Abnormal IgA1 *O*-Glycosylation in a Multi-ethnic Population of IgA Nephropathy Patients in KwaZulu Natal, South Africa

Thesis submitted in partial fulfillment of the requirements for the degree of Master of Medical Science in the Department of Medicine.

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Declaration

The following work has not been submitted previously, to this, or any other University. The following work has been independently conceptualised, conducted, and completed by the candidate.

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Prof. A.G.H Assounga

Date: 19/12/13

Publication

An abstract of the following work entitled: Abnormal IgA1 *O*-Glycosylation in a Multi-ethnic population of IgA Nephropathy Patients in KwaZulu Natal, South Africa, was published in the *Cardiovascular Journal of Africa*, August 2012 supplement vol. 23 no. 7: pg. 16.

Presentations (South Africa; Ghana; Hong Kong; Italy**)**

Oral -

First Roche Renal Expert Forum

The following work was selected for oral presentation at the First Roche Renal Expert Forum. The forum hosted several international presenters and attendees; and was held at the Zimbali Fairmont Resort, in KwaZulu Natal (KZN), South Africa, from the 11th to the 12th of March 2011.

South African Congress of Nephrology

The following work was selected for oral presentation at the South African Congress of Nephrology. The Congress was held in Pretoria at the Council for Scientific and Industrial Research (CSIR) International Convention Centre, from the 30th of August to the 2nd of September 2012. The Congress included presentations from several international delegates.

Poster -

The following work was selected for presentation at the 8th Conference of the Federation of African Immunological Societies in Durban, KZN on the 3rd of December, 2012. The conference hosted national and international presenters and attendees.

The following work was selected for presentation at the African Association of Nephrology-African Pediatrics Nephrology Association Congress in Ghana on the 22nd of February, 2013.

The following work was selected for presentation at the International Society of Nephrology (ISN) World Congress of Nephrology in Hong Kong, on the 1st of June 2013.

The following work was selected for presentation at the International Congress of Immunology in Milan, Italy on the 23rd of August 2013.

Acknowledgements

The grace of **God** has enabled the completion of this work.

Prof. A.G.H. Assounga is the Head of the Department of Nephrology in the Division of Internal Medicine at the Nelson R. Mandela School of Medicine (NRMSM), University of KwaZulu Natal (UKZN); and Chief Nephrologist at the Inkosi Albert Luthuli Central Hospital (IALCH) in Durban, KZN. Prof. A.G.H. Assounga has provided expert supervision; scientific and statistical input; expert advice and guidance; and continued support. Prof. A.G.H Assounga has also provided teachings in the field of Immunology and facilitated and guided the study thereof.

Dr. A.C. Smith is the Senior Research Scientist of the Renal Research Group, at the University of Leicester, in the United Kingdom. She has worked in the Research group since 1989, and has written several papers on the analysis of IgA1 *O*-glycosylation in IgA Nephropathy (IgAN) patients. Dr. Alice C. Smith has offered expert advice and guidance, prior to, and during the experiments.

Mr. S. Naidoo is a Metrologist at a local calibration company. Mr. S. Naidoo has assisted in the provision of some laboratory consumables. Mr. S. Naidoo had also hired a Phlebotomist and provided transport, to access the control subjects and some IgAN patients, for the extraction of blood samples.

Nurse S. Govender of the Renal Clinic, at the IALCH in Durban, KZN, provided visit dates for, and extracted blood samples from the IgAN patients.

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Abstract

Background: The pathogenesis of IgA Nephropathy (IgAN) is poorly understood globally and curative therapy currently does not exist. Variable presentation among IgAN patients globally may be indicative of various underlying pathogenic mechanisms. Pathogenetic data on IgAN in Africa is scarce to nil. The current study provides the first *O*-glycosylation data for IgAN in South Africa or Africa.

Methods: An enzyme-linked immunosorbent assay-type lectin binding assay was used to compare the serum IgA1 *O*-galactosylation in 19 IgAN patients and 20 controls. During 2007, 2009, and 2011, blood was extracted from consenting biopsy-diagnosed South African IgAN patients of African, Caucasian, Indian (predominantly) and mixed-race descent in KwaZulu Natal. The mean absorbance value corresponding to the degree of degalactosylation for the IgAN group was compared to that of the normal control group for each test. A non-parametric Wilcoxon matched-pairs test was used accordingly. The two-tailed p-value was used to assess for statistical significance between the groups. The low number of attending and consenting IgAN patients precluded IgA1 *O*-galactosylation analyses between race, gender, and disease stage.

Results: The average means of the experiments for the IgAN group is 0.3678 ± 0.0790 (SEM) and is statistically significantly greater than the normal control group which is 0.2969 ± 0.0586 (SEM); ($p = 0.0076$).

Conclusion: Thus, IgAN patients exhibited abnormal IgA1 *O*-glycosylation with a greater level of terminal degalactosylation of IgA1 in comparison to controls. Such a finding is consistent with other studies in Caucasian and Asian populations globally. Future specific therapeutic strategies that target the formation of abnormal glycosylation in IgA1 may be potentially beneficial in the study population.

CHAPTER 1 - INTRODUCTION

1.1 Definition of IgAN

IgAN, also eponymously known as Berger's disease, was first described in 1968 by Berger and Hinglais, as the presence of mesangial deposits of immunoglobulin A (IgA) and lesser amounts of complement (C) 3, as detected by immunofluorescence in patients with frequent episodes of asymptomatic

haematuria and proteinuria. Berger subsequently proposed primary glomerulonephritis, with a predominance of mesangial IgA deposits, as being a distinct disease entity (1969).

1.2 Role and Structure of IgA

IgA is the most abundant antibody in mucosal secretions, and plays a significant role as the first line of defence against invading pathogenic organisms and the entry of commensals at the mucosal border (Kerr, 1990). IgA is also present in the serum where it is the second most common antibody and serves as the second line of defence.

Two subclasses of IgA exist, i.e. IgA1 and IgA2. Monomeric IgA1 consists of two identical heavy and two identical light polypeptide chains (Kerr, 1990). The heavy and light chains are held together by disulphide bonds and non-covalent interactions. Dimeric IgA1 consists of two IgA monomers covalently linked by a polypeptide J chain (fig. 1). The cysteine (Cys) 471 in one tailpiece of each Fc fragment is disulphide-bridged with either Cys 14 or Cys 68 of the J chain. Each heavy chain consists of a variable domain, referred to as the variable heavy domain (VH), and 3 constant heavy (CH) domains. Each light chain consists of

1 variable light (VL) and 1 constant light domain (CL) domain. The constant domain contains an amino acid sequence that does not vary between immunoglobulins of a particular class; whilst the variable region differs between immunoglobulins produced by different B-lymphocytes. The variable regions form the antigen binding sites. The region within the antibody that complements the shape of the antigen is referred to as the complementarity determining region (CDR).

The proteolytic enzyme, papain, cleaves the hinge region to yield two fragments of antigen binding or Fab fragments and 1 fragment of crystallisation or Fc fragment, per monomer. Each Fab fragment contains the VH and CH1 domains, and the light chain. The Fc fragment contains the CH2 and CH3 domains.

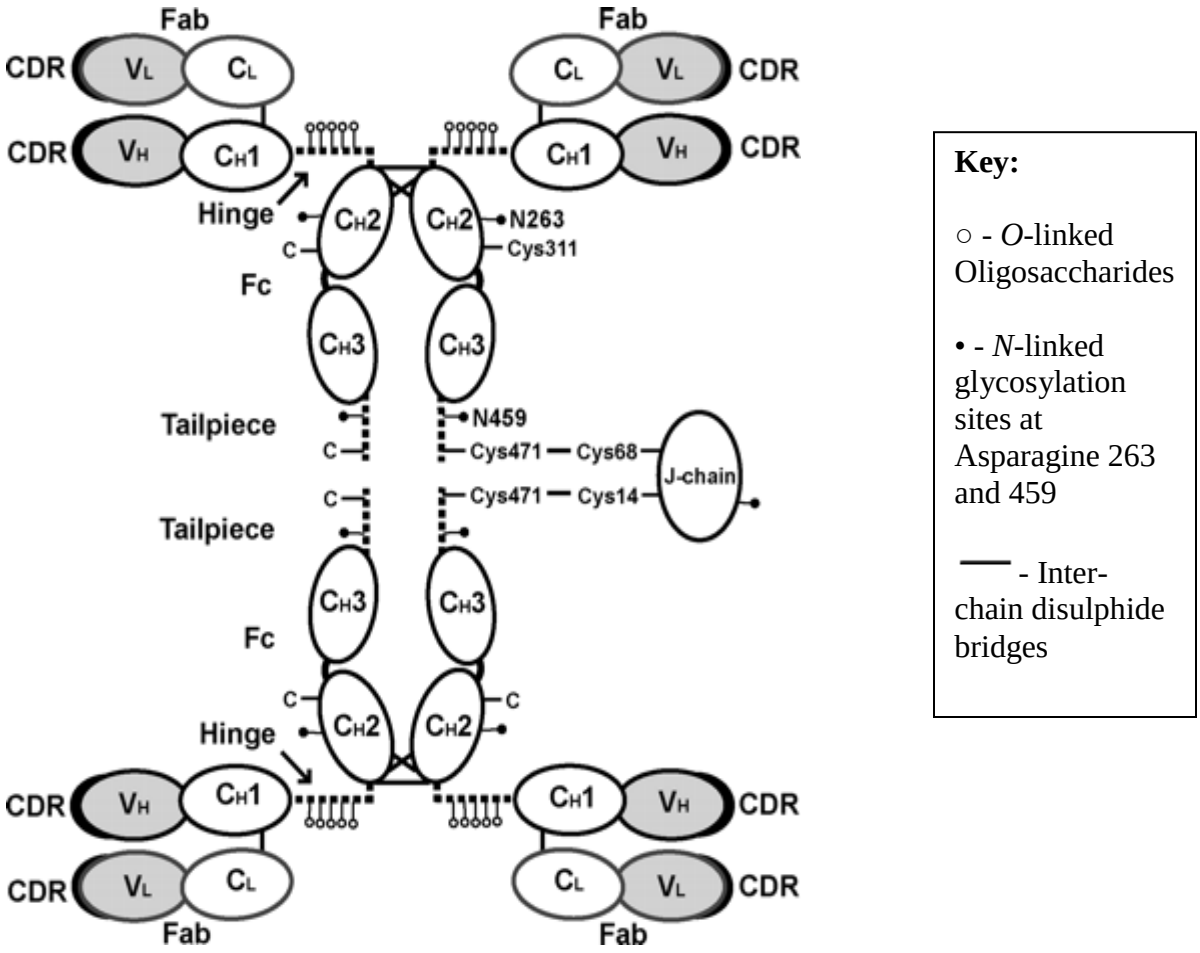

Figure 1: Schematic Representation of the Dimeric IgA1 Molecule (Bonner *et al*, 2008) - r*eproduced following permission from the Journal of Immunology - Copyright 2008. The American Association of Immunologists, Inc.* (Appendix 9)

The hinge region confers flexibility to the antibody molecule and contains the *O*-glycosylation sites. Unlike IgA1, the IgA2 hinge region does not contain *O*-glycosylation sites and is shorter (Furtado *et al*, 2004). The deposited mesangial IgA in IgAN is polymeric IgA1. This difference prompted investigation into the pathogenic role of abnormal IgA1 *O*-glycosylation.

1.3 Rationale for the Analysis of IgA1 *O*-Galactosylation, in a South African population of IgAN patients

1.3.1 The Regulatory and Excretory Roles of the Kidney are affected in IgAN

The kidneys are responsible for the regulation of the acid-base balance; water and electrolyte balances; body fluid osmolarity and electrolyte concentrations; and arterial pressure. The kidneys excrete metabolic waste products, toxins and foreign chemicals; secrete hormones (e.g. erythropoietin); and are involved in gluconeogenesis (Guyton & Hall, 1997).

The nephron is the functional unit of the kidney. Each kidney at birth contains approximately one million nephrons. The nephron contains the initial filtering component (the renal corpuscle) and a tubule specialised for reabsorption and secretion (the renal tubule). The renal corpuscle consists of the glomerulus and the Bowman's capsule. The glomerulus is situated within the Bowman's capsule at the end of a renal tubule (Guyton & Hall, 1997).

The glomerulus is a tuft of capillaries that performs the initial homeostatic filtration step in the formation of urine. IgAN is a primary glomerular disease that is diffuse (i.e. affecting all, or at least 50% of the glomeruli) and global (i.e. affecting the whole glomerulus, usually uniformly; or more than 50% of the glomerular segments) (Lawler, 1991). The kidney cannot regenerate new nephrons and the homeostatic functions are thus disrupted during the disease (Guyton & Hall, 1997).

1.3.2 Diagnosis and Clinical Manifestation of IgAN

The diagnosis of IgAN is made by the immunofluorescence staining of renal biopsy material. In IgAN, generalised, diffuse IgA1 deposition in the mesangia of all glomeruli, as the predominant immunoglobulin or co-dominant immunoglobulin with IgM, IgG, or both, is detected (Anderton & Thomson, 1988).

In patients with a moderate or advanced stage of IgAN, increased immunofluorescence staining of extra-cellular matrix components, such as type IV collagen, laminin and fibronectin, in the glomerular capillary walls and or

mesangia, has also been reported. In addition, C3, C5b-9, properdin and fibrinogen are usually detected. C1q may be present in minimal amounts.

In the absence of disease, the filterability of the solute is dependent on the size and charge of the molecule. Negatively charged, large molecules are filtered less easily than positively charged molecules of equal size. Negatively charged albumin, the most abundant serum protein, is repelled by the negative charges of the basement membrane proteoglycans, and is thus restricted from filtration. As the molecular weight of a molecule approaches that of albumin, the filterability rapidly decreases, approaching zero (Guyton & Hall, 1997). In IgAN, however, insoluble immune complexes become entrapped in the glomeruli, especially in the basement membrane. The entrapment of insoluble immune complexes results in the proliferation of many glomerular cells, mainly the epithelial and mesangial cells that lie between the endothelium and epithelium. In addition, the inflammatory reaction results in the entrapment of large numbers of leukocytes, causing blockage of many glomeruli. Unblocked glomeruli become excessively permeable, allowing both protein and erythrocytes to leave the blood of the glomerular capillaries and enter the glomerular filtrate (Guyton & Hall, 1997).

Clinical features of IgAN include gross haematuria that may follow respiratory infection; malaise; loin discomfort; and low-grade fever. A common finding is microscopic haematuria, with or without proteinuria, upon urinalysis (Anderton & Thomson, 1988).

The glomerular leakage of proteins and cytokines result in the injury and activation of the tubular epithelium. This in turn triggers the infiltration of leukocytes and the associated fibrotic response.

The ultrafiltered protein load of tubular epithelial cells activates mechanisms of tubulointerstitial injury and accelerates the progression of disease to end-stage renal failure (Abbate *et al*, 2006). The load of plasma proteins stimulates the expression of proinflammatory and profibrotic mediators in renal tubular cells (Tang *et al*, 2003; Drumm *et al*, 2002; Yard *et al*, 2001; Wang et *al*, 1997).

Zoja *et* al demonstrated *in vitro* that the plasma protein load induces the synthesis of vasoconstrictor peptide endothelin-1 in proximal tubular cells and mediates progressive renal injury by stimulating renal cell proliferation, extracellular matrix production and the attraction of monocytes (1995). Macrophages which feature prominently in the interstitial inflammatory

infiltrate, mediates the progression of renal injury, and macrophage numbers in renal biopsy may predict renal survival in chronic renal disease (Eardley *et al*, 2006). Macrophages regulate matrix accumulation via the release of growth factors such as transforming growth factor ß (TGF-ß) (Abbate *et al*, 2002). TGF-ß stimulates the transformation of interstitial cells into myofibroblasts. Proximal tubular epithelial cells promote fibrogenesis via the paracrine release of TGF-ß.

Donadelli *et al* demonstrated *in vitro* the induction of fractalkine in proximal tubular cells via protein overload (2003). Fractalkine may contribute to directing mononuclear cells into the peritubular interstitium and enhancing their adhesion property, thus favouring interstitial inflammation and disease progression.

Tang *et al*, demonstrated *in vivo* and *in vitro* the role of albumin in the upregulation of the potent chemokine interleukin (IL) 8 in proximal tubular epithelial cells through nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-Kb)-dependent pathways (2003). Acute exposure to albumin induced interleukin 8 expression in a time and dose-dependent manner. The generation of intracellular reactive oxygen species was also upregulated by albumin.

Excess complement ultrafiltration and complement synthesis in proximal tubular cells may underlie complement-mediated injury in chronic proteinuric renal disease. Tang *et al* demonstrated the upregulation of C3 messenger ribonucleic acid (mRNA) and biosynthesis in proximal tubular epithelial cells due to protein overload at the apical surface (1999).

Erkan *et al* demonstrated a dose and duration-dependent induction of apoptosis of cultured proximal tubular cells following albumin overload; mediated at least in part by the Fas-associated death domain protein-caspase 8 pathway (2001).

The deposition of C3 in the mesangium may be found in association with tissue injury in IgAN. While IgA does not fix complement, it may activate the complement cascade of the alternative pathway (Hiemstra *et al*, 1987). Activated complement may cause tissue injury either directly or indirectly by recruiting effector cells, which in turn may promote local inflammation when activated (Couser, 1985).

Renal IgA deposition is also noted in other disorders, *viz*. Henoch-Schönlein purpura (HSP), systemic lupus erythematosus and liver disease (Sinniah & Feng, 1976; Callard *et al*, 1975; Urizar *et al*, 1968).

1.3.3 Prognosis in IgAN

Raised serum creatinine concentrations, and proteinuria at presentation, are predictive of a poor prognosis. Male gender, late age at onset, and persistent microscopic haematuria, are also predictors of disease progression. Hypertension is a significant prognostic factor for progression in those patients with normal renal function (Ibels & Gyory, 1994).

Tubulointerstitial, vascular and glomerular lesions are histological markers of disease progression (Daniel *et al*, 2000; Mera *et al*, 2000; To *et al*, 2000; Bogenschutz *et al*, 1990). Walsh *et al* have demonstrated that interstitial fibrosis, glomerular sclerosis, and crescent formation, independently predicted adverse outcomes defined as a composite of doubling of serum creatinine levels, end-stage renal disease (ESRD), or death (2010).

Capillary wall IgA deposition rather than purely mesangial deposition is also indicative of more aggressive disease (Yoshimura *et al*, 1987). An increased level of circulatory fibroblast growth factor-23, a regulator of phosphorous homeostasis, was associated with albuminuria and disease progression in chronic IgAN (Lundberg *et al*, 2012).

1.3.4 Incidence and Prevalence of IgAN

Since the diagnosis of IgAN is made by renal biopsy, incidence data is dependent on the practice of biopsy. However, policies governing biopsy-practice varies globally. In addition, many cases of IgAN remain undiagnosed even in regions where biopsy is practiced. Nephrologists may be reluctant to perform biopsies on asymptomatic patients with mild abnormalities upon urinalysis; and lack of access to health care prevents biopsy and diagnosis in certain individuals. Conditions such as shrunken kidneys, for example, may preclude biopsy. Thus, the true incidence of IgAN cannot be determined for a population.

IgAN is the most common type of primary glomerulonephritis in regions where biopsy is conducted. From a review of 40 studies on the incidence of primary glomerulonephritis in Europe, North and South America, Canada, Australasia and the Middle East, the incidence rate for IgAN was the highest (McGrogan *et al*, 2011). In the unit of per 100 000 per year, the incidence for IgAN was 2.5; compared to 1.2 for membranous nephropathy, 0.2 for membrano-proliferative glomerulonephritis, 0.8 for focal segmental glomerulosclerosis, 0.6 for minimal change disease, and 0.2 for mesangio-proliferative glomerulonephritis.

Systematic study for IgAN incidence data in Africa is hampered by restriction of renal biopsy due to cost (Seedat, 1992). In 1992, Oviasu reported IgAN in a Nigerian male and suggested that the rarity of IgAN in African Blacks may be unlikely. In 1997, a similar incidence for Caucasian and African-American children and adolescents has been reported (Sehic *et al*, 1997).

Although all racial groups are affected by IgAN, the highest prevalence has been reported in certain groups of Asian and European descent (Julian *et al*, 1988). Prevalence rates of up to 40% in Asia, 20% in Europe and 10% in North America, were reported among all biopsies carried out for glomerular diseases. In Asia, the reported prevalence is comparatively higher as routine urinalyses are performed for schoolchildren and renal biopsies are performed on patients with asymptomatic haematuria. In Singapore, the mandatory screening practice for urinary abnormalities in army recruits has also resulted in the reporting of a high incidence of IgAN in Singaporean Asian males (Sinniah *et al*, 1976). Reported incidences may thus reflect policies governing biopsy practice, which may vary in different countries.

However, a geospatial analysis of 85 populations revealed that the genetic risk score based on the replicated genome-wide association studies (GWAS) loci was

highest in Asians; intermediate in Europeans; and the least in Africans. Such data reflect current incidence data for IgAN (Kiryluk *et al*, 2013).

1.3.5 Serology in IgAN

The sera of patients with IgAN may be characterised by increased levels of circulating IgA1-containing immune complexes; high levels of polymeric IgA1 in more than 50% of the patients (Tomana *et al*, 1999; Schena *et al*, 1989; Lai *et al*, 1987; Czerkinsky *et al*, 1986; Valentijn *et al*, 1984; Coppo *et al*, 1982); and an increased number of IgA1-bearing B-lymphocytes (Schena *et al*, 1986) and activated T helper cells (Lai *et al*, 1994). *In vitro* studies have demonstrated overproduction of IgA1 by B-lymphocytes from the sera of patients with IgAN (Schena *et al*, 1986; Sakai *et al*, 1979). An increased concentration of C3 may also be found in the serum (Emancipator & Lamm, 1989).

1.3.6 Primary Pathogenic Defect in IgAN

1.3.6.1 The Primary Pathogenic Defect/s Are External to the Kidney

Donor kidneys from patients with sub-clinical IgAN that were transplanted in patients without IgAN became clear of immune deposits over several weeks (Sanfilippo *et al*, 1982; Silva *et al*, 1982). In addition, histological recurrence of mesangial IgA1 deposition may be seen beyond three months post-transplantation in patients with IgAN (Odum *et al*, 1994). Thus, the primary pathogenic defect does not lie within the kidney.

In a retrospective study of 190 IgAN patients and 380 non-diabetic controls, at 15 years, IgAN patients had a higher cumulative incidence of graft failures (Moroni *et al*, 2013). At multivariate analysis, IgAN, delayed graft function and acute rejection were predictive of graft loss.

1.3.6.2 Abnormalities of Production Control Do not Provide a Direct Explanation for Mesangial Deposition

IgAN does not occur in other diseases with raised serum IgA1 levels *viz.* IgA myeloma, unless the IgA1 molecules are aberrantly glycosylated (Tanaka *et al*, 2011; Feehally & Allen, 1999). Hence, abnormalities of control of IgA1 production do not provide a direct explanation for mesangial deposition of IgA1.

1.3.6.3 Role of the *O*-Galactosylation of the IgA1 Glycoprotein

The carbohydrate moiety of the glycoprotein aids in the maintenance of the biologically active conformation and structural stability; decreases immunogenicity; confers protection against proteolysis; and serves as ligands for cell receptors. Immunoglobulins are glycoproteins. The *O*-linked carbohydrate moieties of the IgA1 hinge region (fig. 2) constitute the recognition motif of the asialoglycoprotein receptor (ASGPR) of the liver (Stockett *et al*, 1982). Terminal *O*-linked D-galactose (fig. 2) is the ligand of the hepatic ASGPR, and thus mediates the clearance of IgA1 from the circulation by endocytosis.

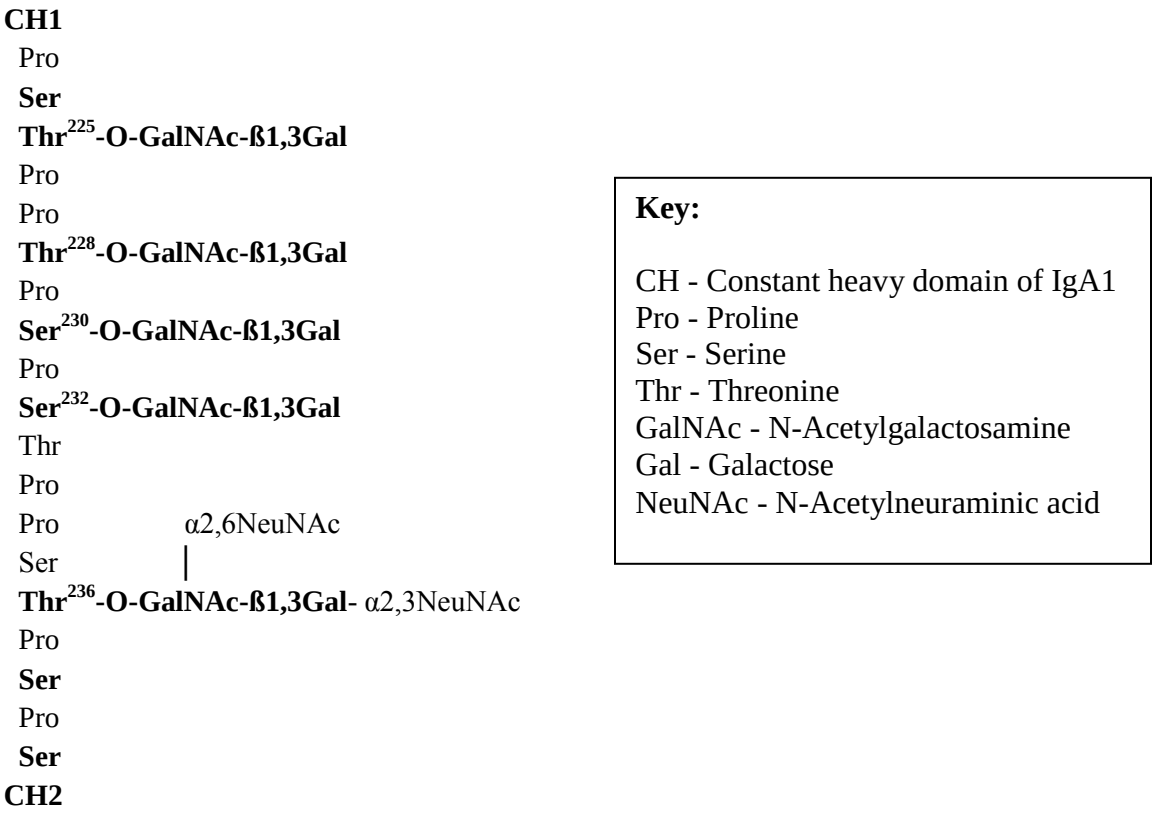

Figure 2: Schematic Representation of the *O*-Galactosylation Sites and *O*-Galactosylation at the Hinge Region between the CH1 and CH2 domains of IgA1

1.3.6.4 Hypogalactosylation of the IgA1 Glycoprotein in IgAN

Several of the pioneering studies on the *O*-galactosylation of IgA1 have demonstrated that the serum IgA1 is aberrantly glycosylated (Allen *et al*, 2001; Amore *et al*, 2001; Allen & Feehally, 2000; Allen *et al*, 1999; Hiki *et al*, 1998; Tomana *et al*, 1997; Hiki *et al*, 1996; Allen *et al*, 1995; Tomana *et al*, 1995;

Mestecky *et al*, 1993). These studies were conducted primarily in Caucasian or Asian populations.

1.3.6.5 Hypogalactosylation of IgA1 and the Resultant Generation of Circulating Immune Complexes

Incomplete *O*-galactosylation results in the exposure of neo-antigenic *N*-Acetylgalactosamine (GalNAc). Neo-antigenic GalNAc may be recognised by GalNAc-specific IgG or IgA1, resulting in the increased level of circulatory immune complexes (Mestecky *et al*, 2002; Tomana *et al*, 1999; Tomana *et al*, 1997; Schena *et al*, 1989; Coppo *et al*, 1982). Hashimoto *et al* used two IgAN mouse models to study the pathological role of complement pathways in IgAN (2012). The mouse model with the significantly greater level of IgA hypogalactosylation correlated with a significantly greater level of circulating immune complexes despite having significantly lesser levels of serum IgA than the latter.

Since terminal D-galactose is the ligand of the hepatic asialoglycoprotein receptor, galactose-deficient IgA1 molecules and galactose-deficient IgA1-containing immune complexes, in the sera of patients with IgAN, may thus

escape hepatic clearance, the major catabolic pathway of IgA1, and persist in the circulation while other ligands of the ASGPR are properly cleared (Roccatello *et al*, 1993). The increased serum levels of IgA1 in IgAN patients may be thus attributed to the lack of clearance by the liver, as opposed to being entirely attributed to the increased antibody production.

There is also enhanced binding of IgA1 to circulatory fibronectin (Davin *et al*, 1991) and bacterial or viral lectins (Coppo, 1988) in IgAN.

1.3.6.6 Galactose-Deficient IgA1-Containing Circulating Immune Complexes are Boosted During Infection which Precedes IgAN Onset

Indeed, the onset and exacerbation of IgAN i.e. recurrent macroscopic haematuria, is preceded by respiratory or gastrointestinal syndromes (Jessen *et al*, 1992; Berger & Hinglais, 1968).

Increased levels of GalNAc-specific antibodies may result from preceding infection by microorganisms which express GalNAc-associated epitopes (Tomana *et al*, 1999). Many viruses such as respiratory syncytial, Epstein-Barr, and herpes viruses, as well as certain strains of *Streptococci*, express GalNAc on

their surface structures which may induce GalNAc-specific IgG, IgM, and IgA. Such GalNAc-specific antibodies in turn recognise and form complexes with galactose-deficient IgA1. Circulatory immune complex formation is thus further compounded in patients with IgAN who have increased levels of galactose-deficient IgA1 in the sera in comparison to healthy controls.

Indeed, urinary levels of IgA/IgG immune complexes were noted to be higher in patients with IgAN than in patients with non-IgAN associated nephropathies and normal controls (Matousovic *et al*, 2006).

Glycosylation confers protection against bacterial proteases through providing increased steric hindrance. Following the molecular graphics modelling of dimeric IgA1, Bonner *et al* hypothesise that reduced galactosylation in IgA1 may enhance susceptibility to bacterial cleavage at the Fab regions resulting in the complete or partial removal thereof, followed by self-aggregation of the IgA molecule (2008). In addition, under-glycosylation of IgA1 may result in the destabilisation of the extended hinge conformation, resulting in the displacement of the Fab regions and the self-association of the Fc regions.

Multimer formation due to IgA1 aggregation following digestion by bacterial proteases was demonstrated in polymeric but not monomeric IgA1 (Almogren & Kerr, 2008). This provides further evidence for the propensity of polymeric IgA1 in IgAN to aggregate and deposit in the mesangium.

Galactose-deficient IgA1 may thus account for the increased levels of circulating immune complexes that remain uncleared from circulation and are hence prone to mesangial trapping.

1.3.6.7 Galactose-Deficient IgA1 Selectively Deposit in the Renal Mesangium

Allen *et al* have demonstrated that the lectin binding of galactose-deficient IgA1, eluted from the glomeruli of IgAN patients, was markedly higher than that of the serum IgA1 of the same individual, and the serum IgA1 of other patients and controls (2001). Such a finding indicates that galactose-deficient IgA1 abnormally and selectively deposit in the glomerular mesangium of IgAN patients. The study finding strongly suggests that abnormal IgA1 *O*-glycosylation is not an epiphenomenon but may be directly implicated in the mesangial deposition of IgA.

Novak *et al* demonstrated that circulating immune complexes from patients with IgAN are less efficiently internalised by hepatoma cells (HepG2) and bind more efficiently to cultured mesangial cells than those from healthy controls (2002).

Galactose-deficient IgA1 from patients with IgAN also have an enhanced *in vitro* affinity for, and bind efficiently to, mesangial matrix components such as type IV collagen, fibronectin, and laminin, due to carbohydrate interactions; resulting in the accumulation of IgA1 deposits in the mesangium (Coppo *et al*, 1995; Coppo *et al*, 1993).

It is noted that galactose-deficient IgA1 in HSP, is restricted to patients with clinical nephritis (Allen *et al*, 1998).

1.3.6.8 Pathogenic Effects of Galactose-deficient IgA1 Deposition in the Renal Mesangium

1.3.6.8.1 Up-regulation of Macrophage Migration Inhibitory Factor and Renin

In vitro studies have demonstrated that the binding of IgA1 to mesangial cells leads to increased expression of growth factors, cytokines, and integrins, which

may enhance inflammatory injury. Polymeric IgA1 from IgAN patients up-regulates gene expression of renin and macrophage migration inhibitory factor in human mesangial cells, in a dose-dependent manner, leading to renal fibrosis (Lai & Leung, 2002).

1.3.6.8.2 Down-regulation of Adiponectin

Adiponectin, an adipocyte-derived secretory factor has anti-inflammatory effects, including the inhibition of adhesion molecules and cytokines (Wolf *et al*, 2004). Inoue *et al* reported suppression of adiponectin secretion in cultured human mesangial cells following stimulation with desialylated and degalactosylated IgA1 (2012). In addition, a down-regulation of adiponectin expression was detected via immunofluorescence staining in the glomeruli of renal biopsy specimens from patients with IgAN compared to those with lupus nephritis. Thus, local suppression of adiponectin by galactose-deficient IgA1 could be involved in the regulation of glomerular inflammation and sclerosis in IgAN.

1.3.6.8.3 Mesangial Cell Proliferation

Novak *et al* reported that enhanced mesangial cell proliferation was caused by galactose-deficient IgA1-containing circulating immune complexes from the sera of IgAN patients than uncomplexed IgA1 or immune complexes from healthy control subjects (2005). In addition, circulating immune complexes containing higher levels of galactose-deficient IgA1 enhanced mesangial cell proliferation more efficiently than complexes with lower levels of galactose-deficient IgA1. Thus galactose-deficient IgA1-containing immune complexes have an enhanced ability to stimulate proliferation in mesangial cells.

Novak *et al* demonstrated that circulating immune complexes from both adults and paediatric IgAN patients are galactose-deficient and that the large immune complexes abundant in galactose-deficient IgA1 play a pathogenic role by stimulating the proliferation of cultured human mesangial cells and increasing the production of the extra-cellular matrix protein, laminin (2011). Complexes from paediatric patients with active disease stimulated the expression of IL-6 and IL-8 at higher levels than those with inactive disease.

1.3.6.8.4 Increased Apoptosis Rate and Nitric Oxide Synthesis

Galactose-deficient IgA1, isolated from patients with IgAN, was shown to significantly increase the apoptosis rate and nitric oxide synthesis activity of cultured mesangial cells, compared to IgA1 from healthy controls (Amore *et al*, 2001). Amore *et al* have hypothesised that the extent of abnormal IgA1 glycosylation, may determine the individual clinical course in IgAN (2001).

1.3.6.8.5 Platelet-Activating Factor Induction

An *in vitro* study demonstrated the pathogenic role of galactose-deficient IgA1 as a mediator of podocyte changes that are associated with proteinuria in IgAN, through the induction of platelet-activating factor in mesangial cells (Coppo *et al*, 2010). Platelet-activating factor effected the down-regulation of nephrin (an adhesion molecule that is critical to the glomerular permselectivity), and cytoskeletal F-actin reorganisation in human podocytes that were cultured with medium from mesangial cells treated with galactose-deficient IgA1 (that was either prepared or extracted from the sera of IgAN patients).

1.3.6.8.6 Complement Activation

A recent study using IgAN mouse models (grouped ddY (gddY) mice and high serum IgA (HIGA) mice) demonstrated the pathogenic role of galactose-deficient IgA1 as a mediator of immune complex formation and complement activation leading to the full-blown progression of IgAN (Hashimoto *et al*, 2012). The study results also demonstrate that the extent of galactose-deficiency could be a significant factor in determining the extent of pathology. The gddY mice contained significantly higher levels of galactose-deficient serum IgA than the HIGA mice and controls and the galactose content was significantly lower in IgA molecules of gddY than HIGA mice.

Glomerular activation of the classical, lectin and alternate pathways of complement was demonstrated by staining of C3, C5b-9, C1q, C4, mannose-binding lectin (MBL)-A/C, MBL-associated serine protease-2, factor B and properdin; the staining was significantly stronger in gddY mice than in HIGA mice. Thus, the greater activation of the complement pathways may be attributed to the greater degree of IgA hypogalactosylation in gddY mice.

Whilst the levels of serum IgA are significantly higher in HIGA mice compared to gddY and control mice, the levels of serum IgA-IgG_2/IgM and IgA-MBL-A/C immune complexes and polymeric IgA in gddY mice was significantly higher than that of HIGA and control mice. The significantly greater number of immune complexes in gddY compared to HIGA mice despite the high serum IgA level in the HIGA mice, may be attributed to the greater degree of IgA hypogalactosylation deficiency which results in the formation of immune complexes.

In addition, the percentage of the IgG and IgM deposition in the glomeruli was significantly higher in gddY than HIGA mice, suggesting that the enhanced degree of immune deposition may be attributed to the greater degree of IgA hypogalactosylation.

Although the degree of glomerular IgA deposition was similar in gddY and HIGA mice, the extent of glomerular injury was more severe in gddY mice (defined by significantly greater albumin-creatinine ratio, severity of glomerular lesions, mesangial matrix expansion and glomerular type IV collagen expression). Thus the greater degree of glomerular injury could be attributed to the greater degree of IgA hypogalactosylation in gddY mice.

1.3.7 Immunoglobulin Glycosylation

1.3.7.1 Carbohydrate Proportion

Depending on the immunoglobulin class, carbohydrate groups may form between two to fourteen percent of the immunoglobulin structure by weight (Wolfe, 1995). IgA1 is highly glycosylated, containing 8% carbohydrate by weight.

1.3.7.2 Enzymatic Addition of Carbohydrate Monomers

Protein glycosylation is the most common and complex form of post-translational modification. Unlike the synthesis of nucleic acids and proteins, polysaccharide synthesis does not depend on template molecules. A specific glycosyltransferase, an integral membrane glycoprotein of the endoplasmic reticulum or Golgi complex, catalyses the addition of a specific carbohydrate monomer (Wolfe, 1995).

1.3.7.3 Transporter Proteins

Before the addition of monosaccharide units to the protein, monosaccharides are activated in the cytoplasm by reaction with one of the nucleoside triphosphates: uridine triphosphate (UTP), cytidine triphosphate (CTP), or guanosine triphosphate (GTP). The activated monosaccharides are transported across the endoplasmic reticulum and Golgi membranes by transporter proteins that exchange a nucleotide-monosaccharide complex from the outside for an unbound nucleotide on the inside.

1.3.7.4 Glycan-Amino Acid Linkages

Over 20 different types of glycan-amino acid linkages exist (Ju *et al*, 2011). The two major types of glycosylation are *N*- and *O*-linked. *N*-linked glycans are attached via *N*-Acetylglucosamine (GlcNAc) or GalNAc to a side chain amino group in an asparagine residue. *O*-linked glycans are attached by an *O*-glycosidic bond between GalNAc and the hydroxyl group of a threonine (Thr) or serine (Ser) residue (fig. 2). *N*-linked glycans are commonly found in serum proteins while *O*-linked glycans are mainly restricted to membrane proteins and only a few serum proteins. IgA1, in contrast to IgG, IgM and IgA2, contains *O*-linked in addition to *N*-linked glycans.

1.3.7.5 *O*-Galactosylation of IgA1

1.3.7.5.1 *O*-Galactosylation Sites

The IgA1 hinge region consists of a unique 18 amino acid sequence (fig. 2). The sequence contains a closely located series of nine potential *O*-glycosylation sites on each heavy chain, i.e. five serine and four threonine residues. *O*-glycans occupy three threonine and two serine residues on each heavy chain (fig. 2).

1.3.7.5.2 Core 1 β3-galactosyltransferase

The intracellular enzyme core 1 β3-galactosyltransferase (β 1,3 GT) also known as T-synthase, catalyses the transfer of galactose from the nucleotide donor, uridine 5' diphosphate-galactose (UDP-Gal), to the acceptor GalNAc that is *O*-linked to serine or threonine residues in the IgA1 hinge region (Allen *et al*, 1997). This addition results in the formation of Galβ1,3GalNAc (fig. 2) which is referred to as the Thomsen-Friendenrich or T antigen; whilst the agalactosylated GalNAc moiety is referred to as the T antigen nouvelle or Tn antigen (Moreau *et al*, 1957). Further extension of Galβ1,3GalNAc may include sialic acid (*N*-Acetylneuraminic acid (NeuNAc)) in α 2,3 linkage with galactose to form

monosialyl-Galβ1,3GalNAc, or further α 2,6 linkage with GalNAc to form disialyl-Galβ1,3GalNAc (fig. 2) (Field *et al*, 1989; Baenziger & Kornfield, 1974).

1.3.7.5.3 Core 1 β3-Gal-T-specific molecular chaperone (Cosmc)

The functionality of β 1,3 GT is dependent on the activity of Cosmc (Ju and Cummings, 2002). The endoplasmic reticulum-localised Cosmc functions as a chaperone in the folding of β 1,3 GT (Ju *et al*, 2008). Cosmc binds to newly synthesised β 1,3 GT and prevents its aggregation and subsequent degradation in the endoplasmic reticulum-associated degradation proteasome pathway.

1.3.8 Abnormal Protein Glycosylation in Other Diseases

In recent decades, abnormal protein glycosylation has become increasingly recognised in disease states.

1.3.8.1 Rheumatoid Arthritis

Axford *et al* described hypogalactosylation of the *N*-linked moieties of circulating IgG, as a result of reduced activity of the *N*-galactosylating enzyme, β 1,4 galactosyltransferase, in patients with rheumatoid arthritis (1992).

Ercan *et al* reported a significant correlation between the levels of abnormal IgG galactosylation and disease activity in rheumatoid arthritis; and that this abnormality predated disease onset and diagnosis (2010).

1.3.8.2 Thomsen-nouvelle (Tn) syndrome

In Tn syndrome, or Tn polyagglutinability syndrome, T- and B-lymphocytes, platelets, and or erythrocyte cell membrane proteins fail to express the T antigen, due to reduced β 1,3 GT activity (Cartron & Nurden, 1979; Cartron *et al*, 1978). Clinical manifestations include uraemia and thrombocytopenia.
Although this failure of terminal galactosylation is similar to that described in IgAN, the antigenic determinants and corresponding antibodies differ. In Tn syndrome, the Tn antigen consists of three adjacent GalNAc residues; a configuration that is not present in the IgA1 hinge region. In Tn syndrome, the

anti-Tn antibodies belong to the IgM class, whilst the GalNAc-specific antibodies in IgAN also include the IgG and IgA1 isotypes. Whilst disease-associated *O*-glycosylation abnormalities have been described in membrane proteins, IgAN is the first disorder that describes such an abnormality in serum proteins.

1.3.8.3 *O*-Glycans and Vascular Development

Fu *et al* have demonstrated the significant role of *O*-glycans in vascular development (2008). Fu *et al* generated mice that lack β 1,3 GT in endothelial and haematopoietic cells. The lack of β 1,3 GT resulted in disorganised and blood-filled lymphatic vessels. The chylomicron deposition, due to misconnected portal vein and intestinal lymphatic systems, caused fatty liver disease in pups. Thus endothelial cell *O*-glycans control the separation of blood and lymphatic vessels during embryonic and postnatal development, in part by regulating the expression of podoplanin, a mucin-type transmembrane glycoprotein that regulates organ development, cell motility, and tumourigenesis and metastasis.

1.3.8.4 Cancer

Expression of the Tn antigen has been associated with many forms of cancer (Ju *et al*, 2011). Tn expression has been shown to positively correlate with the potential for metastasis and poor prognosis.

In colon cancer, the Tn antigen that is present on Mucin 1, a glycoprotein on colon carcinoma cells, was shown to bind to macrophage galactose-type lectin (MGL) that is expressed by macrophages and tolerogenic dendritic cells. The binding of the Tn antigen and MGL may thus lead to immunosuppression and escape of the tumour from immunosurveillance (Saeland *et al*, 2007).

In two human cervical cancer specimens, and colon cancer and melanoma-derived cell lines, the Tn antigen expression was associated with mutations in the gene encoding Cosmc (Ju *et al*, 2008). This mutation could have resulted in inactivity of Cosmc and thus inactivity of the β 1,3 GT resulting in the formation of the Tn antigen.

1.4 Approaches to IgA1 *O*-Glycosylation Analysis

Approaches that enable the analysis of IgA1 *O*-glycosylation include: lectin binding to the *O*-glycans on the complete IgA1 molecule; size analysis of isolated hinge region glycopeptides by matrix assisted laser desorption ionising mass spectroscopy (MALDI-MS); and separation of free *O*-glycans by chromatography or electrophoresis.

1.4.1 Lectin Binding

Lectins are soluble glycoproteins that are derived from plants and animals, and contain multiple binding sites which demonstrate strong and specific affinity for certain carbohydrate ligands (Wolfe, 1995). Lectin binding assays exploit this characteristic in order to elucidate glycan structure. The glycosylation pattern of a molecule is determined by the differential binding of lectins that are specific for individual saccharide residues on the intact molecule. Lectin binding assays constitute the most widely used approach for IgA1 *O*-glycosylation analysis.

Vicia villosa (VV*)* lectin, isolated from the seeds of the plant, binds to GalNAc in the absence of terminal galactose. Thus increased VV binding is an indicator

of terminal D-galactose-deficient GalNAc. Allen *et al* reported a significant increase in VV binding to serum IgA1 in patients with IgAN compared to age-matched controls (1995). Similar results were obtained with alternate GalNAc-specific lectins *viz*. *Helix aspersa* (HA), *Helix pomatia* (HP), *Caragana arborescens* (CA), and *Bauhinia purpurea* (BP) in patients with IgAN (Allen *et al*, 1998; Tomana *et al*, 1997).

Allen *et al* reported no difference in the *N*-glycosylation of IgA1 of IgAN patients and healthy controls with the use of the *N*-glycan specific lectins *viz*. *Triticum vulgaris* (TV) and *Erythrina crystagalli* (EC) (1995).

Lectins with specificity to Galβ1,3GalNAc include *Amaranthus caudatus* (AC) and Peanut agglutinin (PNA) (Baharaki *et al*, 1996; Allen *et al*, 1995). Jacalin lectin, isolated from *Artocarpus integrifolia* binds specifically to the IgA isotype (Roque-Barreira & Campos-Neto, 1985). Jacalin binds to Galβ1,3GalNAc as well as GalNAc (Hiki *et al*, 1996; Tomino *et al*, 1995; Andre *et al*, 1990).

1.4.2 Mass Spectroscopy

The *O*-glycans of IgA1 are concentrated on a short stretch of the amino acid backbone in the hinge region, and therefore constitute a large percentage of the molecular mass of the isolated hinge glycopeptide.

The MALDI-MS approach involves size analysis of the IgA1 hinge peptide with the attached *O*-glycans, following digestion of the IgA1 molecule (Iwase *et al*, 1998; Iwase *et al*, 1996).

Trypsin is used to digest the reduced and recarboxymethylated IgA1 molecule to yield a mixture of peptides, including the *O*-glycosylated hinge peptide. The mass of the glycopeptide is in the range of 3.5 - 9.5 kDa with a carbohydrate content of up to 60% percent by mass. The *O*-glycosylated hinge peptides are separated from the digest mixture by reverse phase high-performance liquid chromatography (HPLC) or Jacalin affinity chromatography. The glycopeptide preparation is then subjected to MALDI-MS. Since the molecular mass of the peptide is constant, the carbohydrate component of each glycopeptide is deduced from the known individual molecular mass of a saccharide unit.

Hiki *et al* reported a lack of terminal galactosylation of the IgA1 hinge *O*-glycans from IgAN patients, following MALDI-MS analysis (1998). Such data support the findings obtained via lectin binding assays.

1.4.3 HPLC and Fluorophore-Assisted Carbohydrate Electrophoresis (FACE)

HPLC or FACE enables the identification and quantification of IgA1 *O*-glycans following the chemical or enzymatic release from IgA1 (Hu, 1995; Iwase *et al*, 1992).

Chemical release of the IgA1 *O*-glycans occurs by hydrazinolysis at 60°C; whilst *N*-glycans are retained, as temperatures in excess of 90 °C is required for *N*-glycan release (Patel *et al*, 1993).

GalNAc and Galβ1,3GalNAc moieties may be released from IgA1 by *N*-Acetylgalactosaminidase (GalNAcase) and endo-*N*-Acetylgalactosaminidase (*O*-glycanase) respectively (Allen *et al*, 1999). Desialylation is a precursor for the release of GalNAc and Galβ1,3GalNAc moieties.

1.4.3.1 Chromatography

Gas liquid chromatography has been used to determine the total carbohydrate composition in individual monosaccharide units of the IgA1 glycan moieties, rather than the intact oligosaccharide chains, in patients with IgAN (Mestecky *et al*, 1993). The vaporised sample constituents interact with the walls of the column and elution times are compared in order to determine the carbohydrate composition. A decrease in the total IgA1 galactose content was demonstrated in IgAN. This was interpreted as reduced *O*-glycosylation, as decreased Jacalin binding to IgA1 in IgAN patients was also observed.

1.4.3.2 FACE

Polyacrylamide gel electrophoresis is used to separate fluorophore-labelled free *O*-glycans. The densities of the resultant bands under ultraviolet light, provide a quantitative measure of the glycans present. This approach has demonstrated a significant increase in the frequency of single GalNAc units in IgAN patients (Allen *et al*, 1999). This finding is in agreement with VV binding data.

1.5 Advantages of the Lectin Binding Approach to IgA1 *O*-Glycosylation Analysis

The lectin binding approach is relatively rapid and inexpensive in comparison to other methods (Feehally & Allen, 1999). The lectin binding assay is useful for screening large numbers of samples to compare group means.

In addition, some lectin binding assays do not require purified IgA1 preparations; while IgA1 purification constitutes the first step in other methods for IgA1 *O*-glycosylation analyses. The purification process involved may influence the data obtained.

In the chromatographic approach, harsh elution conditions which may damage the *O*-glycans are required to displace IgA1 from the columns. In most studies, lectin binding assays are carried out in enzyme-linked immunosorbent assay (ELISA) - type systems. IgA1 is immobilised on plastic immunoplates. Thus, no elution is necessary, as IgA1 remains bound to the plate.

1.6 Heritability of IgA1 *O*-Galactosylation

The lectin binding assay has also been used to facilitate studies which identify genes that are associated with the undergalactosylation of IgA1 (Kiryluk *et al*, 2010). Kiryluk *et al* argue that identifying subgroups based on the galactose-deficient IgA1 level may be reflective of different disease aetiology and may thus reduce heterogeneity in linkage and genetic association studies in IgAN (2010).

Recent serum IgA1 galactosylation studies in IgAN patients and their relatives suggest that abnormal serum IgA1 galactosylation is an inherited trait (Kiryluk *et al*, 2011; Hastings *et al*, 2010; Lin *et al*, 2009; Gharavi *et al*, 2008).

Gharavi *et al* reported high serum galactose-deficient IgA1 in Caucasian patients with familial IgAN (i.e. biopsy proven IgAN in at least 2 family members) and 47% of their at-risk relatives based on the assumption of autosomal dominant inheritance (2008). Serum galactose-deficient IgA1 was also high in 78% of sporadic IgAN (i.e. biopsy proven IgAN in an individual whose family members are all negative upon urinalysis) and in 25% of their blood relatives.

In a cohort of Chinese IgAN patients and their first degree relatives, the level of serum IgA1 hypogalactosylation was significantly higher than that of normal controls, whilst no difference was found between normal controls and the spouses of IgAN patients (Lin *et al*, 2009).

Hastings *et al* reported elevated serum galactose-deficient IgA1 levels in African American IgAN patients in comparison to controls (2010).

Kiryluk *et al* demonstrated that serum hypogalactosylated IgA1 levels are highly inherited in paediatric IgAN patients (2011).

1.7 Anti-galactose-deficient IgA1 Antibodies and IgA1 Glycoform Heterogeneity

Although studies in Caucasian, Asian and African American patients demonstrated elevated levels of galactose-deficient IgA1 in both IgAN patients and their first degree relatives in comparison to controls (Hastings *et al*, 2010; Lin *et al*, 2009; Gharavi *et al*, 2008), an additional requirement for the pathogenic mesangial deposition of IgA1 is the presence of autoantibodies specific for galactose-deficient IgA1. Uncomplexed galactose-deficient IgA1

does not affect cellular proliferation (Novak *et al*, 2007; Novak *et al*, 2005). In IgAN, galactose-deficient-IgA1-specific autoantibodies are not genetically determined and are generated through somatic mutations (Suzuki *et al*, 2009). Suzuki *et al* reported an alanine to serine substitution in the complementarity-determining region 3 of the variable region of the gene encoding the IgG heavy chain in IgAN patients, in comparison to healthy controls (2009). Reversion from serine to alanine via site-directed mutagenesis of the IgG of an IgAN patient resulted in reduced binding of IgG to galactose-deficient IgA1 by 72%. The alanine to serine substitution in the IgG of a normal control resulted in increased binding to galactose-deficient IgA1 to 80% of that of the IgG of the IgAN patient.

Furthermore, a series of IgA1-*O* glycoforms have been identified in polymeric IgA1 myeloma protein (Takahashi *et al*, 2012). The IgA1 hinge regions are equally glycosylated but the *O*-glycosylation attachment sites are different. Mass spectrophotometric analysis of offline fractions revealed two distinct isomeric hinge-region glycopeptides that contain a disaccharide attached to Ser232 in both fractions and a GalNAc monosaccharide attached to either Thr233 or Thr236 in an adjacent fraction. In addition, other combinations of *O*-glycan amino acid positional isomers involving Ser230, Thr233, and Thr236

were shown. It is not known whether such glycoforms exist in undergalactosylated IgA1 from IgAN patients and relatives and if only certain glycoforms are pathogenic.

It is not known, what proportion, if any, of such asymptomatic relatives with elevated levels of galactose-deficient IgA1 that may develop IgAN in the future.

1.8 Severity of IgAN

1.8.1 Most Common Glomerulonephritis and Leading Cause of ESRD in Developed Countries

IgAN is the most common form of primary glomerulonephritis, and a leading cause of chronic kidney disease and end-stage renal disease in developed countries (Levy & Berger, 1988).

ESRD is a major public health problem. It affects 1 in 1000 individuals with an annual death rate of 20%. 10 to 50% of patients with IgAN may progress to ESRD within 20 years of disease onset (Galla, 1995). In certain patients, however, the rate of deterioration is especially rapid. These patients require

either dialysis or kidney transplantation (Julian *et al*, 1999). During dialysis, renal changes may continue (Lawler, 1991). While renal transplantation may return renal function, it does not remove the cause of the recipient's original disease. Renal transplantation is not a cure, but a treatment of ESRD. In centres that conducted routine biopsy following transplantation, 50 to 60 % of the recipients were reported to experience histological recurrence (Odum *et al*, 1994; Berger *et al*, 1984).

1.8.2 Recurrence Post-transplantation

Recurrent IgAN following transplantation has previously been assumed to follow a benign clinical course, but recent studies have suggested that it is a significant contributor to graft loss (Moroni *et al*, 2013). Clinically important recurrence leading to graft loss has been reported in about 5 to 16% of recipients, (Moriyama *et al*, 2005; Freese *et al*, 1999; Frohnert *et al*, 1997; Ohmacht *et al*, 1997; Odum *et al*, 1994). Recurrent IgAN may not be prevented either histologically or clinically, by immunosuppression with corticosteroids, azathioprine, and or cyclosporin A.

1.8.3 Non-specific and Non-Curative Treatment

Current treatment for IgAN includes the use of immunomodulators that suppress the humoural and cell-mediated immune systems. These include steroid therapy (Pozzi *et al*, 1999) which is recommended for nephrotic patients with mild histological changes and preserved renal function; fish oil supplements (FOS) (Donadio *et al*, 1994) which contain a high concentration of Omega 3 fatty acids, and are recommended for patients with slowly progressive disease; and a combination of steroids and cyclophosphamide which may be effective in patients with focal necrotising lesions, often accompanied by crescents and a rapid decline in renal function.

However, steroids, FOS and other immunocytoreductive agents such as cyclophosphamide, are non-specific immunomodulators, and thus fail to target specific components that are unique to the molecular pathogenesis of IgAN. Such agents fail to halt the progression of the disease (Hogg & Wyatt, 2004). FOS may only delay progression for a few years, rather than permanently preventing progressive disease (Novak *et al*, 2002). The use of high dose steroids over long course therapy is associated with significant morbidity (Novak *et al*, 2002).

Angiotensin converting enzyme inhibitors and angiotensin II receptor blockers are used to control blood pressure and reduce proteinuria; and are thus symptomatically therapeutic as opposed to being curative. Such agents do not halt the progression of the disease, and there is no benefit offered in the utilisation of such agents in early IgAN. In an open-label randomised control trial, Li *et al* demonstrated that treatment with 2.5 mg daily of ramipril, an ACE inhibitor, for 5 years is non-beneficial in early IgAN patients with minimal proteinuria, and normal blood pressure and renal function (2013). Li *et al* suggest starting ACE inhibitors angiotensin-receptor blocker treatment when the proteinuria level is above 0.5 g/day (2013). Throughout the study period, the blood pressure of the treatment group was slightly lower than that of the control group. At 60 months, the event-free survival was slightly higher for the treatment group at 60 months of follow up. The proteinuria-free survival was similar in both groups; impaired renal function did not develop in either group but the glomerular filtration rate decline was similar.

Thus, no specific therapies for IgAN have been developed, and there is currently no cure (Hall *et al*, 2004).

1.8.4 The Pathogenesis of IgAN is Incompletely Understood

IgAN is a complex multi-factorial disorder for which the underlying pathogenetic mechanisms are incompletely understood. The mesangial deposition of IgA1 is the initiating event for the pathology in IgAN (Barratt *et al*, 2004). The elucidation of the key events that enable or promote mesangial deposition is critical for the development of specific therapies that target the underlying aberrancies.

1.9 Study Aim and Hypothesis

The significant contribution of IgAN to ESRD worldwide, and the absence of curative therapy, reflects the importance of elucidating the key pathological mechanisms responsible for mesangial IgA1 deposition.

In the case of such a complex multi-factorial disorder, the determination and comparison of the underlying pathogenetic mechanisms among various populations globally, is essential for the advancement of the understanding of the disorder and the eventual development of specific therapies. Hitherto,

experimental data on the pathogenesis of IgAN for any country in the African continent is scarce.

It is hypothesised that there is a difference in the *O*-glycosylation patterns of serum IgA1 between IgAN patients and healthy controls of different ethnic origins in the South African population. It is hypothesised that the serum IgA1 from patients with IgAN are hypogalactosylated and thus abnormal glycosylation is a pathogenetic mechanism in IgAN patients in the study population.

IgA1 *O*-galactosylation comparisons between racial groups; between end-stage and pre-dialysis patients; and between gender, was investigated, although it is noted that the sample size for such comparisons are too low for hypothesis generation and valid conclusions in this study. Nonetheless, in a statistically valid sample size, it would be hypothesised that IgA1 *O*-galactosylation is significantly higher in males than females, based on a worse IgAN prognosis for males and the pathogenic role of galactose-deficient IgA1 in promoting mesangial deposition and renal injury and hence a progression of the disease; it would be hypothesised that IgA1 *O*-galactosylation is significantly higher in the end-stage than pre-dialysis IgAN patients due to the pathogenic role of

galactose-deficient IgA1 in the promotion of mesangial deposition and injury and hence a progression to end-stage IgAN. Hypotheses based on racial comparison are precluded by the paucity of data in the literature on IgA1 *O*-galactosylation studies among different racial groups; although since the IgAN prognosis was reported to be more severe in an Asian group in comparison to non-Asians (Barbour *et al*, 2013), it may be hypothesised that there is a significantly greater level of IgA1 *O*-galactosylation in Indian IgAN patients in comparison to other race groups.

1. South Africa consists of a uniquely multi-ethnic population. The study aims to elucidate and comparatively analyse the *O*-glycosylation patterns of serum IgA1 between IgAN patients and healthy controls of different ethnic origins in KZN, South Africa, and thus provide pathogenetic data for IgAN. The study thus aims to provide the first *O*-glycosylation data for IgAN for the African continent.

Since the study aims to investigate an initial event which may be responsible for the mesangial deposition of IgA1, data from the study may form the basis for research into the development of specific therapies.

CHAPTER 2 - METHODS

2.1 Concise Study Design

Following submission of the study proposal and ethics application, approval was attained.

Subsequently, blood samples were obtained following informed consent from South African adult biopsy-proven IgAN patients. Such patients were of African, Caucasian, Indian, Coloured and Mixed-Race descent, and were attending the Inkosi Albert Luthuli Central Hospital in Durban, KZN.

IgAN patients that were on dialysis or received a transplant were noted from a database at the IALCH.

Blood and urine samples were obtained following informed consent from adult volunteers of African, Caucasian, Indian, and Coloured descent, from various locations in Durban, KZN. Volunteers were screened for renal disease via the urine-dipstick test.

Serum IgA1 *O*-glycosylation analysis was performed on the serum samples, using the VV lectin binding approach in an ELISA-type system. The statistical analysis of the raw data was performed using the GraphPad Instat® 3.0 statistical package (La Jolla, California; United States of America (USA)).

2.2 Ethical and Study Approval

Ethical approval was attained following submission of the study proposal and ethics application to the Biomedical Research Ethics Committee. Study approval was attained following submission of the study proposal and ethics approval to the Postgraduate Education Committee of the NRMSM, the IALCH Manager, and the KZN Department of Health.

2.3 Participant Recruitment

2.3.1 IgAN Patient Participants

The Chronic Renal Programme database at the renal clinic at the IALCH in Durban, KZN, was first accessed in 2006 to identify patients with IgAN. The

database contained a list of renal patients that were either accepted, or not accepted, onto the chronic renal programme at the clinic. The database contained 1769 renal patients. Biopsy was not carried out in all of the patients. 30 patients had biopsy-proven IgAN.

However, a significant proportion of the 30 IgAN patients declined participation in the study, whilst some were deceased, or transferred out to another hospital. IgAN patients have unique clinic visit dates, i.e. all patients do not attend the clinic on the same day. The frequency of visits is as low as once a year for some patients. The recruitment process for patient participants thus spanned a period of 6 years; in order to access patients, and to attain new IgAN patients to obtain a statistically significant number of participants. Patients were recruited during 2007, 2009, and 2011.

IgAN patients were approached when they attended the Renal Clinic. A Phlebotomist was hired for the extraction of the blood samples from some patients that were accessed at their homes. IgAN patients were informed about the study verbally and through the provision of detailed information documents in the spoken language of the patient. Each patient participant signed an informed consent form.

A blood sample of 4 ml was extracted at the clinic by the attending Sister from each participant. An exception included one participant who provided 2 samples: 1 in 2007 and subsequently in 2009.

The blood sample was immediately transported in a polystyrene box to the Medicine Laboratory at the NRMSM, UKZN, for processing.

2.3.2 Control Participants and Urinalysis

Control participants were recruited from two independent work institutions in the Durban area. The potential participants were informed about the study verbally and via the provision of detailed information documents in the spoken language of the potential participant. Each volunteer signed an informed consent form. Since the presence of blood and or protein in the urine may be an indicator of renal disease, a fresh urine sample was obtained from each control volunteer and immediately subjected to urinalysis at the work site. Urinalysis was conducted via the Makromed urine dipstick test which enables the detection of blood and protein that may not be visible to the naked eye. A colour change of the indicator dye at the relevant areas on the test strip indicates the presence of blood

or protein in the urine. The colour change was noted between 45 to 60 seconds following insertion of the strip into the urine sample.

Control volunteers with negative urinalysis results were recruited as control participants, from whom blood samples were subsequently obtained. Volunteers with positive urinalysis results for the presence of blood and or protein were excluded from the study and were referred to seek medical attention.

A Phlebotomist was hired for the extraction of a 4ml blood sample from each of the control participants at the work site. Recruitment of control participants took place during 2008 and 2009.

2.4 Participant Description

2.4.1 Control Population Description

The control population consisted of 20 participants. The control population included participants of African, Caucasian, Coloured, and Indian descent (Appendix 2; table 1) in the proportion of 30%, 15%, 5% and 50% respectively (Appendix 3; table 2). The median and mean age of the control population was

32 and 31 respectively (Appendix 4; table 3). The age range was 19 to 49 (Appendix 3; table 2). Males constituted 45% of the control population (Appendix 4; table 3).

2.4.2 Experimental Population Description

The experimental population i.e. the IgAN patient population consisted of 19 participants. The patient population contained participants of African, Caucasian, Coloured, and Indian descent, and one individual of mixed race (Indian and Caucasian) (Appendix 5; table 4) in the proportion of 10.53, 10.53, 15.79, 57.89 and 5.26% respectively (Appendix 6; table 5). The median and mean age of the experimental population was 43 and 46 respectively (Appendix 4; table 3). The age range was 29 to 66. Males constituted 63 % of the experimental population (Appendix 4; table 3).

Of the 19 patients, 53% were at the end-stage, i.e. on dialysis and or having been transplanted. The end-stage IgAN patient population included participants of African, Caucasian, Coloured and Indian descent (Appendix 5; table 4). The end-stage IgAN patients were predominantly male (80%).

2.5 Whole Blood Extraction and Separation

2.5.1 Whole Blood Extraction

4 ml of whole blood was extracted from each participant into two EDTA anticoagulated tubes by the attending Sister or Phlebotomist. The purple top tubes were labeled with the name of the participant and transported immediately to the NRMSM Medicine laboratory in a polystyrene box for whole blood separation into plasma and serum.

2.5.2 Whole Blood Separation

2.5.2.1 Sample Encoding

For the maintenance of participant confidentiality, each participant was allocated a unique code consisting of letters and numbers (Appendix 2; Appendix 5). This unique code was used to label the sample tubes and cryogenic vials. Participant codes were maintained during the experimental procedures and subsequent data processing.

2.5.2.2 Whole Blood Separation and Serum Storage

Under the laminar flow cabinet, whole blood from the participant's pair of purple top tubes was pipetted into the appropriately labeled 15 ml polypropylene centrifuge tube (Corning; New York, USA).

Centrifuge tubes were placed opposite each other in the centrifuge (Hettich Universal/K25; Tuttlingen, Germany) for separation of whole blood into plasma and serum at room temperature (25ºC) and a rotational speed of 2500 revolutions per minute (rpm) for 10 minutes.

Following centrifugation, the serum from the centrifuge tube was pipetted into a series of appropriately labelled cryogenic vials (Corning; New York, USA) and stored in the Medicine laboratory freezer at -70ºC. IgA glycosylation may remain unchanged for more than 10 years in a stored frozen serum sample (Smith, 2008; personal communication).

2.6 IgA1 *O*-Glycosylation Analysis

2.6.1 Experimental Design

The analysis of serum IgA1 *O*-glycosylation was carried out using a lectin-binding assay in an ELISA-type system (Allen *et al*, 2001).

96-well immunoplates were coated with rabbit anti-human IgA antibody, followed by washing, and blocking with 2% fetal calf serum (FCS) in phosphate-buffered saline (PBS). Serum samples were diluted to 1:100 in PBS and 50µl of each sample was applied to duplicate wells to capture IgA1. Biotinylated VV lectins were applied to each well. Lectin binding was detected with horseradish peroxidase (HRP)-conjugated avidin. The reaction was developed with o-Phenylenediamine (OPD)/H_2O_2 substrate and stopped with 1 mol/l H_2SO_4. The results were read as absorbance (A) at 490 nm. The lectin binding of serum IgA1 from patient and control groups were compared using unpaired *t* tests.

2.6.2 Reagent Preparation and Storage

2.6.2.1 Autoclaving

Glass bottles, measuring cylinders, deionised water, and pipette tips were autoclaved for 20 minutes at 121 ºC (Erma; Tokyo, Japan).

2.6.2.2 Preparation of Primary Antibody in Coating Buffer

Rabbit anti-human IgA (5g/l) (Dako; Glostrup, Denmark) was diluted to 1:500 (10μg/ml) in coating buffer.

One capsule of 0.05M carbonate/bicarbonate buffer (pH 9.6) (Sigma; St. Louis, USA) was opened to release the carbonate/bicarbonate powder into an appropriately labelled autoclaved bottle with 100 ml autoclaved deionised water, as per reconstitution instructions on the product specification sheet. The bottle was swirled for 5 minutes to achieve dissolution of the carbonate/bicarbonate buffer.

The carbonate/bicarbonate buffer solution and carbonate/bicarbonate buffer capsules were stored at room temperature.

24.950 ml of carbonate/bicarbonate coating buffer was added to the appropriately labelled 50 ml polypropylene tube (Sterilin; Newport, United Kingdom).

Storage conditions for the primary antibody i.e. Rabbit anti-human IgA includes refrigeration at 2-8ºC. The primary antibody was stored at 4ºC. The vial containing 1 ml of Rabbit anti-human IgA was removed from the refrigerator and tapped gently before being opened. 50 µl of Rabbit anti-human IgA was added to the polypropylene tube containing the 24.950 ml of carbonate/ bicarbonate buffer to produce the primary antibody in coating buffer in a 1:100 dilution. The tube containing the primary antibody/coating buffer mix was gently tapped at the bottom followed by vortexing (Vortex Genie; New York, USA) for approximately 2 seconds at speed setting '3'.

The primary antibody/coating buffer was stored in the refrigerator at 4ºC.

2.6.2.3 Preparation of PBS Solution

PBS solution (pH 7.4) was prepared by dissolving 1 PBS tablet (Sigma; St. Louis, USA) in 200 ml of autoclaved deionised water (as per instruction from the product specification sheet) in an appropriately labelled autoclaved glass bottle, using a magnetic stirrer (Ikamag; Staufen, Germany) at room temperature for approximately 10 minutes.

The PBS solution was stored in the refrigerator at 4ºC. The PBS tablets were stored at room temperature.

2.6.2.4 Preparation of Washing Buffer (PBS/0.3M NaCl/ 0.1% Tween 20)

PBS solution (pH 7.4) was prepared by dissolving 5 PBS tablets in a litre of autoclaved deionised water in an appropriately labelled autoclaved glass bottle, using a magnetic stirrer at room temperature for approximately 10 minutes.

The PBS solution was stored in the refrigerator at 4ºC. The PBS tablets were stored at room temperature.

20.75 g of NaCl (Sigma-Aldrich; Milwaukee, USA) was massed using a mass balance (Adam AFP- 3100 L; Danbury, USA) in an autoclaved glass bottle. 1 litre of PBS solution was added to the bottle, and the contents was dissolved using a magnetic stirrer at room temperature for approximately 8 minutes. Following dissolution, 1 ml of Tween 20 (Sigma; Steinheim, Germany) was added and allowed to dissolve for approximately 5 minutes using the magnetic stirrer.

The washing buffer was stored in the refrigerator at 4ºC.

2.6.2.5 Preparation of Blocking Agent (2% FCS in PBS)

FCS (Highveld Biological; Johannesburg, South Africa) was stored at -70ºC. The FCS was placed in the refrigerator to thaw overnight prior to use.

500µl of FCS was added to an appropriately labelled 50 ml polypropylene tube containing 24.500 ml of PBS. The tube was then vortexed for approximately 5 seconds at speed setting '3'.

The 2% FCS in PBS was stored in the refrigerator at 4ºC.

2.6.2.6 Reconstitution of Biotinylated Lectin (VV) and Dilution of Biotinylated VV Lectin in PBS (1:500)

Biotinylated VV lectin (2 mg) (Vector; California, USA) was stored refrigerated at 4ºC. 1ml of autoclaved deionised water was added to the vial containing Biotinylated VV lectin, for reconstitution. The vial was gently tapped and vortexed for approximately 3 seconds at speed setting '3'.

Reconstituted Biotinylated VV lectin was stored refrigerated at 4ºC.

10 µl of reconstituted Biotinylated VV lectin was added to 4990µl of PBS in a 15 ml tube (1:500). The tube was gently tapped and vortexed for approximately 2 seconds at speed setting '3'.

2.6.2.7 Dilution of HRP-conjugated Avidin in PBS (1:2000)

Horseradish Peroxidase Avidin D Concentrate (5 mg) (Vector; California, USA) was stored refrigerated at 4ºC.

19.990 ml of PBS was added to an appropriately labelled 50 ml polypropylene tube. 10 µl of HRP-avidin was added to the tube. The tube was gently tapped and vortexed for approximately 5 seconds at speed setting '3'.

The HRP-avidin/PBS was stored refrigerated at 4ºC.

2.6.2.8 Preparation of OPD/ H_2O_2 Substrate

OPD tablets (Dako; Glostrup, Denmark) were stored refrigerated at 4ºC. The temperature range for storage is 2-8 ºC.

The bottle of OPD tablets was removed from refrigeration and allowed to stand for 10 minutes at room temperature prior to opening.

The tablet was removed with the aid of a pair of tweezers. The tablet was placed in an appropriately labelled, foil-covered 15 ml polypropylene tube containing 3ml of deionised autoclaved water. The foil-covered tube was used to protect the substrate solution from direct light. The vial was gently tapped at the bottom and vortexed for approximately 4 seconds at speed setting '3'.

30% H_2O_2 solution was stored refrigerated at 4ºC and protected from light, to preserve the activity. Immediately before use, 1.2µl of 30% H_2O_2 solution (500 ml) (Sigma; Steinheim, Germany) was pipetted into the OPD solution. The substrate solution was used within an hour of preparation, as per instructions from the Dako OPD specification sheet.

2.6.2.9 Preparation of 1M H_2SO_4 (Stopping Solution)

At the fume cupboard, 20ml of autoclaved deionised water was added to a 100 ml measuring cylinder. 5.6 ml of H_2SO_4 (95-97%) (Merck; Darmstadt, Germany) was added to the 100 ml measuring cylinder. Autoclaved deionised water was added to the 100 ml measuring cylinder to the final volume of 100 ml. The H_2SO_4 solution was poured into an appropriately labelled 100 ml glass bottle. The bottle containing the H_2SO_4 solution was gently swirled and stored at room temperature.

2.6.3 Methodology

2.6.3.1 Coating of the Plate with Primary Antibody

Costar 96-well plates (Corning; New York, USA) were stored at room temperature.

Prior to each experiment, a sketch of the plate containing the wells designated to specific samples and positive and negative controls, was prepared.

At room temperature at the bench top, 100 µl of refrigerated primary antibody in coating buffer was added to the wells designated to receive samples and controls.

The plate was immediately sealed with a polyethylene wrap (Glad; Babelegi, South Africa) and placed in the refrigerator for incubation at 4ºC for 24 hours.

2.6.3.2 Blocking

The polyethylene wrap was carefully removed and discarded following the 24 hour incubation period. The plate was washed 4 times with 300 µl of wash buffer per well with the aid of a microplate washer (Anthos Fluido; Cambridge, United Kingdom) at room temperature.

The plate was turned over onto a stack of paper towel to remove excess fluid.

The base of the tube containing 2% FCS in PBS was tapped gently for approximately 4 seconds. 100µl of refrigerated 2% FCS in PBS was added to each well. The plate was then sealed with polyethylene wrap and left to incubate for 1 hour on the bench-top at room temperature.

Bovine serum albumin is generally used as the blocking agent in *O*-glycosylation lectin binding studies of IgA1 (Allen *et al*, 2001). However, in this study, fetal calf serum albumin was used instead. For plate 12 (Appendix 8), however, bovine serum albumin was used instead of fetal calf serum in the preparation of the blocking agent, and similar results were achieved.

2.6.3.3 Serum Sample Preparation

The cryogenic vials containing the desired frozen serum samples were removed from storage and left to thaw on the bench-top at room temperature for approximately 10 minutes.

1.5 ml microcentrifuge tubes (Eppendorf; Hamburg, Germany) were labelled according to the serum sample and the designated well.

495µl of PBS was added to each microcentrifuge tube. 5µl of serum was added to the appropriately labelled microcentrifuge tube containing PBS (1:100).

Each tube was vortexed for approximately 6 seconds at speed setting '4'. The base of the tube was tapped 22 times.

50µl of serum/PBS was added to the designated wells in duplicate. 50 µl of PBS was added to the negative control wells.

The plate was carefully sealed with polyethylene wrap and incubated overnight (16 hours) at 4ºC in the refrigerator.

2.6.3.4 Lectin Application

Following the 16 hour incubation period, the plastic wrap was carefully removed and discarded. The plate was washed 4 times with 300 µl of wash buffer per well, with the aid of a microplate washer, at room temperature. The plate was turned over onto a stack of paper towel to remove excess liquid.

50 µl of Biotinylated VV lectin/PBS was added to each well. The plate was sealed with polyethylene wrap and left to incubate for 90 minutes at room temperature on the bench-top.

2.6.3.5 Horseradish Peroxidase/Avidin Application

Following the 90 minute incubation period with the Biotinylated VV lectin/PBS, the polyethylene wrap was carefully removed and discarded. The plate was washed 4 times with 300 µl of wash buffer per well, with the aid of a microplate washer, at room temperature.

The plate was turned over onto a stack of paper towel to remove excess fluid.

50µl of HRP/Avidin/PBS was added to each well. The plate was sealed with polyethylene wrap and left to incubate for 90 minutes at room temperature on the bench-top.

2.6.3.6 Development

Following the 90 minute incubation period with HRP/Avidin/PBS, the polyethylene wrap was carefully removed and discarded. The plate was washed 4 times with 300 µl of wash buffer per well, with the aid of a microplate washer, at room temperature.

Prior to the washing of the plate, the vial containing the OPD was removed from the fridge and left to stand for 10 minutes on the bench-top at room temperature.

3ml of autoclaved deionised water was added to a 15 ml foil-covered propylene tube. Using a pair of tweezers, an OPD tablet was added to the tube. The tube was gently tapped and vortexed for approximately 4 seconds at speed setting '3'.

1.2µl of 30% H_2O_2 solution was added to the vial containing the OPD solution immediately before the addition of OPD/ H_2O_2 substrate to each well. The vial

was gently tapped and vortexed again for approximately 3 seconds at speed setting ‘3’.

The substrate solution was applied to the plates at 50µl per well. Excess substrate solution was discarded as hazardous waste.

The plate was covered with foil and placed on the microplate shaker (IKA Schuttler MTS 4; Staufen, Germany) at 100 rpm at room temperature for 10 minutes for the colour development. An olive-green/brown colour results (fig. 3). The reaction was stopped with the addition of 1M H_2SO_4. A light pink/orange colour results upon addition of stopping solution (fig. 4).

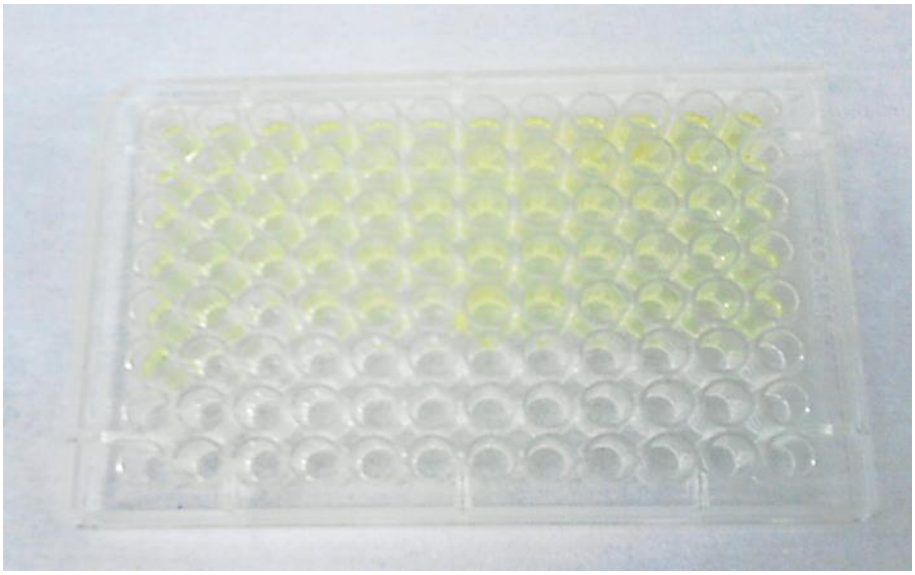

Figure 3: 96 well Immunoplate Following Agitation after Addition of Substrate Solution (OPD/ H_2O_2)

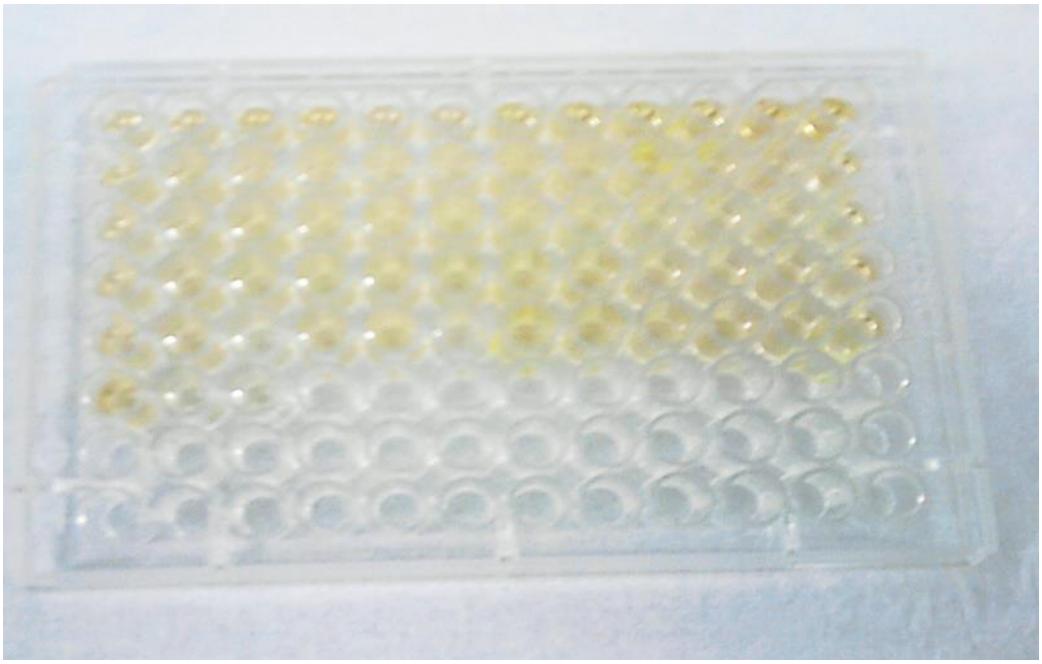

Figure 4: 96 well Immunoplate Following Addition of Stopping Solution (1M H_2SO_4)

2.6.3.7 Plate Reading

Following the addition of stopping solution, the plate was transferred to the plate reader (Anthos 2010; Cambridge, United Kingdom). The plate was read at 490 nm at room temperature.

A print-out of the absorbance values at 490 nm was attained. The plate was wrapped in excess paper towel and discarded as hazardous waste.

2.6.4 Statistical Analyses of the Absorbance Data

Since the VV lectin binds to ungalactosylated GalNAc i.e. the Tn antigen, greater lectin binding is an indicator of IgA1 *O*-hypogalactosylation, which results in a higher absorbance value.

The mean absorbance value for each duplicate sample was calculated and used for the analyses (Appendix 8; table 7, table 8).

The GraphPad Instat 3.0 statistical software was used to compare the absorbance data between and within experimental and control groups. Where the distribution of data did not pass the normality test in the groups to be compared, a non-parametric test called the Wilcoxon matched-pairs test, was used for comparison. The Mann-Whitney test was used to assess for a statistically significant difference between groups; a p value of less than 0.05 was considered to be statistically significant. The Spearman's correlation test was used to assess for a correlation between age and IgA1 *O*-hypogalactosylation.

2.6.4.1 IgAN and IgA1 *O*-Galactosylation

For each experiment, the mean absorbance value for the IgAN group and control group was compared.

2.6.4.2 IgA1 *O*-Galactosylation and Race

Since the IgAN group was predominantly Indian (table 1), for each experiment the mean absorbance value for the Indian IgAN group was compared to that of the other race groups combined.

2.6.4.3 IgA1 *O*-Galactosylation and Gender

The mean absorbance value for the male and female IgAN sub-groups was compared for each experiment.

2.6.4.4 IgA1 *O*-Galactosylation in Pre-dialysis versus End-stage IgAN Patients

For each experiment, the mean absorbance value for the pre-dialysis IgAN patients was compared to that of the end-stage renal disease patients.

2.6.4.5 IgA1 *O*-Galactosylation and Age in IgAN Patients

The Spearman correlation test was used to assess for a possible correlation between age and the level of IgA1 *O*-galactosylation in IgAN patients.

CHAPTER 3 - RESULTS

3.1 IgAN and IgA1 *O*-Galactosylation

The distribution of the data did not pass the normality test. The average means of the experiments for the IgAN group is 0.3678 ± 0.0790 (standard error of the

mean (SEM) and is statistically significantly greater than the normal control group which is 0.2969 ± 0.0586 (SEM); (p = 0.0076) (fig. 5). Thus, in this population of IgAN patients, the serum IgA1 is hypogalactosylated.

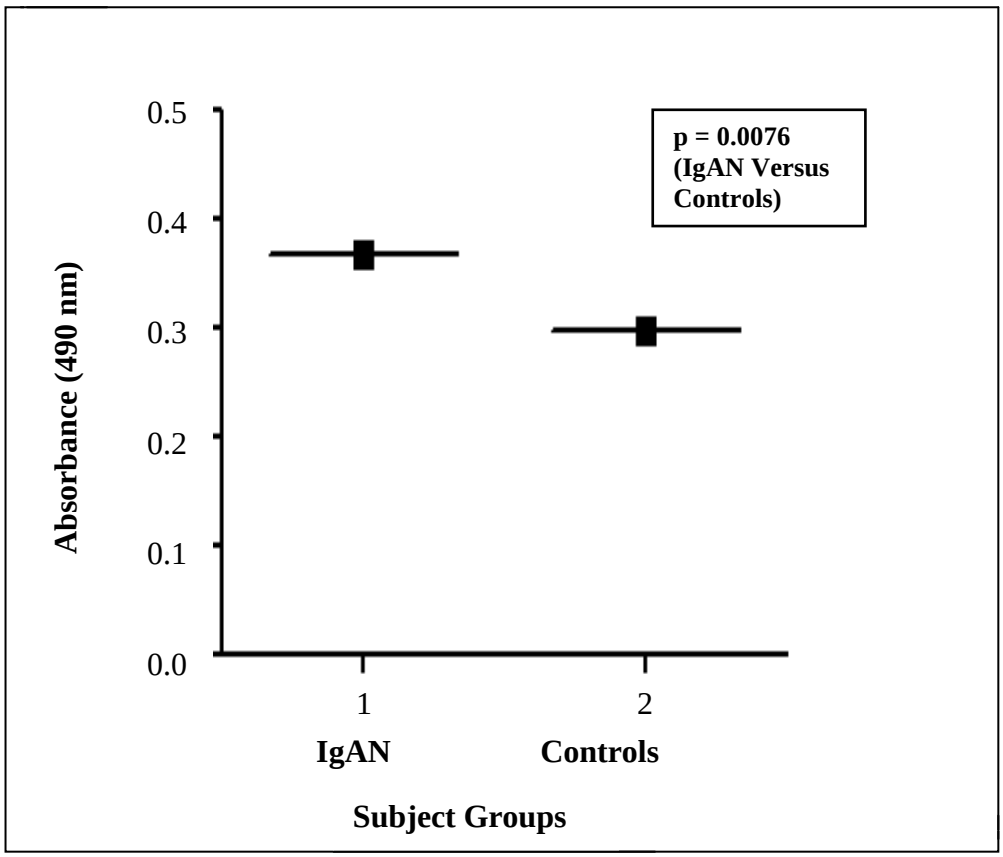

Figure 5: IgA1 *O*-Hypogalactosylation in IgA Nephropathy patients Versus Controls

3.2 Participant Description

Table 1: Race, Age and Gender Distribution in IgAN Patients and Controls

	IgAN	Control
No. Participants	19	20
Male (%)	63	45
Mean Age	46	32
IgAN Patients at ESRD (%)	53	N/A
African	2	6
Caucasian	2	3
Coloured (Mixed)	3	1
Indian/Caucasian (Mixed)	1	0
Indian	11	10

Number (No.); Not Applicable (N/A); End Stage Renal Disease (ESRD).

3.3 IgA1 *O*-Galactosylation and Race

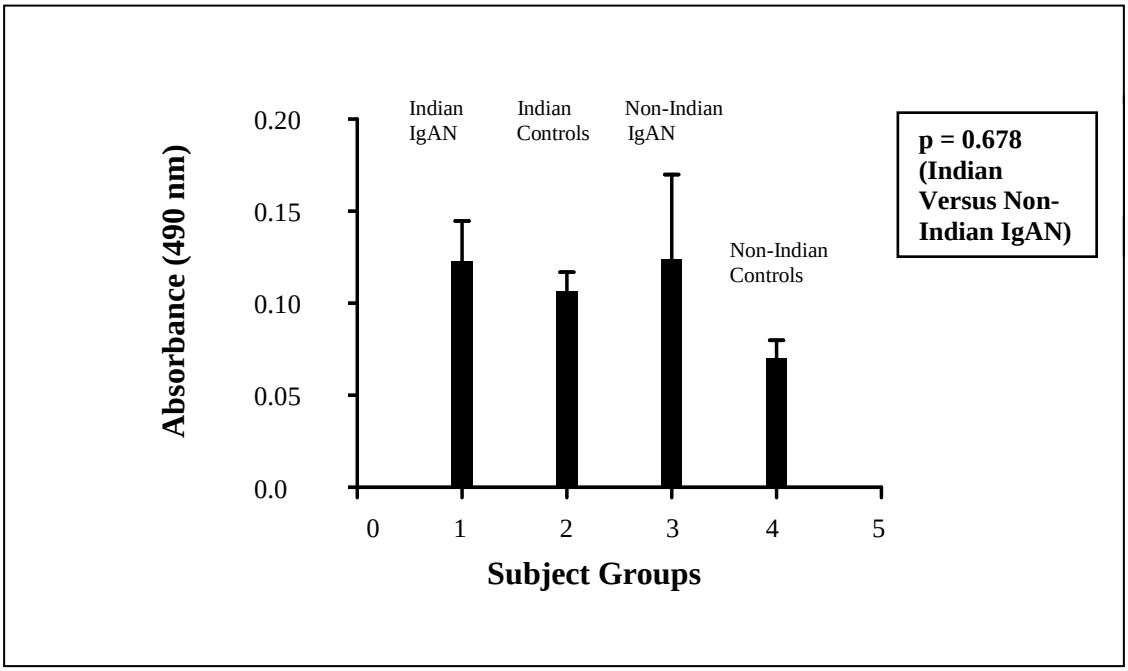

Figure 6: IgA1 *O*-Hypogalactosylation in Indian Versus Non-Indian IgA Nephropathy Patients

The distribution of the data did not pass the normality test. There was no statistically significant difference between the average means of the experiments for the IgAN patients of the Indian group and that of the Non-Indian group ($p = 0.678$) (fig. 6). The mean for the Indian IgAN patients was: 0.1225 ± 0.02220. The mean for the Indian control group was 0.10657 ± 0.01039. The mean for the non-Indian IgAN patient group was 0.12368 ± 0.04616. The mean for the

non-Indian control group was 0.07020 ± 0.00968.

3.4 IgA1 *O*-Galactosylation and Gender

The distribution of the data did not pass the normality test. Although the average of the absorbance means, corresponding to the level of IgA1 *O*-hypogalactosylation, was higher in male than female IgAN patients, there was no statistically significant difference between the level of IgA1 *O*-hypogalactosylation in male and female IgAN patients (fig 7) (p = 0.8501). Fig. 7 and fig. 8 demonstrate that the level of IgA1 *O*-hypogalactosylation is higher in IgAN patients than in controls.

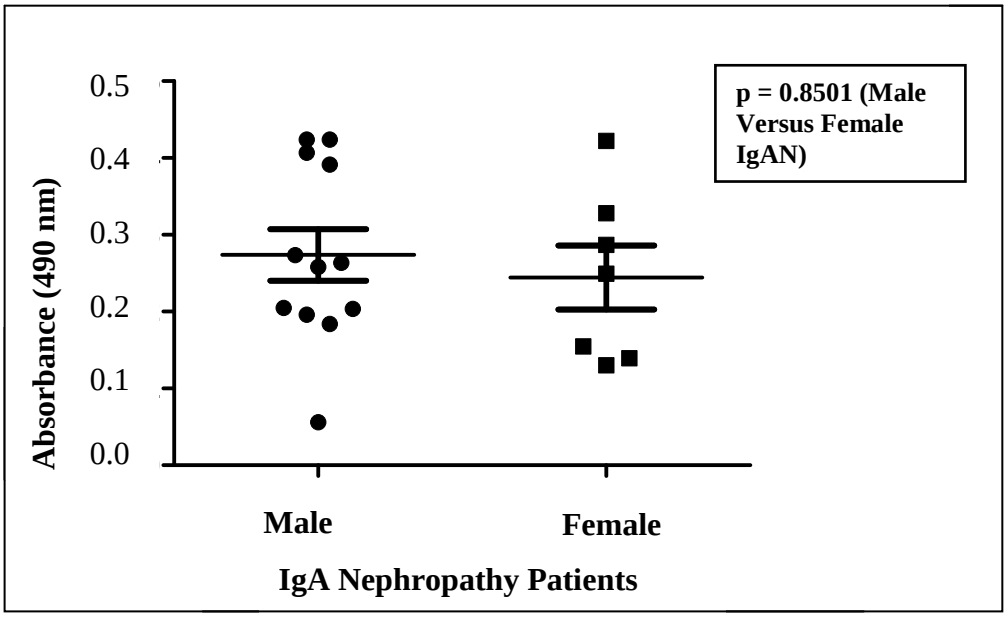

Figure 7: IgA1 *O*-Hypogalactosylation in Male Versus Female IgAN Patients

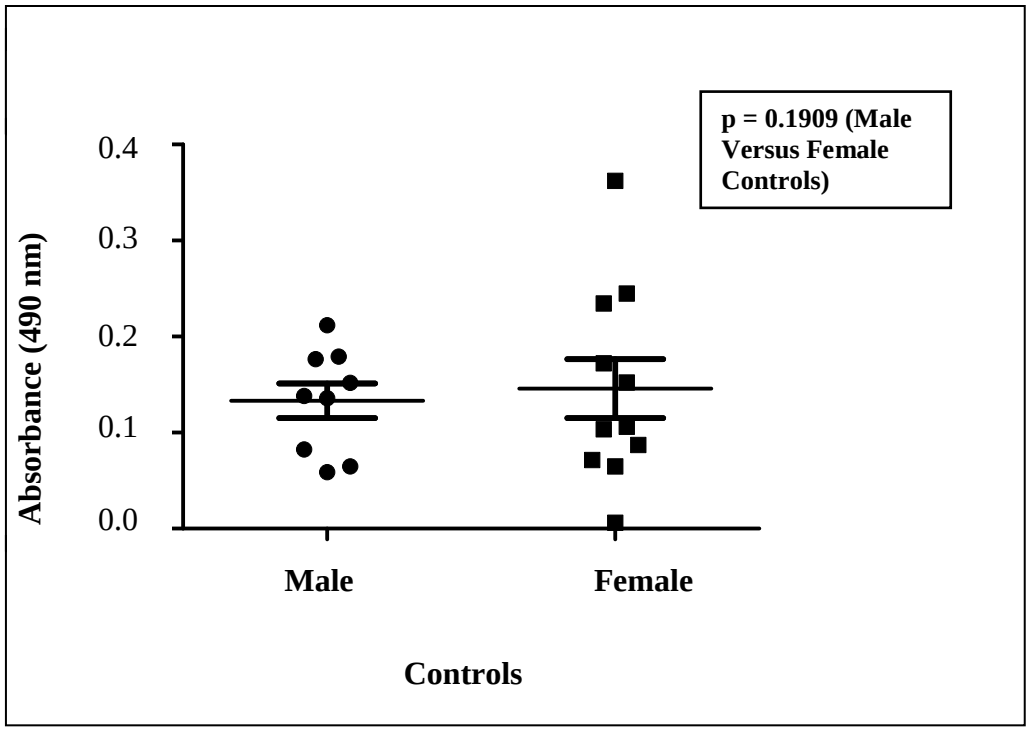

Figure 8: IgA1 *O*-Hypogalactosylation in Male Versus Female Controls

3.5 IgA1 *O*-Galactosylation in Pre-dialysis Versus End-stage IgAN Patients

The distribution of the data did not pass the normality test. Although the level of IgA1 *O*-hypogalactosylation was higher in end-stage IgAN patients compared to pre-dialysis patients, there was no statistically significant difference between the level of IgA1 *O*-hypogalactosylation in pre-dialysis and end-stage IgAN patients (fig. 9). The p value is 0.6685.

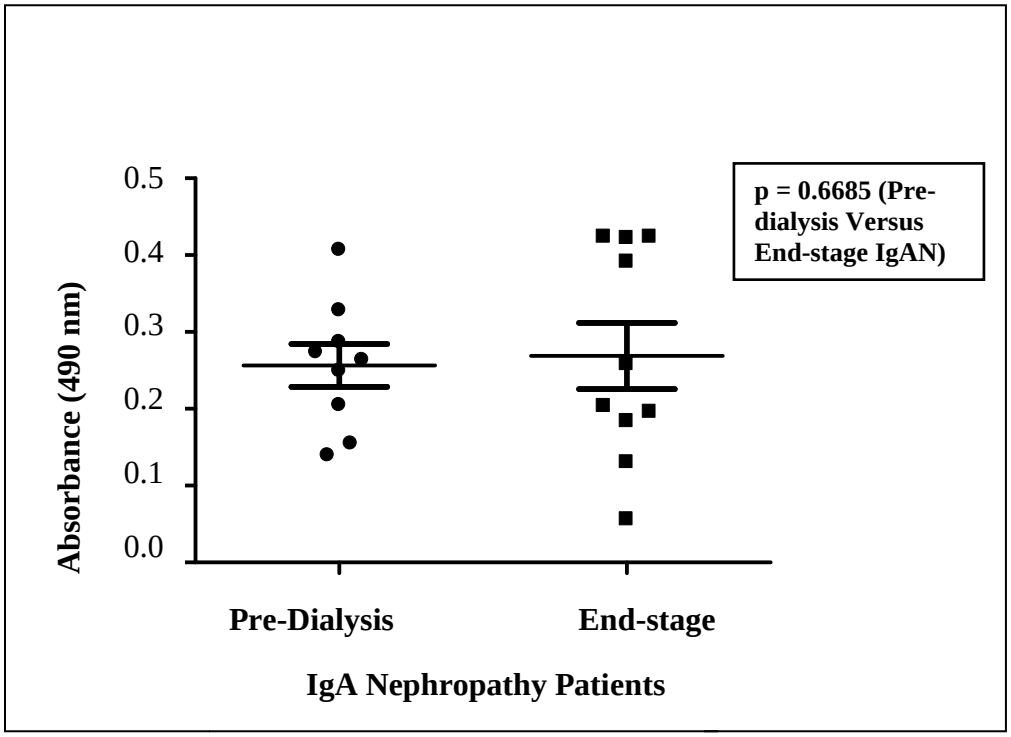

Figure 9: IgA1 *O*-Hypogalactosylation in End-stage Versus Pre-dialysis IgAN Patients

3.6 IgA1 *O*-Galactosylation and Age in IgAN Patients

No correlation was found between IgA1 *O*-galactosylation and age in IgAN patients (IgAN: Spearman R = 0.1258; p = 0.5973 (fig. 10); Controls: Spearman R = 0.2475; p = 0.2927 (fig. 11)).

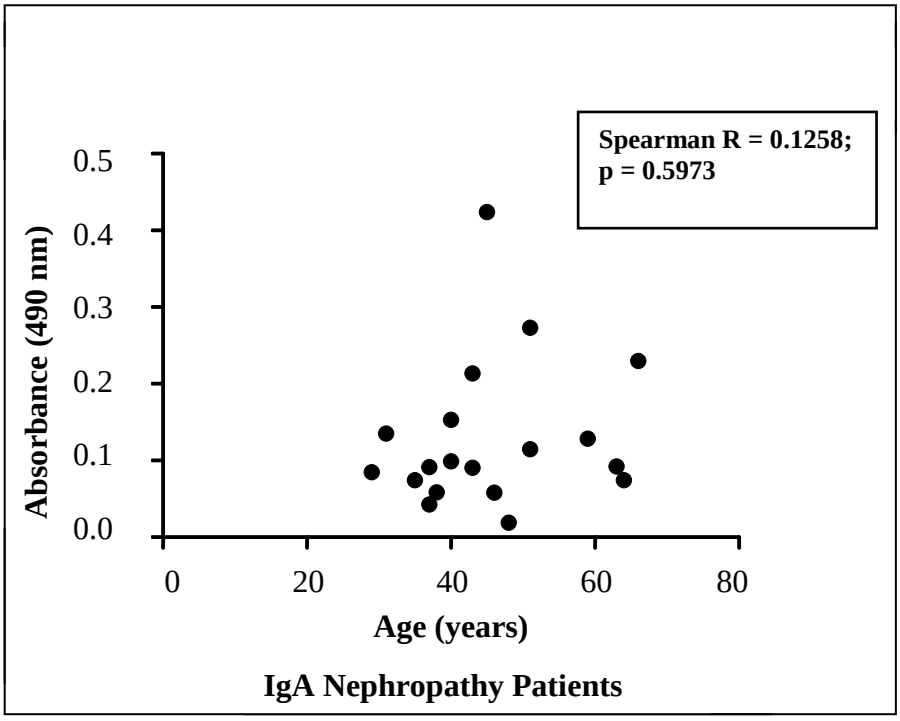

Figure 10: IgA1 *O*-Hypogalactosylation and Age in IgAN Patients

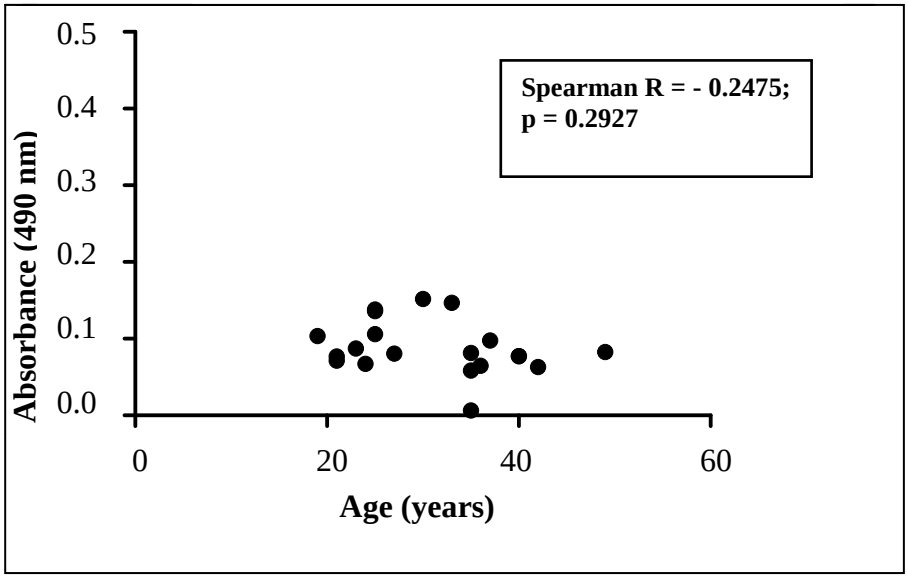

Figure 11: IgA1 *O*-Hypogalactosylation and Age in Controls

CHAPTER 4 - DISCUSSION

4.1 Abnormal Serum IgA1 *O*-Galactosylation in a Multi-ethnic IgAN Population in KZN, South Africa

In this study, the *O*-galactosylation of serum IgA1 in IgAN patients from KZN, South Africa was analysed using an ELISA-type lectin binding assay. In the study population, the IgAN patients exhibited abnormal serum IgA1 *O*-galactosylation, as defined by a statistically significantly greater level of hypogalactosylation in comparison to that of the controls ($p < 0.05$) (fig. 5). Terminal D-galactose is the ligand of the hepatic ASGPR which mediates endocytotic clearance of IgA1 from the circulation following recognition of D-galactose. In IgAN however, the galactose-deficient IgA1-containing immune complexes, may thus escape hepatic clearance and pathogenetically deposit in the renal mesangium, effecting inflammation and injury.

4.2 IgA1 *O*-Galactosylation and Race in IgAN

4.2.1 Sample Size

The recruitment of IgAN patients for the study was hindered by: the number of individuals with IgAN that presented to the IALCH, the number of renal patients attending the IALCH Renal Unit that were biopsied, and the number of IgAN patients at the IALCH that consented to the study. Some individuals with IgAN remain undiagnosed until an advanced stage of the disease is reached. Not every renal patient at the IALCH Renal Unit was biopsied. Certain conditions such as shrunken kidneys, for example, may preclude biopsy. Thus, statistically comparable numbers of IgAN patients for each ethnic group was not attained.

Since the IgAN group in this study was predominantly of Indian ethnicity, the level of serum IgA1 *O*-galactosylation of the Indian sub-group was compared to that of the non-Indian IgAN sub-group. There was no statistically significant difference between the level of IgA1 *O*-hypogalactosylation in the Indian and non-Indian IgAN sub-groups (fig. 6).

Further studies should include a larger cohort with a greater representation of the

various ethnic groups in order to make valid conclusions regarding the relationship between race and IgA1 *O*-galactosylation in IgAN.

4.2.2 Incidence Data for Black and Indian IgAN Patients in KwaZulu Natal

Seedat *et al* conducted an analysis of the primary glomerular diseases in 252 Black and 75 Indian patients that presented to the Renal Unit at the King Edward VIII Hospital, over a period of 6 years i.e. 1981 to 1986 (1988). This unit was the only specialised unit investigating renal diseases in African and Indian patients in Natal, the former KZN. The six year time period matches that of the recruitment period of the current study (2006 to 2011). It is noteworthy that the study by Seedat *et al* revealed a similar race and gender distribution to this study: 2 Africans and 10 Indians, versus 2 Africans and 11 Indians in this study; with a preponderance of males. However, incidence data should be interpreted with caution as various factors, including access to health care, may determine the number of individuals that present for diagnosis; and various factors preclude biopsy and hence diagnosis in the number of presenting individuals.

4.2.3 IgA1 *O*-Galactosylation Data for Indian IgAN Patients

Studies from North America (in Caucasian populations and a few in African Americans), Europe (in Caucasian populations) and Asia (in Chinese and Japanese populations) have demonstrated elevated serum levels of hypogalactosylated IgA1 in IgAN patients (Hastings *et al*, 2010; Shimozato *et al*, 2008; Lau *et al*, 2007; Moldoveanu *et al*, 2007; Renfrow *et al*, 2005; Amore *et al*, 2001; Allen *et al*, 2001; Hiki *et al*, 2001; Allen & Feehally, 2000; Allen *et al*, 1999; Hiki *et al*, 1999; Hiki *et al*, 1998; Tomana *et al*, 1997; Hiki *et al*, 1996; Allen *et al*, 1995; Tomana *et al*, 1995; Mestecky *et al*, 1993).

Following a review of the literature to date (June, 2013), using PubMed and Science Direct databases, and the Google search engine, such *O*-galactosylation studies of serum IgA1 in IgAN patients of Indian descent were not found. In addition, studies on the pathogenesis of IgAN in India are limited (Minz *et al*, 2010). The current study provides IgA1 *O*-galactosylation data for Indian IgAN patients. The study demonstrates that abnormal IgA1 *O*-galactosylation exists in IgAN patients of Indian descent. Such a finding thus contributes to the understanding of the disease pathogenesis in individuals of Indian descent. Since the pathogenesis of IgAN is incompletely understood, it is not known whether

causal or pathogenic factors vary between race groups. It is thus essential for IgA1 glycosylation studies to be conducted in various race groups in order to elucidate the pathogenic mechanisms involved, and to establish if such mechanisms are race-specific. Furthermore, the role of environmental factors and the effect thereof on the IgAN pathogenesis is not completely understood; thus studies in different populations are required globally.

4.3 IgA1 *O*-Galactosylation and Gender in IgAN

4.3.1 Sample Size

In the study population, there was no statistically significant difference between the level of serum IgA1 *O*-hypogalactosylation in male and female IgAN patients. However, it is noteworthy that the number of male and female participants in this study population are too low for statistical comparison (12 males versus 7 females). Further studies should include a larger cohort in order to assess for a statistically valid relationship between gender and IgA1 *O*-galactosylation in IgAN.

4.3.2 IgA1 *O*-Galactosylation in Male IgAN Patients

The IgAN study population was predominantly male. Indeed, IgAN is more common in males than females with a 2:1 ratio, and males are associated with a worse prognosis than females (Neugarten *et al*, 2000; Ibels & Gyory, 1994).

For each IgAN participant, the average of each absorbance value (corresponding to the level of IgA1 *O*-hypogalactosylation) from each experiment was obtained. When the average of these absorbance values for the male and female IgAN patients were compared, the level of serum IgA1 *O*-hypogalactosylation was higher in males than females (fig. 7). Linossier *et al* reported that the statistically significantly reduced terminal *O*-galactosylation of serum IgA1 was predominant in male IgAN patients (2003). The gene encoding Cosmc is located on the X-chromosome i.e. Xq23 (Ju & Cummings, 2002). Since males contain a single X-chromosome, there may be potential for dominance of any mutation in the gene encoding Cosmc, thus predisposing males to abnormal IgA1 *O*-galactosylation via the pathogenetic mechanism of non-functional Cosmc.

4.4 IgA1 *O*-Galactosylation in Pre-dialysis Versus End-stage IgAN Patients

4.4.1 Sample Size

In the IgAN study population, there was no statistically significant difference between the level of IgA1 *O*-hypogalactosylation in pre-dialysis and end-stage IgAN patients. However, it is noteworthy that the number of pre-dialysis and end-stage IgAN patients is too low for a statistically valid comparison (10 end-stage versus 9 pre-dialysis IgAN patients). Further studies should include a larger cohort with a greater representation of pre-dialysis and end-stage IgAN patients, in order to assess for a statistically significant difference in the IgA1 *O*-hypogalactosylation between such groups.

4.4.2 IgA1 *O*-Galactosylation and IgAN Progression

For each IgAN participant, the average of each absorbance value (corresponding to the level of IgA1 *O*-hypogalactosylation) was obtained for each experiment. When the average of these absorbance values for the end-stage and pre-dialysis

IgAN patients were compared, the level of serum IgA1 *O*-hypogalactosylation was higher in the end-stage patients (fig. 9). IgA1 *O*-hypogalactosylation may result in prevention of IgA1 clearance from the serum and the generation of galactose-deficient IgA1-specific antibodies. A greater degree of IgA1 *O*-galactose-deficiency may result in enhanced glomerular pathology and thus disease progression due to the enhanced induction of galactose-deficient IgA1-specific mediated effects *viz*. complement activation (Hashimoto *et al*, 2012; Couser, 1985), mesangial cell proliferation (Novak, *et al* 2011), macrophage migration inhibitory factor and renin up-regulation (Lai & Leung, 2002), increased apoptosis rate and nitric oxide synthesis (Amore *et al*, 2001), platelet-activating factor induction (Coppo *et al*, 2010), and down-regulation of adiponectin (Inoue, 2012).

Berthoux *et al* have demonstrated that autoantibodies targeting hypogalactosylated IgA1 are associated with the progression of IgAN (2012). Serum IgG autoantibody levels predicted dialysis or death. In patients with worse clinical outcomes, the levels were higher in a stepwise pattern. In patients with IgAN, the mean serum levels of total autoantigen, normalised IgG autoantibody, and total IgA autoantibody were significantly higher than in the combined controls (Berthoux *et al*, 2012).

4.4.3 Race and IgAN Progression

Although the incidence of IgAN in India is low, the severity is markedly greater than that of other populations globally (Chacko, 2011). The reasons for this difference are unknown and Chacko argues that more basic studies and multi-centre clinical trials are needed to address this pattern (2011). In this study, the percentage of Indian IgAN patients were higher in both the end-stage (60%) and pre-dialysis (67%) sub-groups. It is noteworthy that the number of patients in the end-stage and pre-dialysis IgAN sub-groups are too low to describe associations between race and disease progression.

Indeed, Barbour *et al* reported an increased risk of ESRD in a longitudinal observational study of a cohort of 202 individuals of self-reported Pacific Asian origin, in comparison to a non-Pacific Asian cohort of 467 individuals. The non-Pacific Asian cohort comprised of 360 Caucasian, 24 Black and 83 individuals classified as "Other" (2013).

Individuals of Pacific Asian origin had a significantly increased rate of estimated glomerular filtration rate decline with an absolute difference of 1.62 ml/min/1.73m^2/year and an increased risk of a 50% reduction in estimated

glomerular filtration rate.

4.4.4 Gender and IgAN Progression

The end-stage IgAN group was predominantly male (8 (80%)). Indeed, the number of end-stage and pre-dialysis IgAN patients is too low to describe associations between gender and IgAN disease progression. In a meta-analysis, male IgAN patients were significantly associated with a rapid rate of disease progression in comparison to female IgAN patients (Neugarten *et al*, 2000).

4.5 IgA1 *O*-Galactosylation and Age in IgAN Patients

In the study, no correlation was found between age and IgA1 *O*-hypogalactosylation in IgAN patients. A meaningful measure of a correlation of IgA1 *O*-hypogalactosylation and age should be a comparison thereof within an individual across time. Such data may also reveal the effect of hypogalactosylation on disease progression and provide a comparison for hypogalactosylation within periods of remission.

Following a review of the literature to date (June, 2013), using PubMed and

Science Direct databases, and the Google search engine, no studies on the effect of age on the *O*-galactosylation of IgA1 in IgAN were conducted.

4.6 IgA1 Concentration and Galactose-Deficiency Per IgA1 Molecule

In the study, the IgA1 concentration per serum sample was not measured and the IgA1 concentration was thus not standardised for each sample. Therefore, based on a principle of ELISA i.e. the saturation of specific binding points for a concentrated test sample, each absorbance value may possibly be an underestimation of the galactose-deficiency per IgA1 molecule.

Further studies merit investigation of the galactose-deficiency per IgA1 molecule in the study population. It is essential to assess the level of hypogalactosylation per IgA1 molecule in relation to IgAN disease progression; and to compare the extent of deficiency among race groups, across gender, and within the same individual over time. Such data may be compared between IgAN populations globally; and thus contribute to a greater understanding of the disease pathogenesis.

4.7 Screening and Prognostic Potential of the Lectin Binding Assay for IgAN

Okazaki *et al* sought to examine the impact of the duration between disease onset and first nephrologist consultation on renal prognosis in a retrospective study in a Japanese IgAN cohort whose medical records were available for a 10 year period (2011).

The rate of increase in serum creatinine levels over the 10 year follow-up period was used as a prognostic indicator. IgAN patients were categorised into histological prognostic stages according to the "Clinical guidelines for IgA Nephropathy in Japan, 2nd version" (Tomino & Sakai, 2003). Group 1, 2, 3 and 4 referred to patients with good prognosis, relatively good prognosis, relatively poor prognosis, and poor prognosis respectively.

Okazaki *et al* reported a significant positive correlation between the duration from disease onset to the first medical intervention i.e. a nephrologist

consultation and renal prognosis in group 3 patients i.e. patients with relatively poor prognosis (2011). Thus early medical intervention may result in improved renal prognosis. Early screening is required for the subsequent diagnosis via renal biopsy in order to provide early medical attention.

The global standardisation of a lectin binding assay for the specific measurement of IgA1 *O*-hypogalactosylation per sample of a specific IgA1 concentration may hold promise for a screening or prognostic test for IgAN. Future studies based on global comparative analyses of standardised assay results may serve to ascertain a pathogenic level of IgA1 *O*-hypogalactosylation in IgAN. Such a level may serve as a possible prognostic indicator or a means of screening for IgAN. The lectin-binding assay is relatively rapid and inexpensive in comparison to other assays for IgA1 *O*-galactosylation analysis. Therefore more people may be screened for IgAN than is currently possible.

4.8 Mechanisms Responsible for Abnormal IgA1 *O*-Galactosylation

This study provides evidence for a pathogenic mechanism of abnormal IgA1 *O*-galactosylation in a population of IgAN patients in South Africa. For the development of specific therapies, further studies need to assess the precise mechanism or mechanisms responsible for abnormal IgA1 *O*-galactosylation in such a population. IgAN is very likely the result of an interplay of multiple factors and or mechanisms.

Boyd *et al* argue that the lack of a clear understanding of the origins of galactose-deficient IgA1 and an incomplete understanding of immune complex formation have hampered the development of specific therapeutic strategies for the prevention of mesangial IgA deposition (2012).

4.8.1 Evaluated Hypotheses for Abnormal IgA1 *O*-Glycosylation

4.8.1.1 Altered Amino Acid Sequence of the IgA1 Hinge Region

It has been hypothesised that a change in the amino acid sequence of the α 1 gene encoding the IgA1 hinge region, could alter the template available for *O*-glycosylation. Greer *et al* however, reported that the hinge region nucleotide sequence was identical in IgAN patients and controls (1998). Furthermore, Hiki *et al* demonstrated via mass spectrometry that the isolated deglycosylated IgA1 hinge region peptide is of identical molecular mass in IgAN patients and controls (1998). Such data dispute an abnormality of the hinge-region amino acid sequence and provide evidence that the *O*-glycosylation defect is post-translational.

4.8.1.2 Removal of Terminal Galactose via Glycosidases

It has been hypothesised that the reduced terminal galactosylation of GalNAc could be due to its removal by abnormal glycosidase activity in circulation. However, C1 inhibitor, one of the few serum proteins with *O*-glycosylation, does not display the same lectin binding pattern as IgA1 from IgAN patients (Allen *et al*, 1995). The difference in VV binding to C1 inhibitor and IgA1 from patients with IgAN

disputes a widespread glycosidase defect. Such data indicate that abnormal IgA1 *O*-glycosylation is a synthetic defect rather than a degradative one.

4.8.1.3 Galactose Addition Defect – β 1,3 GT Functionality

A defect in the addition of galactose to GalNAc may be responsible for abnormal IgA1 *O*-glycosylation in IgAN patients (Allen *et al*, 1997). Allen *et al* compared the functional activity of β 1,3 GT, the enzyme responsible for the galactosylation of *O*-linked glycans, in cell lysates of IgAN patients and healthy controls (1997). Regalactosylation of degalactosylated IgA1 hinge region fragments from healthy controls was measured following incubation with T-cell, B-cell and monocyte lysates from IgAN patients and controls. Hypogalactosylation of the IgA1 hinge region was detected following incubation with B-cell lysates from patients with IgAN. Such data demonstrate that a defect in the activity of β 1,3 GT is responsible for abnormal IgA1 *O*-galactosylation in IgAN patients.

4.8.2 Functionality of Cosmc and β 1,3 GT is Essential for Normal Galactosylation of IgA1

The activity of β 1,3 GT is dependent on the activity of Cosmc which functions as a molecular chaperone (Ju and Cummings, 2002). Aryal *et al* demonstrated partial activity restoration *in vitro* of denatured β 1,3 GT using Soluble N-terminal 6×His-tagged Cosmc (2010). Aryal *et al* argue that it is unclear whether the molecular chaperone Cosmc requires co-chaperones *in vivo* (2010).

In a Chinese cohort of IgAN patients, down regulation of Cosmc due to external suppression was reported; whilst no genetic mutations for Cosmc were detected (Qin *et al*, 2007). Similarly, in a European cohort of IgAN patients, no mutations in the gene encoding Cosmc were detected; the study did not assess for mRNA levels (Malycha *et al*, 2008).

A deficiency in Cosmc and or β 1,3 GT may be responsible for the abnormal IgA1 *O*-galactosylation in the South African IgAN study population. The specific deficiency or deficiencies contributing to abnormal IgA1 *O*-galactosylation may vary in different populations globally and may be influenced by various genetic and or environmental factors. Thus, further studies are required to assess the

functionality of Cosmc and β 1,3 GT and factors which may influence the functionality thereof in the South African study population of IgAN patients.

4.8.3 Factors that may Influence Cosmc or β 1,3 GT Functionality

4.8.3.1 Interleukin 4

Yamada *et al* demonstrated that the Th2 cytokine IL- 4 down-regulates mRNA expression of both β 1,3 GT and Cosmc in the human IgA1-positive B-cell line (2010). Stimulation of B-cells with IL-4 also resulted in proliferation of the B-cell line, increased IgA1 production and hypogalactosylation of the IgA1 hinge region. The precise mechanism whereby IL-4 influences hypogalactosylation is unknown and merits further study.

Although IL-2 increased IgA1 secretion, there was no effect on cell proliferation or glycosylation. Whilst IL-5 stimulated significant cell proliferation, there was little or no effect on IgA1 secretion or glycosylation. Yamada *et al* thus argue that increased cell proliferation and the rate of IgA1 synthesis may not be critical determinants for altered glycosylation activity (2010).

Blood mononuclear cells from patients with IgAN produce more IL-4 upon mitogen stimulation; express higher levels of mRNA encoding IL-4 and demonstrate increased IL-5 activity compared to normal controls (Scivittaro *et al*, 1994; Lai *et al*, 1991). Compared to patients with mild disease, patients with severe renal dysfunction are more likely to hyperproduce IL-4, although they have normal IL-4 responses during remission (Lai *et al*, 1994; Scivittaro *et al*, 1994; Lai *et al*, 1991).

The levels of IL-4 in the South African IgAN study population have not been assessed; further studies are required to assess the levels thereof and to establish if hyper-production of IL-4 may be a contributory pathogenic mechanism in the study population. A possible mechanism whereby IL-4 could alter IgA1 *O*-galactosylation may include an abnormally increased IgA1 production per B-cell versus cellular proliferation. Renz *et al* demonstrated a reciprocal regulatory effect of IL-4 on cell growth and immunoglobulin production in human B-cell lines (1992). Such an increase in IgA1 production per B-cell in relation to B-cell proliferation, may increase the available IgA1 for galactosylation in relation to the level of Cosmc and β 1,3 GT and thus result in pathogenic hypogalactosylated IgA1. The hypothesis of an increase in hypogalactosylation via the mechanism of increased IgA1 production per B-cell, differs from the pathogenic mechanism of

increased IgA production in IgA myeloma wherein the mechanism responsible for increased levels of IgA is clonal expansion of myeloma cells. It is essential to assess the level of IgA1 production per B-cell versus B-cell proliferation in patients with IgAN. Such an investigation was not conducted in the South African IgAN study population or in IgAN populations globally; and merits further study in such populations.

4.8.3.2 Hypermethylation

A recent study revealed a mechanism of epigenetic silencing of Cosmc in the Tn4 cell line i.e. immortalised B-cells from a male patient with a Tn-syndrome-like phenotype (Mi *et al*, 2012). These cells lack transcripts for Cosmc due to epigenetic silencing by abnormal hypermethylation of the promoter for the Cosmc gene. The gene encoding Cosmc is located on chromosome Xq24 (Ju and Cummings, 2002). The Tn4 cells also lack β 1,3 GT activity and express the Tn antigen. Treatment of the Tn4 cells with a deoxyribonucleic acid (DNA) methylation inhibitor 5-aza-2'-deoxycytidine restored the transcription of Cosmc and β 1,3 GT activity.

Qin *et al* recently reported significantly lower levels of Cosmc mRNA in cultured

peripheral B-lymphocytes from IgAN patients, compared to that of normal controls (2011). The level of Cosmc mRNA however, increased significantly upon demethylation. Epigenetic hypermethylation may thus play a role in the hypogalactosylation of IgA1 in IgAN patients in which levels of Cosmc expression is low.

In mammals, epigenetic mechanisms regulate gene expression, cellular differentiation and development, chromosomal integrity preservation, parental imprinting, and X-chromosome inactivation (Hermann *et al*, 2004). Methylation of DNA is carried out by DNA methyltransferases (DNMTs).

In further studies, the state of methylation of the Cosmc promoter should be investigated in different populations of IgAN cohorts in which Cosmc expression is low.

The level of Cosmc expression in the study population or any South African IgAN population has not been investigated; nor has the effect of demethylation on Cosmc expression been examined; further studies are required for the investigation thereof. If such a mechanism of abnormal hypermethylation of the Cosmc promoter in IgAN is established, then further studies to elucidate the mechanisms responsible

for hypermethylation should be conducted. Such pathogenic mechanisms that may be responsible for hypermethylation include the upregulation of DNMTs; loss of protection of the promoter by DNA binding proteins or transcription factors (Mi *et al*, 2012); loss of protection due to hypermethylation of the Alu elements that surround the promoter; or the recognition of the methylated promoter by methyl-CpG (cytosine-phosphate-guanine)-binding domain proteins that recruit the histone deacetylase-containing transcription repression complex which stops Cosmc transcription (Bogdanovic & Veenstra, 2009; Patra & Bettuzzi, 2009).

In addition, investigations into possible mutations in the gene encoding Cosmc in the South African study IgAN population or any South African IgAN population have not been conducted; further studies are required for the establishment thereof. Inactive Cosmc may result in inactivity of β 1,3 GT and hence abnormal IgA1 *O*-galactosylation.

4.8.3.3 MicroRNA (miR) - 148b

Serino *et al* recently identified a new regulatory factor for the expression of β1,3 GT, and hence the levels of IgA1 *O*-galactosylation, in peripheral blood mononuclear cells (PBMCs) of IgAN patients (2012). High throughput miRNA

profiling revealed a significant up-regulation of miR-148b in the PBMCs of IgAN patients in comparison to healthy controls and patients with other renal diseases i.e. 3 membranoproliferative glomerulonephritis type I patients, 5 focal segmental glomerulosclerosis patients, and 10 HSP-nephritis patients.

The β 1,3 GT expression was significantly lower in IgAN patients in comparison to healthy controls and there was a significant negative correlation between β 1,3 GT mRNA levels and miR-148b expression levels.

Endogenous β 1,3 GT mRNA levels were significantly reduced by 3-fold in PBMCs from healthy controls following transfection with a miR-148b mimic. Transfection of PBMCs from the IgAN patients with a miR-148b inhibitor led to a significant 3-fold endogenous increase in β 1,3 GT mRNA levels.

The level of IgA1 *O*-hypogalactosylation was significantly higher in IgAN patients than controls and was significantly positively correlated with the miR-148b expression level; thus supporting the role of miR-148b as a down-regulator of β1,3 GT. To support the role of up-regulated miR-148b in the hypogalactosylation of IgA1, IgA1-producing human B lymphoma cells were transfected with the miR-148b mimic and inhibitors. Transfection with the miR-148b mimic led to

a significant increase in hypogalactosylated IgA1whilst transfection with the miR-148b inhibitor led to a significant decrease in hypogalactosylated IgA1.

Serino *et al* argue that the manipulation of miR-148b levels may provide a potential therapeutic strategy in IgAN (2012).

The expression of β 1,3 GT was not investigated in the study population or any South African IgAN population. In order to elucidate the mechanisms responsible for the abnormal IgA1 *O*-galactosylation demonstrated in the study population, it is essential to investigate the levels of expressed β 1,3 GT. A deficiency thereof may warrant further investigation into miR-148b levels. It is also essential to assess for possible mutations in the gene encoding β 1,3 GT; it may be possible that inactivity of β 1,3 GT due to genetic mutation may be responsible for the abnormal IgA1 *O*-galactosylation demonstrated in the South African IgAN study population.

4.9 Proposed Therapeutic Strategy for an IgAN Population with Hypogalactosylated IgA1

Kobayashi *et al* reported a reduction in serum IgA levels and an increase in the sialylation and galactosylation thereof in ddY mice following IL-12 administration

(2002). IL-12 induces a shift from the Th2 to Th1 response, which may have resulted in the reduction of Th2 cytokine levels such as IL-4 and IL-5, resulting in increased sialylation and galactosylation of IgA. Kobayashi *et al* argue that such data confirm a down-regulation of the clonal expansion of IgA-producing B-cells and thus a reduced level of IgA1 (2002).

However, Kobayashi *et al* reported a slight but not significant worsening of renal function and crescent formation following IL-12 administration (2002). Kobayashi *et al* argue that although the duration of the study was too short to make conclusions about a detrimental impact of IL-12 administration on glomerular pathology, further up-regulation of TGF-β due to prolonged IL-12 administration may accelerate nephrosclerosis (2002).

Thus, following review of the literature, it can be newly hypothesised that monoclonal antibody therapy against IL-4 may serve as a possible therapeutic approach in patients with IgAN; as opposed to the possible use of recombinant IL-12 therapy which although may create a shift away from the Th2 response, may also result in nephrosclerosis due to the cell-mediated Th1 response. In the South African IgAN study population the levels of IL-12 in addition to IL-4 were not studied; investigation thereof may provide significant information regarding the

pathology of IgAN in the study population with abnormal hypogalactosylated IgA1.

4.10 Proposed Therapeutic Strategies for the Amelioration of Renal Mesangium Pathology in an IgAN Population with Hypogalactosylated IgA1

Based on the pathogenic effects of galactose-deficient IgA1 on the renal mesangium, potential therapeutic strategies for the amelioration thereof, may include the following in mesangial cells: down-regulation of renin and macrophage migration inhibitory factor (refer to 1.3.6.8.1); up-regulation of adiponectin secretion in mesangial cells (refer to 1.3.6.8.2); and suppression of platelet-activating factor (refer to 1.3.6.8.5).

4.11 Mycophenolic Acid and Up-regulation of Cosmc

A recent study demonstrated that mycophenolic acid, an immunosuppressant used to prevent allograft rejection, can up-regulate the expression of Cosmc in B-lymphocytes of IgAN patients *in vitro* and increase the IgA1 *O*-galactosylation

level (Xie *et al*, 2013).

Mycophenolic acid induces a cytostatic effect in lymphocytes (Allison & Euqui, 1996).

The precise mechanism whereby mycophenolic acid may result in the up-regulation of Cosmc is unknown.

Currently, evidence supporting the use of mycophenolic acid in treating IgAN is inconclusive. Trials to date are of small size, and longer term studies are required (Tang *et al*, 2010).

4.12 Study Strengths and Limitations

4.12.1 Strengths

4.12.1.1 The study addresses the paucity of data on the pathogenesis of IgAN from Africa.

4.12.1.2 IgAN is a disorder for which the pathogenetic mechanisms are not fully

understood worldwide. The study provides evidence for a pathogenetic mechanism of abnormal IgA1 *O*-galactosylation in IgAN patients from KwaZulu Natal, South Africa. The study is thus an essential contribution towards the understanding of the disease pathogenesis.

4.12.1.3 Since the study was based on an initiating event in the pathogenesis of IgAN, i.e. the hypogalactosylation of IgA1, such data may inform the research into specific therapies for IgAN which currently do not exist.

4.12.1.4 Since abnormal IgA1 *O*-glycosylation was demonstrated to be a pathogenic mechanism in the IgAN study population, the study data may lend support to the potential usefulness of specific therapies targeting the pathogenetic mechanism of aberrant IgA1 *O*-galactosylation in the study population of IgAN patients from KwaZulu Natal South, Africa.

4.12.1.5 The study was based on broad inclusion criteria. The lack of homogeneity in the study group due to the inclusion of various race groups, a wide age distribution, both genders, and both pre-dialysis and end-stage IgAN patients, precludes restrictive applicability of the study data to a select patient sub-group.

4.12.1.6 The utilisation of a relatively rapid and inexpensive assay for the investigation of the IgA1 *O*-galactosylation i.e. the *Vicia villosa* lectin-binding assay, enables the production of comparable pathogenetic data in other resource-restricted countries, particularly in Africa.

4.12.1.7 The study highlights the potential future utility of the lectin binding assay for the determination of abnormal IgA1 *O*-galactosylation as a future screening tool for IgAN.

4.12.1.8 The study provides IgA1 *O*-glycosylation data for IgAN patients of Indian ethnicity. Following review of the literature to date, IgA1 *O*-glycosylation data does not exist for IgAN patients of Indian ethnicity.

4.12.1.9 The study highlights a new hypothetical therapeutic strategy targeting abnormal IgA1 *O*-galactosylation, which merits investigation, i.e. monoclonal antibody therapy against IL-4.

4.12.1.10 The study highlights avenues for further study for the elucidation of the mechanisms responsible for abnormal IgA1 *O*-galactosylation in the study population.

4.12.1.11 The study highlights therapeutic strategies that target the amelioration of galactose-deficient IgA1-induced glomerular pathology.

4.12.2 Limitations

4.12.2.1 The number of patients diagnosed with IgAN at the IALCH i.e. the hospital from which IgAN patients were recruited, and the lack of consent to the study by some IgAN patients dictated the sample size and sub-group demographics; and hence, precluded sub-group IgA1 *O*-galactosylation analyses.

Thus, the IgA1 *O*-galactosylation analyses between racial groups, between genders, and between pre-dialysis and terminal IgAN patients were precluded by a low IgAN patient sub-group sample size.

4.12.2.2 The level of IgA1 *O*-hypogalactosylation per IgA1 molecule and the level of IgA1 per sample was not standardised; however this does not influence the study, and does not prevent general comparative analyses globally.

4.13 Conclusion

Prior to this study, the *O*-glycosylation state of IgA1 in South African IgAN patients had not been defined. This study provides pathogenetic data on IgAN, which is scarce or nil in African countries to date. The data of this study is thus an essential contribution towards the global advancement in the understanding of the pathogenesis of IgAN and the eventual development of specific therapies.

In this study, the hypothesis of abnormal serum IgA1 *O*-galactosylation in IgAN patients was proven. Thus, specific therapies developed against the pathogenic mechanism of IgA1 *O*-hypogalactosylation may potentially be of benefit in this study population.

Further studies are required to elucidate the specific mechanisms responsible for abnormal IgA1 *O*-galactosylation in the South African population. Based on the data of this study, further investigation into the levels of Cosmc and β 1,3 GT, and possible genetic mutations thereof are required in the IgAN study population. Serum IL-4 levels merits investigation in the study population. The mechanisms whereby IL-4 may decrease galactosylation of IgA1 in patients with IgAN is currently unknown; investigation into the mechanisms thereof should

be conducted in IgAN populations globally. Increased IgA1 production per B-cell in relation to B-cell proliferation, due to possible enhanced IL-4 levels is hypothesised as a possible mechanism responsible for abnormal IgA1 *O*-galactosylation in IgAN.

It is further hypothesised that monoclonal antibody therapy against IL-4 may serve as a potential therapeutic agent for IgAN. The effect of monoclonal antibody therapy against IL-4 on IgA1 *O*-galactosyltion in cultured B-lymphocytes from patients with IgAN merits further study in the study population and in other IgAN populations globally.

IgAN is a multifactorial disease and is "not the same disease" (Feehally, 2010; personal communication) in all parts of the world (differing prognoses and presentation), but this finding of abnormal IgA1 *O*-galactosylation is consistent with that of other populations globally; and thus supports universal strategies for therapeutic agents that target this abnormality.

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Appendix 1

Abbreviations -

Absorbance (A)

Asialoglycoprotein receptor (ASGPR)

core 1 β3-galactosyltransferase (β 1,3 GT)

Complement (C)

Complementarity determining region (CDR)

Constant Heavy (CH)

Constant Light (CL)

Council for Scientific and Industrial Research (CSIR)

Cysteine (Cys)

Deoxyribonucleic acid (DNA)

DNA methyltransferases (DNMTs)

End-stage renal disease (ESRD)

Enzyme-linked immunosorbent assay (ELISA)

Ethylenediaminotetraacetic acid (EDTA)

Fetal calf serum (FCS)

Figure (fig.)

Fish oil supplements (FOS)

Fluorophore-assisted carbohydrate electrophoresis (FACE)

Fragment antigen binding (Fab)

Fragment crystallisable (Fc)

Grouped ddY (gddY)

Helix aspersa (HA)

Henoch-Schönlein Purpura (HSP)

High performance liquid chromatography (HPLC)

High serum IgA (HIGA)

Horse radish peroxidase (HRP)

Immunoglobulin A1 (IgA1)

Immunoglobulin A Nephropathy (IgAN)

Inkosi Albert Luthuli Central Hospital (IALCH)

Interleukin (IL)

International Society of Nephrology (ISN)

KwaZulu Natal (KZN)

Macrophage galactose-type lectin (MGL)

Mannose-binding lectin (MBL)

Matrix assisted laser desorption ionising mass spectroscopy (MALDI-MS)

Messenger Ribonucleic Acid (mRNA)

MicroRNA (miRNA)

N-Acetylgalactosamine (GalNAc)

N-Acetylglucosamine (GlcNAc)

N-Acetylneuraminic acid (NeuNAc)

Nelson R. Mandela School of Medicine (NRMSM)

Nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB)

Number (no.)

Not applicable (N/A)

o-Phenylenediamine (OPD)

Peripheral blood mononuclear cells (PBMCs)

Phosphate-buffered saline (PBS)

Revolutions per minute (rpm)

Serine (Ser)

Standard Error of the Mean (SEM)

Threonine (Thr)

Transforming growth factor ß (TGF-ß)

United States of America (USA)

University of KwaZulu Natal (UKZN)

Variable Heavy (VH)

Variable Light (VL)

Vicia villosa (VV)

Appendix 2

Table 1: Control Population Encoding, Age, Race, and Gender

Blood Extraction Date	Code	Age At Extraction (Years)	Race	Gender
2009 - 6 May	CAM37	37	African	Male
2008 - 18 Aug	CAF24273	35	African	Male
2009 - 1 Oct	CAF42	42	African	Female
2008 - 18 Aug	CAF24289	25	African	Female
2008 - 18 Aug	CAF24282	35	African	Female
2008 - 18 Aug	CAF24262	36	African	Female
2009 - 22 May	CCF40	40	Caucasian	Female
2009 - 6 May	CCF27	27	Caucasian	Female
2009 - 6 May	CCF24	24	Caucasian	Female
2008 - 18 Aug	CCLF24284	23	Coloured	Female
2009 - 22 May	CIM11	40	Indian	Male
2009 - 12 May	CIM33	33	Indian	Male
2009 - 12 May	CIM21	21	Indian	Male
2008 - 18 Aug	CIM24250	49	Indian	Male
2008 - 18 Aug	CIM24261	30	Indian	Male
2008 - 18 Aug	CIM24288	25	Indian	Male
2008 - 18 Aug	CIM24281	25	Indian	Male
2009 - 22 May	CIF35	35	Indian	Female
2008 - 18 Aug	CIF24283	21	Indian	Female
2008 - 18 Aug	CIF24280	19	Indian	Female

Appendix 3

Table 2: Control Population: Age Range and Age, Race, and Gender Proportion

	Indian	African	Caucasian	Coloured
Number of Controls	10	6	3	1
Proportion of Controls (%)	50	30	15	5
Proportion Male (%)	70	33.3	0	0
Mean Age	37	35	30	N/A
Age range	19-49	25-42	24-40	23
Median Age	28	35	27	N/A
Female Mean Age	25	35	30	N/A
Female Median Age	21	36	27	N/A
Male Mean Age	33	36	N/A	N/A
Male Median Age	30	36	N/A	N/A
Age Range Female	19-35	25-42	N/A	N/A
Age Range Male	21-49	35 & 37	N/A	N/A

Appendix 4

Table 3: Experimental and Control Population: Age Range and Age, Race, and Gender Proportion

	Experimental Population	Control Population
Age Range	29-66: 29, 31, 35, 37, 37, 38, 40, 40, 43, 43, 45, 46, 48, 51, 51, 54, 59, 63, 66	19-49: 19, 21, 21, 23, 24, 25, 25, 25, 27, 30, 33, 35, 35, 35, 36, 37, 40, 40, 42, 49
Median Age	43	32
Mean Age	46	31
Mean Age Male	44	33
Mean Age Female	41	30
Percentage Male (%)	63	45

Appendix 5

Table 4: Experimental Population Encoding and Descriptors

Extraction Date	Code	Age (Years)	Race	Gender	End-stage IgAN
2009 - 4 June	PAM13072	29	African	Male	No
2011- 3 March	PAM02011	45	African	Male	Yes - dialysis
2009 - 4 June	PCM04952	40	Caucasian	Male	Yes - transplant
2009 - 4 June	PCF02620	40	Caucasian	Female	Yes - transplant
2009 - 26 March	PCLM08931	54	Coloured	Male	Yes - transplant
2009 - 26 March	PCLM03297	37	Coloured	Male	No
2007 - 28 June	PCLF2024	46	Coloured	Female	No
2009 - 3 September	PIF5300	59	Indian/Caucasian	Female	No
2007 - 12 July	PIM8434	43	Indian	Male	No
2007 - 19 July	PIM1829	48	Indian	Male	Yes - transplant
2009 - 2 June	PIM2407	63	Indian	Male	Yes - transplant
2009 - 25 August	PIM2945	51	Indian	Male	Yes - transplant
2009 - 2 June	PIM1971	38	Indian	Male	Yes - transplant
2009 - 2 June	PIM26745	35	Indian	Male	Yes - dialysis
2009 - 4 June	PIM2386	43	Indian	Male	No
2007 - 28 June; 2009 - 4 June	PIF03104	64/66	Indian	Female	No
2009 - 2 June	PIF1274	37	Indian	Female	No
2009 - 2 June	PIF1868	31	Indian	Female	No
2007 - 19 July	PIF1522	51	Indian	Female	Yes - transplant

Appendix 6

Table 5: IgAN Patient Population: Age Range and Age, Race, and Gender Proportion

	Indian	African	Caucasian	Coloured	Mixed Race Indian/Caucasian
Number of Experimental Participants	11	2	2	3	1
Proportion of Experimental Participants (%)	57.89	10.53	10.53	15.79	5.26
Proportion Male (%)	70	100	50	66.7	0
Mean Age	46	37	40	46	N/A
Age range	31-66	29 & 45	40 & 40	37-54	59
Median Age	43	N/A	N/A	46	N/A
Female Mean Age	46	N/A	N/A	N/A	N/A
Female Median Age	43	N/A	N/A	N/A	N/A
Male Mean Age	46	N/A	N/A	46	N/A
Male Median Age	48	N/A	N/A	N/A	N/A
Age Range Female	31-66	N/A	40	46	59
Age Range Male	35-63	N/A	N/A	37 & 54	N/A

Appendix 7

Table 6: Consumables/Reagents and Manufacturer

Consumables/Reagents	**Manufacturer**
15 ml polypropylene centrifuge tube	Corning; New York, USA
Cryogenic vials (2.0 ml)	Corning; New York, USA
1.5 ml microcentrifuge tube	Eppendorf; Hamburg, Germany
50 ml polypropylene tube	Sterilin; Newport, United Kingdom
Costar 96-well plate	Corning; New York, USA
Polyethylene Wrap	Glad; Babelegi, South Africa
Rabbit Anti-human IgA (1 ml)	Dako; Glostrup, Denmark
Carbonate/bicarbonate buffer capsules	Sigma; St. Louis, USA
PBS tablets	Sigma; St. Louis, USA
NaCl	Sigma-Aldrich; Milwaukee, USA
Tween 20	Sigma; Steinheim, Germany
FCS	Highveld Biological; Johannesburg, South Africa
Biotinylated VV lectin (2mg)	Vector; California, USA
Horseradish Peroxidase Avidin D Concentrate (5 mg)	Vector; California, USA
OPD tablets	Dako; Glostrup, Denmark
30% H_2O_2 solution (500 ml)	Sigma; Steinheim, Germany
H_2SO_4 (95-97%)	Merck; Darmstadt, Germany

Appendix 8

Table 7: Absorbance Data for Each IgAN Patient Participant

IgAN Patient	Plate	Serum Volume (µl)/PBS volume (µl)	Mean Absorbance Without Plate Background	Average of the Means Across the Plates for 5 µl serum and 495 µl PBS at 490 nm
PAM13072	2	25/25	-0.0865	0.084875
	6	50/0	0.1885	
	6	25/25	0.085	
	6	12.5/37.5	0.0545	
	7	50/0	0.5195	
	8	50/0	0.948	
	10	5/495	0.03025	
	12	5/495	0.019	
	13	5/495	0.07475	
	14	5/495	0.2155	
PAM02011	14	5/495	0.4238	0.4238
PCM04952	2	25/25	0.276	0.153041667
	7	50/0	1.103	
	8	50/0	1.518	
	9	5/495	0.1365	
	10	5/495	0.07175	
	11	5/495	0.06325	
	12	5/495	0.175	
	13	5/495	0.05925	
	14	5/495	0.4125	

PCLM08931	4	25/25	-0.0015	-0.00045
	8	50/0	1.2905	
	9	5/495	0.0275	
	10	5/495	0.01875	
	11	5/495	-0.05525	
	12	5/495	-0.0535	
	13	5/495	0.06025	
PCLM03297	3	25/25	0.03075	0.04265
	7	50/0	1.0985	
	8	50/0	1.9135	
	9	5/495	0.11	
	10	5/495	0.02675	
	11	5/495	0.03525	
	12	5/495	-0.042	
	13	5/495	0.08325	
PIM8434	1	25/25	0.0537	0.213625
	8	50/0	0.854	
	9	5/495	0.188	
	10	5/495	0.09775	
	11	5/495	0.06825	
	12	5/495	0.2895	
	13	5/495	0.21775	
	14	5/495	0.4205	
PIM1829	1	25/25	-0.0658	0.01895
	8	50/0	0.3625	
	9	5/495	0.069	
	10	5/495	0.04475	
	11	5/495	-0.04525	
	12	5/495	-0.055	
	13	5/495	0.08125	

PIM2407	2	25/25	0.3215	0.092458333
	8	50/0	1.1895	
	9	5/495	0.1225	
	10	5/495	0.01575	
	11	5/495	0.01225	
	12	5/495	-0.034	
	13	5/495	0.07075	
	14	5/495	0.3675	
PIM2945	4	25/25	0.0125	0.272958333
	7	50/0	0.5305	
	8	50/0	1.3415	
	9	5/495	0.254	
	10	5/495	0.15675	
	11	5/495	0.19925	
	12	5/495	0.058	
	13	5/495	0.27425	
	14	5/495	0.6955	
PIM1971	6	50/0	0.2875	0.058375
	6	25/25	0.161	
	6	12.5/37.5	0.048	
	8	50/0	1.1125	
	9	5/495	0.047	
	10	5/495	0.03075	
	11	5/495	-0.00775	
	12	5/495	-0.021	
	13	5/495	0.04525	
	14	5/495	0.256	
PIM26745	5	25/25	-0.015	0.074541667
	8	50/0	1.1955	
	9	5/495	0.0825	

	10	5/495	0.05025	
	11	5/495	-0.00625	
	12	5/495	0.002	
	13	5/495	0.05975	
	14	5/495	0.259	
PIM2386	8	50/0	1.301	0.090625
	9	5/495	0.0745	
	10	5/495	0.04275	
	11	5/495	0.02125	
	12	5/495	0.047	
	13	5/495	0.06275	
	14	5/495	0.2955	
PCF02620	3	25/25	0.40375	0.09875
	7	50/0	0.864	
	8	50/0	1.292	
	10	5/495	0.07175	
	12	5/495	-0.0595	
	13	5/495	0.08725	
	14	5/495	0.2955	
PCLF2024	8	50/0	0.546	0.05825
	9	5/495	0.1225	
	10	5/495	0.07275	
	11	5/495	0.01225	
	12	5/495	-0.042	
	13	5/495	0.12575	
PIF5300	3	25/25	0.00675	0.1285
	7	50/0	0.219	
	8	50/0	0.344	
	10	5/495	0.06725	
	12	5/495	-0.0525	

	13	5/495	0.11825	
	14	5/495	0.381	
PIF03104	8	50/0	0.805	0.074166667
2007	10	5/495	0.05975	
	12	5/495	-0.016	
	13	5/495	0.17875	
PIF03104	4	25/25	0.0045	0.229875
2009	8	50/0	1.5255	
	9	5/495	0.063	
	10	5/495	0.05625	
	11	5/495	0.04075	
	12	5/495	0.8435	
	13	5/495	0.11175	
	14	5/495	0.264	
PIF1274	4	25/25	0.0025	0.091291667
	8	50/0	1.7445	
	9	5/495	0.0865	
	10	5/495	0.06275	
	11	5/495	0.04675	
	12	5/495	-0.0665	
	13	5/495	0.06225	
	14	5/495	0.356	
PIF1868	5	25/25	-0.0195	0.135375
	8	50/0	1.201	
	9	5/495	0.107	
	10	5/495	0.06475	
	11	5/495	0.00275	
	12	5/495	0.168	
	13	5/495	0.09375	
	14	5/495	0.376	

PIF1522	1	25/25	0.0502	0.114708333
	8	50/0	0.303	
	9	5/495	0.1185	
	10	5/495	0.06225	
	11	5/495	0.03525	
	12	5/495	0.077	
	13	5/495	0.09975	
	14	5/495	0.2955	

Table 8: Absorbance Data for Each Control Participant

Participant	Plate	Serum Volume (µl)/PBS volume (µl)	Mean Absorbance Without Plate Background	Average of the Means Across the Plates
CAM 37	2	25/25	-0.276	0.0649375
	4	25/25	0.0045	
	6	50/0	0.1075	
	6	25/25	0.0595	
	6	12.5/37.5	0.0575	
	8	50/0	0.241	
	9	5/495	0.066	
	10	5/495	0.05375	
	11	5/495	0.02725	
	12	5/495	0.0165	
	13	5/495	0.14675	
	14	5/495	0.275	
CAM24273	10	5/495	0.05875	0.05875
CAF42	1	25/25	0.0132	0.23455
	4	25/25	0.0045	
	7	50/0	0.591	
	8	50/0	1.1865	

	9	5/495	0.0705	
	10	5/495	0.06125	
	11	5/495	0.07425	
	12	5/495	-0.042	
	13	5/495	0.15175	
CAF24289	10	5/495	0.03725	0.1061875
	11	5/495	0.01275	
	13	5/495	0.10125	
	14	5/495	0.2735	
CAF24282	10	5/495	0.00625	0.00625
CAF24262	10	5/495	0.06475	0.06475
CCF40	1	25/25	0.0597	0.172095
	4	25/25	0.0035	
	7	50/0	0.4945	
	8	50/0	0.7	
	9	5/495	0.0565	
	10	5/495	0.04675	
	11	5/495	-0.01075	
	12	5/495	0.035	
	13	5/495	0.11125	
	14	5/495	0.2245	
CCF27	2	25/25	-0.0305	0.24478125

	8	50/0	1.505	
	9	5/495	0.0625	
	10	5/495	0.05975	
	11	5/495	0.03125	
	12	5/495	-0.027	
	13	5/495	0.10525	
	14	5/495	0.252	
CCF24	3	25/25	0.02975	0.152
	4	25/25	0.0125	
	7	50/0	0.3945	
	8	50/0	0.5945	
	9	5/495	0.087	
	10	5/495	0.08875	
	11	5/495	0.04925	
	12	5/495	-0.057	
	13	5/495	0.16875	
CCLF24284	9	5/495	0.095	0.0871875
	10	5/495	0.05125	
	11	5/495	0.05925	
	13	5/495	0.14325	
CIM11	2	25/25	-0.171	0.179027778
	7	50/0	0.6295	

	8	50/0	0.689	
	9	5/495	0.056	
	10	5/495	0.06225	
	11	5/495	-0.00075	
	12	5/495	0.02	
	13	5/495	0.09475	
	14	5/495	0.2315	
CIM33	1	25/25	0.0302	0.1765375
	5	25/25	-0.0315	
	6	50/0	0.211	
	6	25/25	0.1805	
	6	12.5/37.5	0.098	
	8	50/0	0.749	
	9	5/495	0.073	
	10	5/495	0.04625	
	11	5/495	0.02475	
	12	5/495	0.3045	
	13	5/495	0.12125	
	14	5/495	0.3115	
CIM21	3	25/25	0.04175	0.211888889
	7	50/0	0.174	
	8	50/0	1.2295	

	9	5/495	0.056	
	10	5/495	0.04775	
	11	5/495	0.01625	
	12	5/495	0.0145	
	13	5/495	0.10675	
	14	5/495	0.2205	
CIM24250	9	5/495	0.057	0.08255
	10	5/495	0.02625	
	11	5/495	0.00225	
	13	5/495	0.06925	
	14	5/495	0.258	
CIM24261	10	5/495	0.05725	0.151833333
	13	5/495	0.13425	
	14	5/495	0.264	
CIM24288	10	5/495	0.05725	0.135833333
	13	5/495	0.10875	
	14	5/495	0.2415	
CIM24281	9	5/495	0.1	0.13825
	10	5/495	0.06625	
	13	5/495	0.11575	
	14	5/495	0.271	
CIF35	5	25/25	-0.0145	0.362083333

	7	50/0	1.047	
	8	50/0	1.738	
	9	5/495	0.0725	
	10	5/495	0.02925	
	11	5/495	0.03525	
	12	5/495	0.1745	
	13	5/495	0.08825	
	14	5/495	0.0885	
CIF24283	9	5/495	0.034	0.07145
	10	5/495	0.02975	
	11	5/495	-0.00625	
	13	5/495	0.08725	
	14	5/495	0.2125	
CIF24280	9	5/495	0.0905	0.10335
	10	5/495	0.03575	
	11	5/495	0.02325	
	13	5/495	0.10975	
	14	5/495	0.2575	

Appendix 9

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March 29, 2013

Prishani Nansook
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