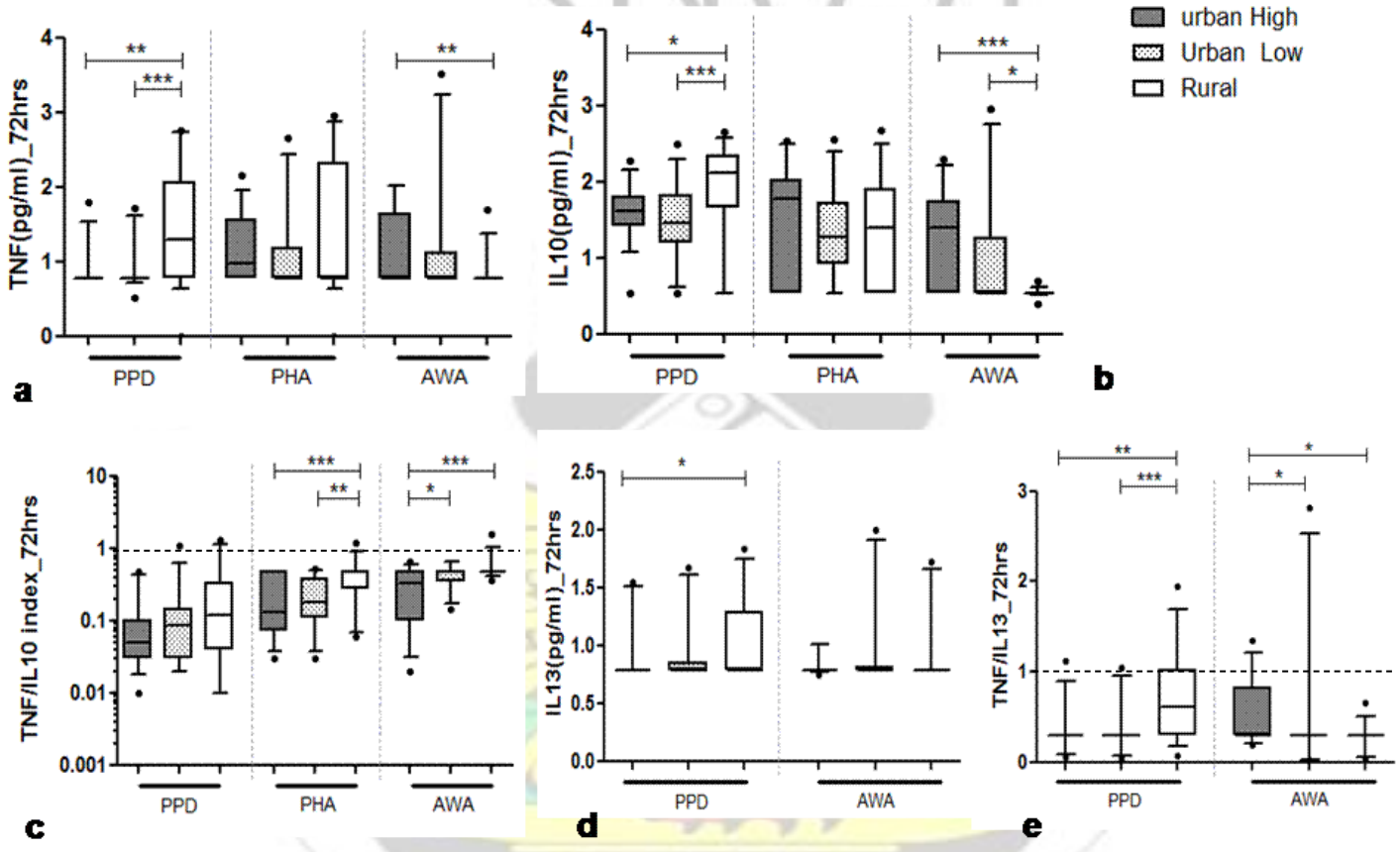

Figure 3: Urban-rural differences in the measurement of pro- and anti-inflammatory cytokine production in whole blood after 72hr stimulation. (a) Urban-rural comparisons of TNF-α production following 72h stimulation with PPD, PHA, and AWA. **(b)** Urbanrural comparisons of IL-10 production following 72h stimulation with the same stimuli. **(c)** Urban-rural comparisons of TNF/IL-10 cytokine indices following 72h incubation with adaptive stimuli. The Kruskal-Wallis and Dunn's post tests were employed in analysis. An asterisk (*) indicates a p-value less than 0.05, two asterisks (**) represents a p-value less than 0.01, and three asterisks (***) denotes a pvalue less than 0.001. Broken line is set at threshold value (i.e. 1).

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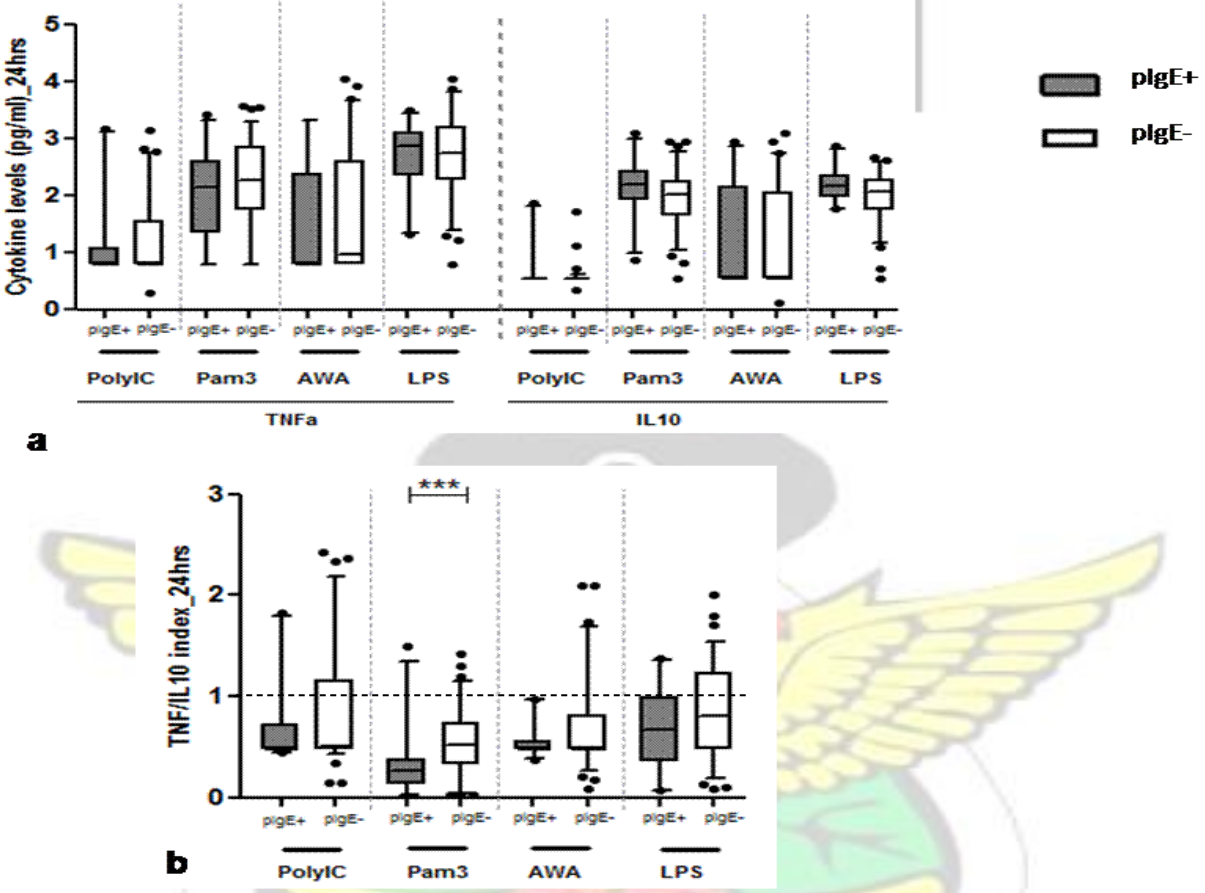


Figure 4: Differences in the measurement of pro- and anti-inflammatory cytokine production between pIgE-positive and pIgEnegative subjects in whole blood after 24hr stimulation. (a) Comparisons of TNF-α production following 24h stimulation with Poly(I:C), Pam3, AWA and LPS,. **(b)** Comparisons of IL-10 production following 24h stimulation with the same stimuli. **(c)** Comparisons of TNF/IL-10 cytokine indices following 24h incubation with innate stimuli. The Mann Whitney U test was employed in analysis. An asterisk (*) indicates a p-value less than 0.05, two asterisks (**) represents a p-value less than 0.01, and three asterisks (***) denotes a pvalue less than 0.001. Broken line is set at threshold value (i.e. 1)..

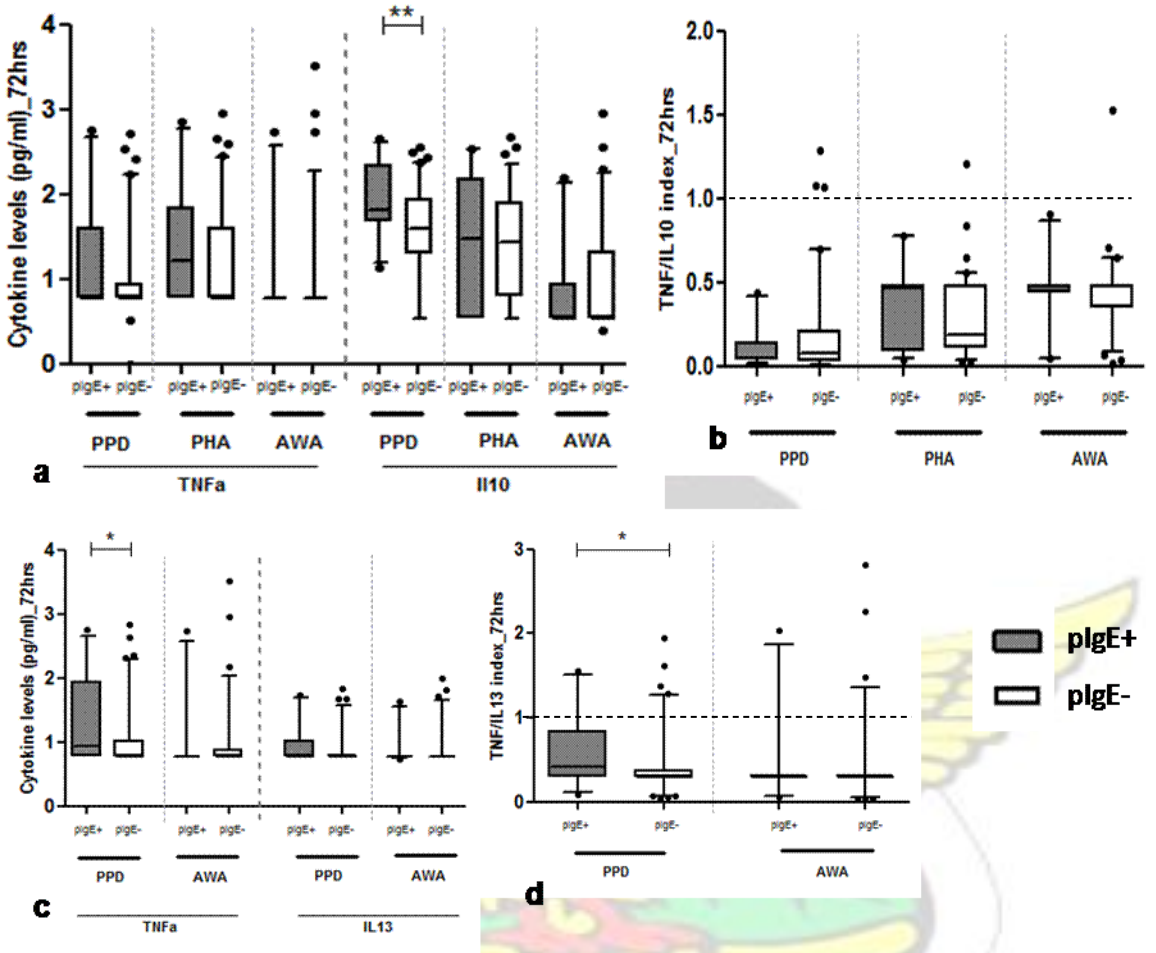


Figure 5: Differences in the measurement of pro- and anti-inflammatory cytokine production between pIgE-positive and pIgE-negative subjects in whole blood after 72hr stimulation. (a) Comparisons of TNF-α and IL-10 production following 72h stimulation with adaptive stimuli. **(b)** Comparisons of TNF/IL-10 cytokine indices following 72h incubation with innate stimuli. (c) Comparisons of TNF- α and IL-13 production following 72h stimulation with PPD and AWA. (d) Comparisons of TNF/IL-13 cytokine indices with respect to PPD and AWA. The Mann Whitney U test was employed in analysis. An asterisk (*) indicates a p-value less than 0.05, two asterisks (**) represents a p-value less than 0.01, and three asterisks (***) denotes a p-value less than 0.001. Broken line is set at threshold value (i.e. 1)

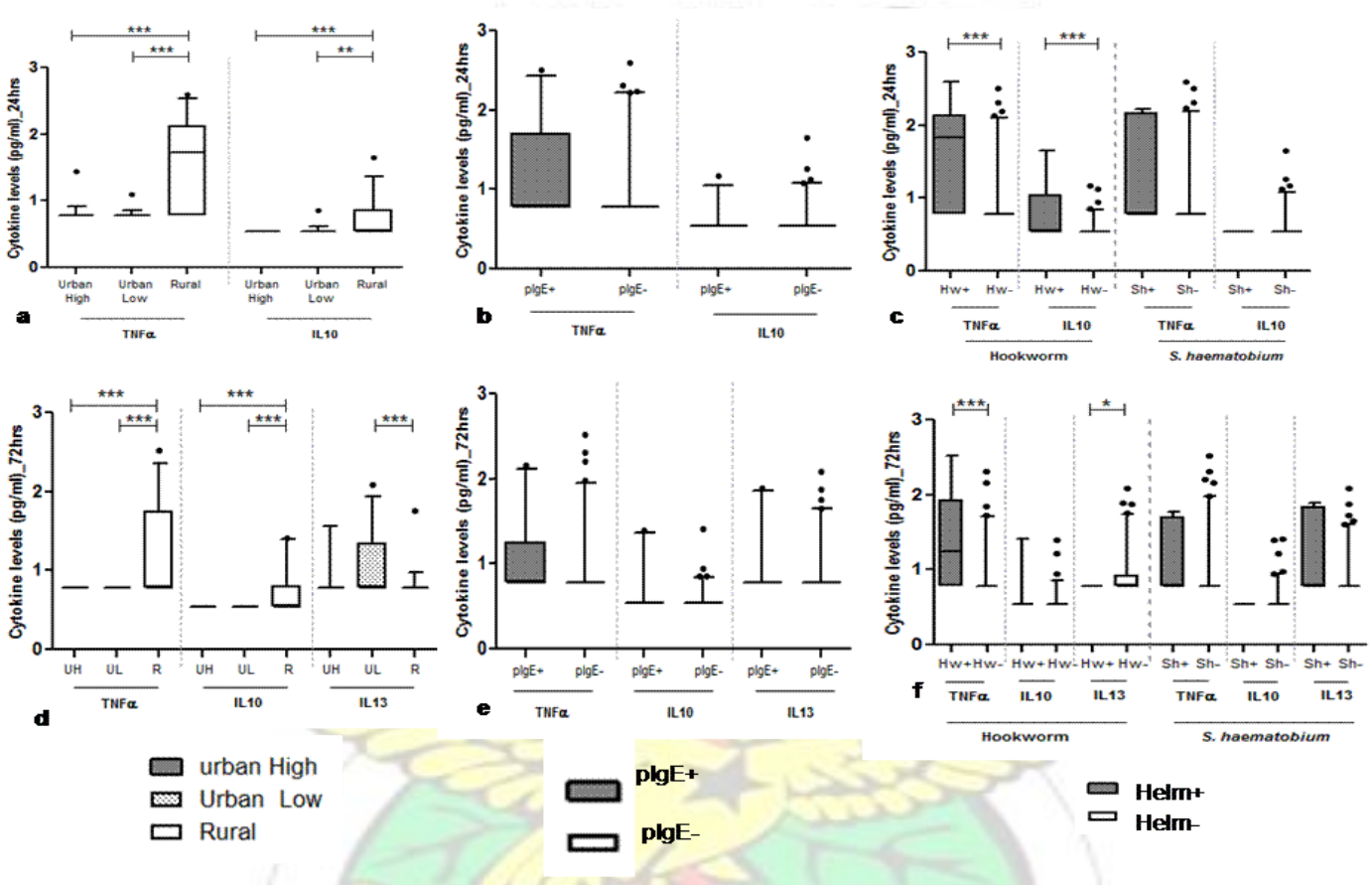
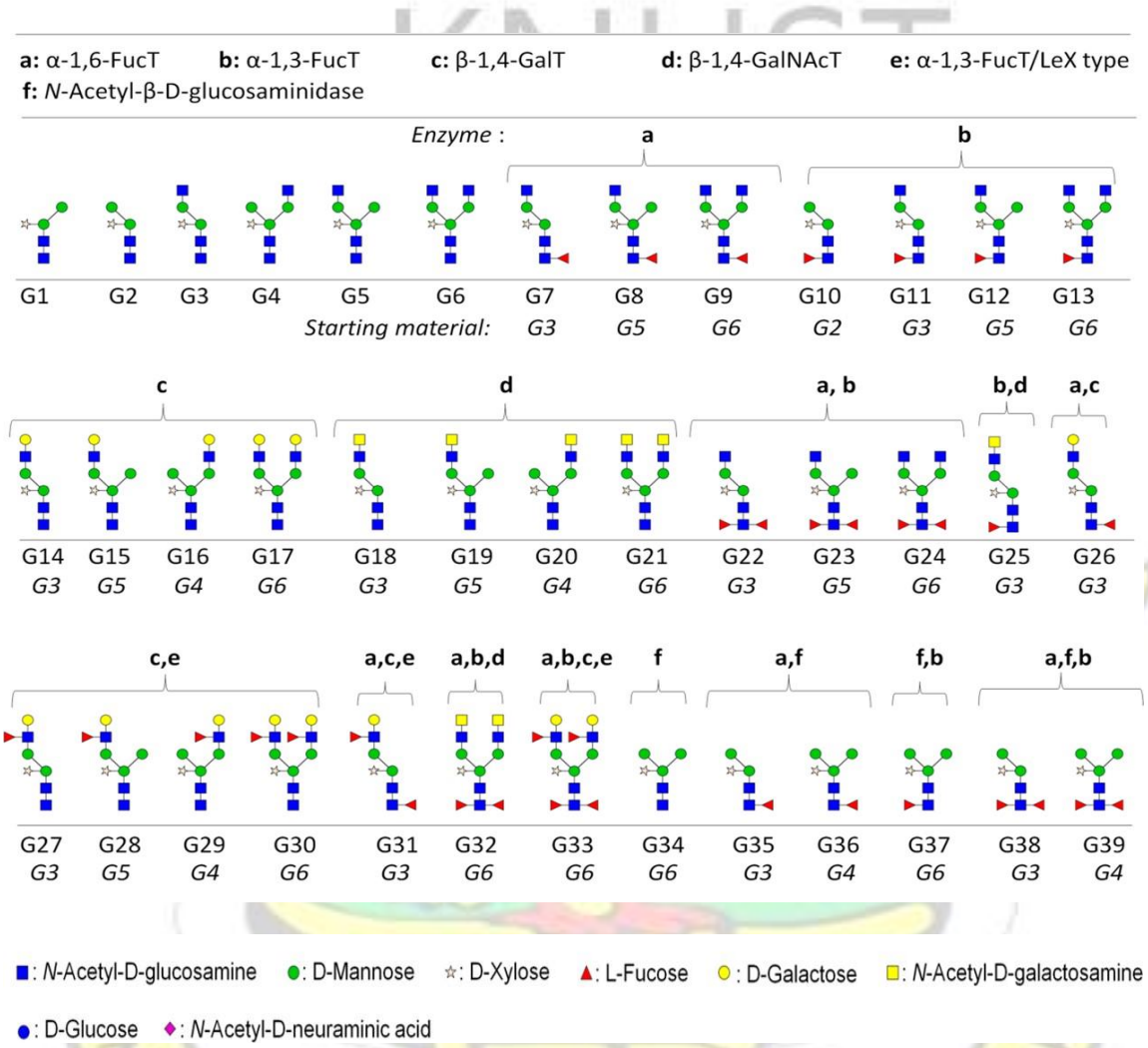


Figure 6: Differences in measured pro- and anti-inflammatory cytokine production in unstimulated whole blood obtained from study participants. **(a)** Urban-rural differences in spontaneous cytokine production after incubation for 24h. **(b)** Differences in spontaneous cytokine production between pIgE-positive and negative participants following 24h incubation. **(c)** Comparison of spontaneous cytokine production between helminth-positive and negative subjects after incubation for 24h. **(d)** Urban-rural differences in cytokine production following unstimulated incubation for 72h. **(e)** Differences in spontaneous cytokine production between pIgE-positive and negative participants following 72h unstimulated incubation. **(f)** Comparison of spontaneous cytokine production between helminthpositive and negative subjects after unstimulated incubation for 72h. An asterisk (*) indicates a p-value less than 0.05, two asterisks (**) represents a p-value less than 0.01, and three asterisks (***) denotes a p-value less than 0.001.

APPENDIX III: ILLUSTRATION OF THE ARRAY OF 128 GLYCANS WITH ASSOCIATED CODES AND ENZYMES INVOLVED IN THEIR SYNTHESIS (ref: Brzezicka *et al*., 2015)



Enzymes:

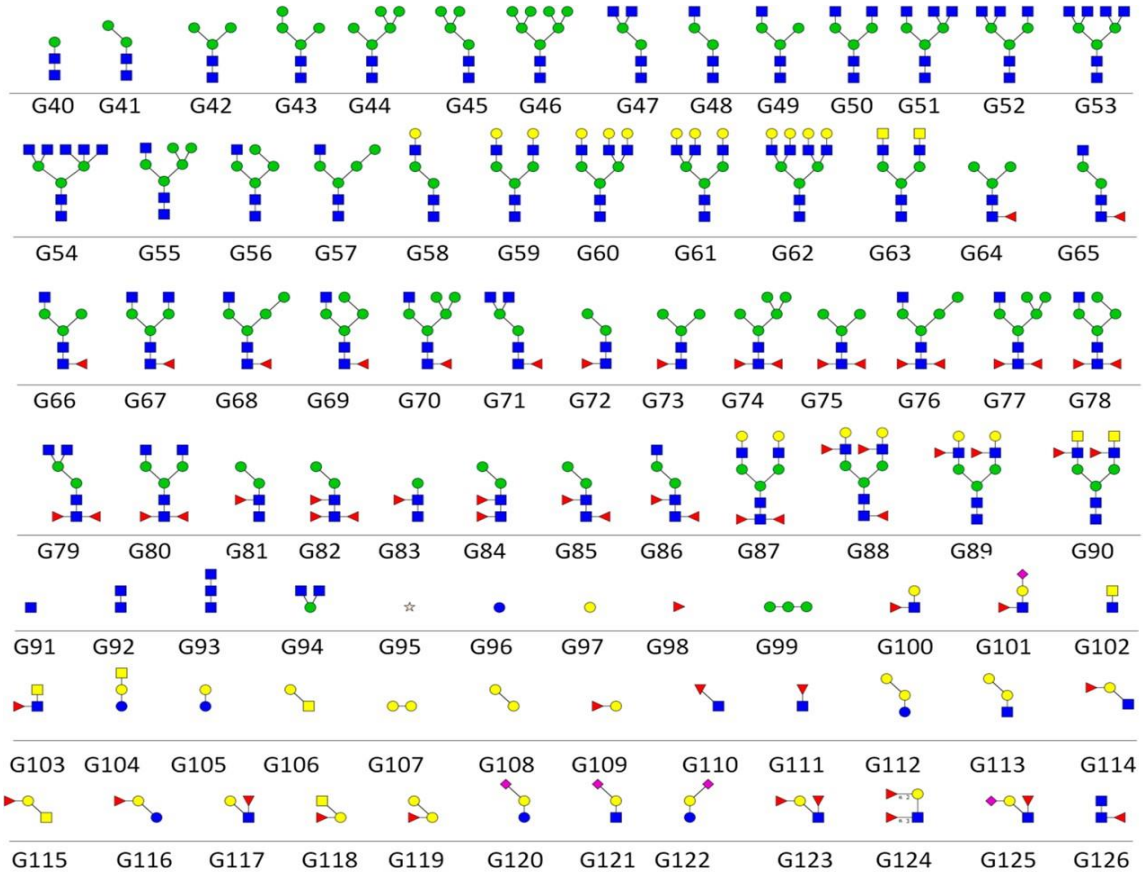
a. α-1,6-FucT = α-1,6-fucosyltransferase;

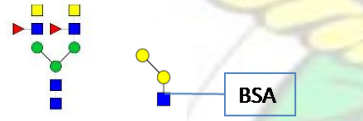
b. α-1,3-fucT = α-1,3-fucosyltransferase;

c. β-1,4-GalT = β-1,4-galactosyltransferase;

d. β-1,4-GalNAcT = N-Acetyl-β-1,4-Galactosaminidase;

e. α-1,3-fucT/LeX type = α-1,3-fucosyltransferase/Lewis X-type transferase

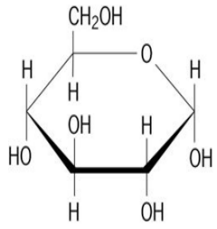
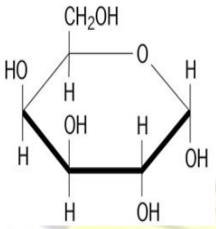
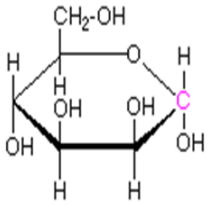
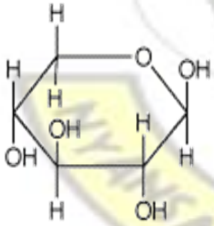


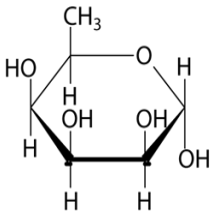
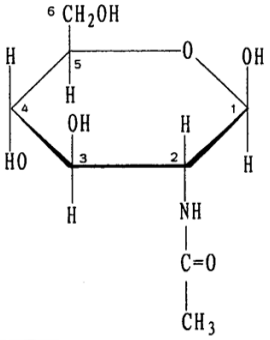
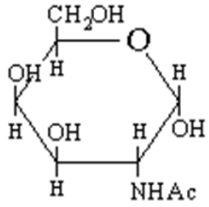


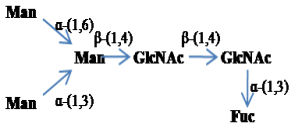
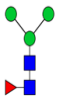
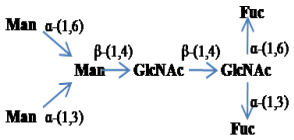
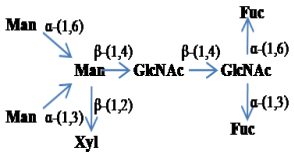
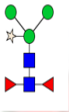
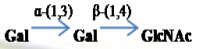
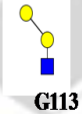
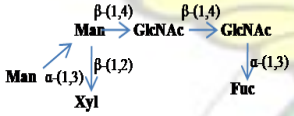
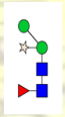
■: *N*-Acetyl-D-glucosamine ●: D-Mannose ☆: D-Xylose ▲: L-Fucose ○: D-Galactose □: *N*-Acetyl-D-galactosamine

●: D-Glucose ◆: *N*-Acetyl-D-neuraminic acid

APPENDIX IV: CHEMICAL STRUCTURES OF GLYCANS UNTILIZED IN THIS STUDY

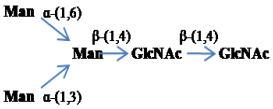
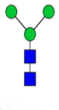
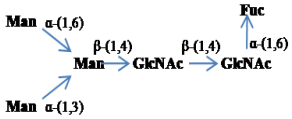
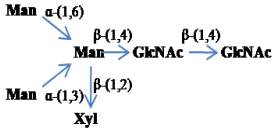
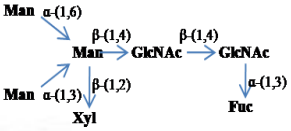
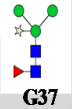
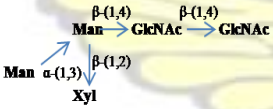
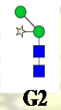
Chemical Structure	Structure in Symbols (Code Name)	Chemical Name
	 G96	α-D-glucose
	 G97	α-D-galactose
		α-D-mannose
	 G95	α-D-xylose

Chemical Structure	Structure in Symbols (Code Name)	Chemical Name
	 G98	α-D-fucose
	 G91	N-Acetyl-D-glucosamine
		N-Acetyl-D-galactosamine

Chemical Structure	Structure in Symbols (Code Name)	Chemical Name
	 G73	Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAcβ1-
	 G75	Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-3)(Fucα1-6) GlcNAcβ1-
	 G39	Manα1-3(Manα1-6)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3) (Fucα1-6)GlcNAcβ1-
	 G113	Galα1-3Galβ1-4GlcNAcβ1-
	 G10	Manα1-3(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAcβ1-

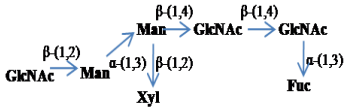
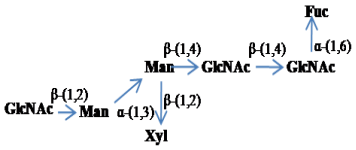
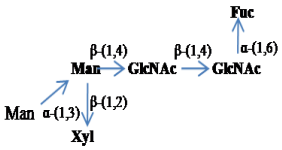
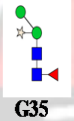
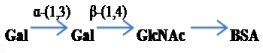
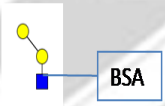
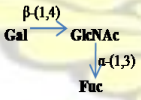
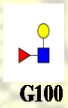
■: *N*-Acetyl-D-glucosamine ●: D-Mannose ☆: D-Xylose ▲: L-Fucose ○: D-Galactose □: *N*-Acetyl-D-galactosamine

●: D-Glucose ◆: *N*-Acetyl-D-neuraminic acid

Chemical Structure	Structure in Symbols (Code Name)	Chemical Name
	 G42	Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ1-
	 G64	Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ1-
	 G34	Manα1-3(Manα1-6)(Xylβ1-2)Manβ1-4GlcNAcβ1-4GlcNAcβ1-
	 G37	Manα1-3(Manα1-6)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3) GlcNAcβ1-
	 G2	Manα1-3(Xylβ1-2)Manβ1-4GlcNAcβ1-4GlcNAcβ1-

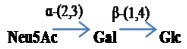
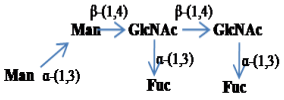
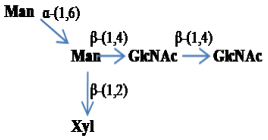
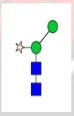
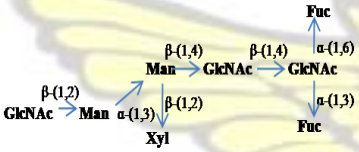
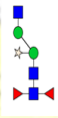
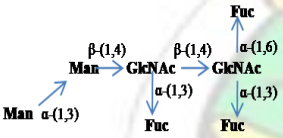
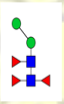
■: *N*-Acetyl-D-glucosamine ●: D-Mannose ☆: D-Xylose ▲: L-Fucose ○: D-Galactose □: *N*-Acetyl-D-galactosamine

●: D-Glucose ♦: *N*-Acetyl-D-neuraminic acid

Chemical Structure	Structure in Symbols (Code Name)	Chemical Name
	 G11	(GlcNAcβ1-2)Manα1-3(Xylβ1-2)Manβ1-4GlcNAcβ1-4 (Fucα1-3)GlcNAcβ1-
	 G7	(GlcNAcβ1-2)Manα1-3(Xylβ1-2)Manβ1-4GlcNAcβ1-4 (Fucα1-6)GlcNAcβ1-
	 G35	Manα1-3(Manα1-6)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3) (Fucα1-6)GlcNAcβ1-
		Galα1-3Galβ1-4GlcNAcβ1-BSA
	 G100	Galβ1-4(Fucα1-3)GlcNAcβ1-

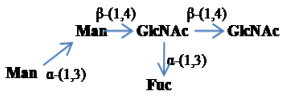
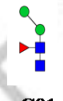
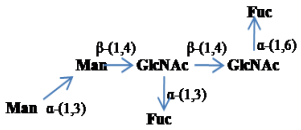
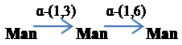
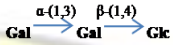
■: *N*-Acetyl-D-glucosamine ●: D-Mannose ☆: D-Xylose ▲: L-Fucose ○: D-Galactose □: *N*-Acetyl-D-galactosamine

●: D-Glucose ◆: *N*-Acetyl-D-neuraminic acid

Chemical Structure	Structure in Symbols (Code Name)	Chemical Name
	 G120	Neu5Acα2-3Galβ1-4GlcNAcβ1-
	 G84	Manα1-3Manβ1-4(Fucα1-3)GlcNAcβ1-4 (Fucα1-3)GlcNAcβ1-
	 G1	(Manα1-6)(Xylβ1-2)Manβ1-4GlcNAcβ1-4GlcNAcβ1-
	 G22	(GlcNAcβ1-2)Manα1-3(Xylβ1-2)Manβ1-4GlcNAcβ1-4 (Fucα1-3) (Fucα1-6)GlcNAcβ1-
	 G82	Manα1-3Manβ1-4(Fucα1-3)GlcNAcβ1-4 (Fucα1-3) (Fucα1-6)GlcNAcβ1-

■: *N*-Acetyl-D-glucosamine ●: D-Mannose ☆: D-Xylose ▲: L-Fucose ○: D-Galactose □: *N*-Acetyl-D-galactosamine

●: D-Glucose ◆: *N*-Acetyl-D-neuraminic acid

Chemical Structure	Structure in Symbols (Code Name)	Chemical Name
	 G81	Manα1-3Manβ1-4(Fucα1-3)GlcNAcβ1-4 GlcNAcβ1-
	 G85	Manα1-3Manβ1-4(Fucα1-3)GlcNAcβ1-4 (Fucα1-6)GlcNAcβ1-
	 G99	Manα1-3(Manα1-6)Manβ1-
	 G112	Galα1-3Galβ1-4Glcβ1-
	 G111	(Fucα1-3)GlcNAcβ1-

■: *N*-Acetyl-D-glucosamine ●: D-Mannose ☆: D-Xylose ▲: L-Fucose ○: D-Galactose □: *N*-Acetyl-D-galactosamine

●: D-Glucose ◆: *N*-Acetyl-D-neuraminic acid