



UNIVERSITY OF
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PSYCHOLOGY**

DEPARTMENT OF INFECTION, IMMUNITY AND INFLAMMATION

IGA1 O-GLYCOSYLATION AND IGA NEPHROPATHY

Thesis submitted for the degree of Doctor of Philosophy

by

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Abstract

IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis worldwide diagnosed by observing deposition of IgA in the mesangium of a renal biopsy. It is known that levels of abnormally glycosylated IgA1 with a reduction in galactose at the hinge region (galactose-deficient IgA1, Gd-IgA1) are elevated in the disease in both circulation and mesangial deposits. Gd-IgA1 is seen as auto-antigenic and leads to immune complex formation which can be deposited in the kidneys leading to renal injury. Higher levels of Gd-IgA1 have also been linked to progressive forms of IgAN.

While much research effort has been devoted to the study of Gd-IgA1 in IgAN, the biological basis for this aberrancy is still not well understood. This project aimed first to examine how genetic variation can influence O-glycosylation of the IgA1 hinge region. Secondly, how the mechanism behind IgA1 hinge region O-glycosylation can be altered during maturation of IgA1 producing B cells. Thirdly, how IgA1 hinge region O-glycosylation can be modulated by the action of cytokines and chemokines.

This work has for the first time identified a disparity in serum Gd-IgA1 levels between Caucasian and Chinese populations; in both health and IgAN. Gender differences in serum Gd-IgA1 levels in IgAN have also been observed for the first time. A haplotype of C1GALT1, the gene for the galactose-adding C1GalT1 enzyme, associated with higher serum Gd-IgA1 levels has also been discovered. Maturation-dependent transcriptional regulation of glycosylation enzymes in B cells has been confirmed, and the C1GALT1 haplotype was found to strongly affect C1GALT1 transcript levels. Novel cytokines were also found to be able to modulate Gd-IgA1 production in IgA1 producing cells. The work in this thesis serves to further elucidate control of the mechanism behind O-glycosylation at the IgA1 hinge region and provides new research questions in the study of IgAN.

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Statement of originality

I confirm that except for the following and unless otherwise stated, all of the work and data presented in this thesis were performed and analysed by the candidate.

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Publications and presentations

Journal publication arising from this work

Galactosylation of IgA1 Is Associated with Common Variation in C1GALT1.

*Gale, D.; Molyneux, K.; **Wimbury, D.**; Higgins, P.; Levine, A.; Caplin, B.; Ferlin, A.; Yin, P.; Nelson, C.; Stanescu, H.; Samani, N.; Kleta, R.; Yu, X. and Barratt, J.*

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Conference presentations arising from this work

The H1 risk haplotype significantly accentuates maturation-dependent transcriptional regulation of the C1GALT1 gene in IgA committed B cells

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O-glycosylation of IgA1 is associated with genetic variation of C1GALT1

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Systematic Analysis of IgA1 Glycosylation in IgA Nephropathy, Membranous Nephropathy and Healthy Subjects and the Effects of Ethnicity

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Gender differences in serum IgA1 O-galactosylation levels and risk of developing progressive IgA nephropathy

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The levels of aberrantly galactosylated IgA1 are associated with the risk of developing progressive renal disease in IgA nephropathy

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Serum IgA1 hinge region O-galactosylation is a heritable trait in Caucasians

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Other journal publications during this work

β 1,4-galactosyltransferase 1 is a novel receptor for IgA in human mesangial cells

*Molyneux, K.; **Wimbury, D.**; Pawluczyk, I.; Muto, M.; Bhachu, J.; Mertens, P.; Feehally, J. and Barratt, J.*

Kidney Int. 2017 Dec;92(6):1458-1468. doi: 10.1016/j.kint.2017.05.002

The Effect of Resistance Exercise on Inflammatory and Myogenic Markers in Patients with Chronic Kidney Disease.

*Watson, E.; Viana, J.; **Wimbury, D.**; Martin, N.; Greening, N.; Barratt, J. and Smith, A.*

Front Physiol. 2017 Jul 28;8:541. doi: 10.3389/fphys.2017.00541

Other conference presentations during this work

The confounding effects of loss of excretory renal function and development of non-selective proteinuria on serum biomarker levels and a potential role for B cell survival factors in renal progression in IgAN

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Further evidence for mucosal immune dysregulation in IgA nephropathy: a specific association between secretory IgA and soluble BCMA levels in IgAN
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An analysis of serum biomarkers in IgA nephropathy

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<https://www.luminexcorp.com/eu/blog/webinar-kidney-function-study-suggests-biomarkers-associated-iga-nephropathy/>

Serum BCMA levels are elevated in IgA Nephropathy

Wimbury, D.; Molyneux, K. and Barratt, J.

Poster presentation, American Society of Nephrology Kidney Week 31/10-05/11/2016

A systematic analysis of novel serum biomarkers in IgA nephropathy

Wimbury, D.; Molyneux, K. and Barratt, J.

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Serum BAFF & BCMA levels are elevated in progressive IgA nephropathy

Wimbury, D.; Molyneux, K. and Barratt, J.

Poster presentation, UK Kidney Week 07-10/062016

Resistance exercise does not increase markers of muscle protein degradation in patients with advanced CKD

*Watson, E.; **Wimbury, D.**; Viana, J.; Greening, N.; Barratt, J. and Smith, A.*

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List of abbreviations

ACCS	1-aminocyclopropane-1-carboxylate synthase-like protein 1
APRIL	a proliferation inducing ligand
APS	ammonium persulphate
BAFF	B cell activating factor
BAFFR	BAFF receptor
BCA-1	B cell chemoattractant 1
BSA	bovine serum albumin
C1GalT1	core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1
CagA	Cytotoxin associated gene A protein
CARD9	caspase recruitment domain-containing protein 9
CD	cluster of differentiation
cDNA	complementary DNA
CFHR1	complement factor H-related protein 1
CFHR2	complement factor H-related protein 2
CKD	chronic kidney disease
Cosmc	C1GalT1-specific chaperone 1
Ct	cycle threshold
CV	coefficient of variation
DEFA	defensin alpha 1
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
eGFR	estimated glomerular filtration rate
ELISA	enzyme-linked immunosorbent assay
ERG	endogenous reference gene
ESRD	end-stage renal disease
Fab	fragment, antigen binding
FACS	fluorescence activated cell sorting
FBS	foetal bovine serum

Fc	fragment, crystallisable
FPLC	fast protein liquid chromatography
g	g force
Gal	galactose
GalNAc	N-acetylgalatosamine
GalNT2	polypeptide N-acetylgalactosaminyltransferase 2
GALT	gut associated lymphoid tissue
Gd-IgA1	galactose deficient IgA1
GRAPHIC	Genetic Regulation of Arterial Pressure of Humans in the Community
GWAS	genome-wide association study
HA	<i>Helix aspersa</i>
HBSS	Hanks' balanced salt solution
HORMAD2	HORMA domain-containing protein 2
HRP	horseradish peroxidase
hrs	hours
HSP	Henoch-Schönlein purpura
Ig	immunoglobulin
IgA	immunoglobulin A
IgAN	IgA nephropathy
IgAV	IgA vasculitis
IGHA1	immunoglobulin heavy constant alpha 1
IGHD	immunoglobulin heavy constant delta
IL	interleukin
ITGAM	integrin α M
ITGAX	integrin α X
J chain	immunoglobulin joining chain
KLF10	Kruppel-like factor 10
MAdCAM-1	mucosal vascular addressin cell adhesion molecule 1
MALT	mucosa associated lymphoid tissue
MEST	mesangial hypercellularity, endocapillary hypercellularity, segmental sclerosis/adhesion and tubular atrophy/interstitial fibrosis

MHC	major histocompatibility complex
MN	membranous nephropathy
mRNA	messenger RNA
n	number
NIBSC	National Institute for Biological Standards and Control
NS	not significant
OD	optical density
OPD	o-phenylenediamine dihydrochloride
ORF1	outer dense fiber protein 1
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PenStrep	penicillin-streptomycin
qPCR	quantitative polymerase chain reaction
r	coefficient of regression
RNA	ribonucleic acid
SC	secretory component
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sIgA	secretory IgA
SMC	Single Molecule Counting
SNP	single nucleotide polymorphism
ST3Gal	β -galactoside- α -2,3-sialyltransferase
ST6GalNAcII	N-acetylgalactosaminide alpha-2,6-sialyltransferase 2
TACI	transmembrane activator and CAML interactor
TBS	Tris buffered saline
TEMED	<i>N,N,N,N</i> -Tetramethylethane-1,2-diamine
TGF- β	transforming growth factor beta
TNFSF13	tumor necrosis factor ligand superfamily member 13
TNF α	tumour necrosis factor alpha
TTBS	tris buffered saline/0.1% TWEEN20
UDP	uridine diphosphate

UKGNDB	UK Glomerulonephritis DNA Bank
UTR	untranslated region
VAV3	vav guanine nucleotide exchange factor 3
VV	<i>Vicia villosa</i>

Chapter 1 – Introduction

IgA nephropathy (IgAN), the most common form of primary glomerulonephritis in the world, is an immune-mediated disease and accounts for around 10% of renal patients on dialysis in developed countries. IgAN is characterised by the deposition of IgA in the glomerulus of the renal mesangium. IgA1 from patients with IgAN is known to be aberrantly glycosylated at the hinge region, with a reduction in the amount of galactose present; and this is the case for IgA1 in circulation or isolated from mesangial deposits. This galactose-deficient IgA1 (Gd-IgA1) is seen as a major factor in the pathogenesis of IgAN; it has a tendency to be antigenic and lead to the formation of immune-complexes which are then deposited in the renal mesangium leading to renal injury.

This thesis aims to examine:

1. How genetic variation can influence O-glycosylation of the IgA1 hinge region.
2. How the mechanism behind IgA1 hinge region O-glycosylation can be altered during maturation of B cells.
3. How IgA1 hinge region O-glycosylation can be modulated by the action of cytokines and chemokines.

Individually or together, these factors have the potential to influence the severity and risk of progression of IgAN.

1.1 – IgA Nephropathy

Immunoglobulin A nephropathy (IgAN) was first described in 1968 (Berger and Hinglais, 1968) and is now recognised as the most common form of primary glomerulonephritis worldwide (D'Amico, 1987). The worldwide incidence of IgAN has been found to be around 2 cases per 100,000 people per year (McGrogan, Franssen and de Vries, 2011; Schena and Nistor, 2018). The disease is characterised by the histological observation of IgA deposition in the renal mesangium, which is in itself the only current method of diagnosing IgAN. Many factors have led to the belief that IgAN is an immune-mediated disease.

Despite much research into the pathophysiology of IgAN, the disease is still not well understood; with multiple genetic and environmental factors having

been found to contribute to the disease. It is also unclear as yet whether IgAN is a single disease, or whether it is multiple diseases with similar presentations in different ethnicities or age groups. As it stands, with no specific cure or treatment currently available, IgAN accounts for around 10% of patients on dialysis and recurs in up to half of patients with a renal allograft.

1.1.1 – Clinical manifestation

IgAN patients will often present with persistent microscopic haematuria and proteinuria, but are often asymptomatic. Frequently, patients will present with visible haematuria during or after mucosal infections at the upper respiratory or gastrointestinal tracts. These can present as worsening symptoms of IgAN, or as the initial symptoms eventually leading to a diagnosis of IgAN (Floege and Feehally, 2016).

1.1.2 – Histological diagnosis

At present, the only method by which IgAN can be diagnosed is by histological observation of IgA and IgA-containing immune complexes in a renal biopsy, specifically in the renal mesangium (Figure 1.1). The Oxford classification was established in 2009 for pathological characterisation of renal biopsies in glomerular diseases, in order to assist in predicting prognosis for IgAN (Working Group of the International IgA Nephropathy Network and the Renal Pathology Society *et al*, 2012). The classification utilises a scoring system that has been found to be highly reproducible in many centres worldwide. This system scores mesangial hypercellularity, endocapillary hypercellularity, segmental sclerosis/adhesion and tubular atrophy/interstitial fibrosis (MEST score) in sections of a renal biopsy. However, as a renal biopsy is a very invasive procedure, there is a strong need to find alternate methods of diagnosis and prognosis to minimise the risk to patients.

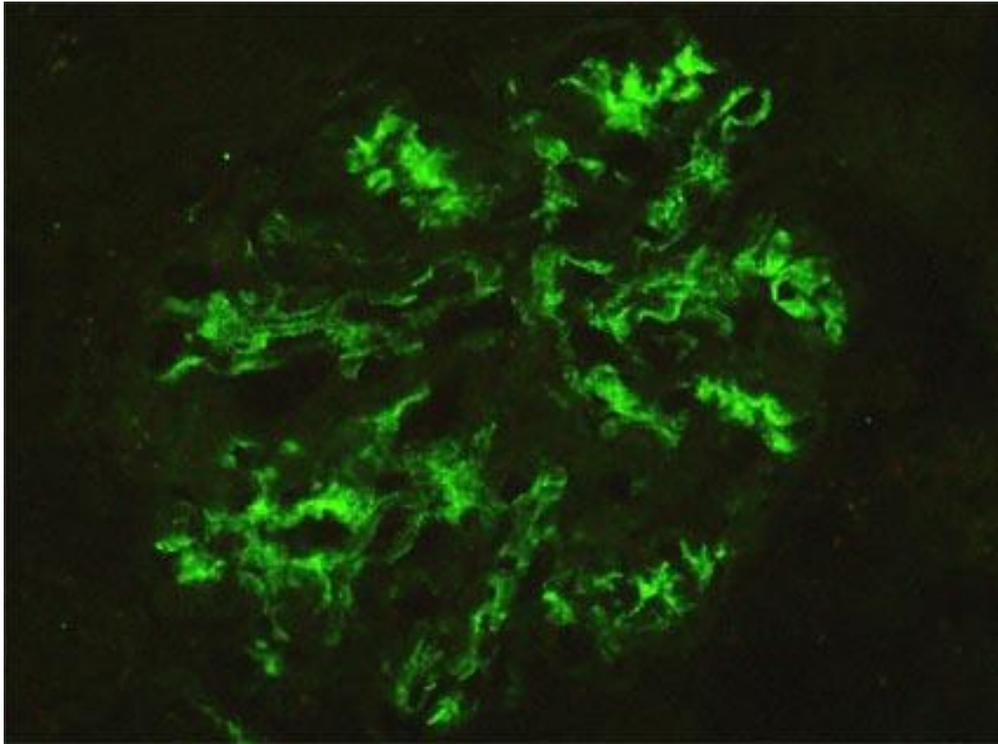


Figure 1.1: Immunofluorescent staining of IgA deposition in the glomerulus: Immunofluorescence staining of a section of a renal biopsy from an IgAN patient with fluorescein-conjugated anti-IgA antibodies. Positive staining of IgA deposited in the renal mesangium evident, specifically in the glomerulus, which is the hallmark of IgAN.

1.1.3 – Pathogenesis

1.1.3.1 – Epidemiology

As a renal biopsy is required for diagnosis, the prevalence of IgAN is hard to measure. However, the reported incidence of IgAN varies depending on ethnicity and geographical location (Geddes *et al*, 2003). In European countries, IgAN has been found to account for around 20% of renal biopsies, in North America this figure is around 5-10%, while in Asian countries this figure rises to around 40% and in central African countries IgAN is diagnosed in less than 5% of renal biopsies (Mestecky *et al*, 2013) (Figure 1.2). These differences could be due to variability in clinical practices across different countries. Countries which regularly screen for urinary abnormalities in the population, such as Japan, biopsy more people which leads to more diagnoses of IgAN. Indeed, a correlation between the incidence of primary glomerulonephritis, including IgAN, and the number of renal biopsies performed in country has been observed (McQuarrie *et al*, 2009).

In Caucasians, the incidence of IgAN is twice as high in males as females. This gender difference is not seen in Asian populations, with equal proportions of males and females being diagnosed with IgAN (Barratt *et al*, 2012; Wyatt and Julian, 2013). The reasons behind differences in the incidence of IgAN in ethnicities and in genders has not as yet been well researched.

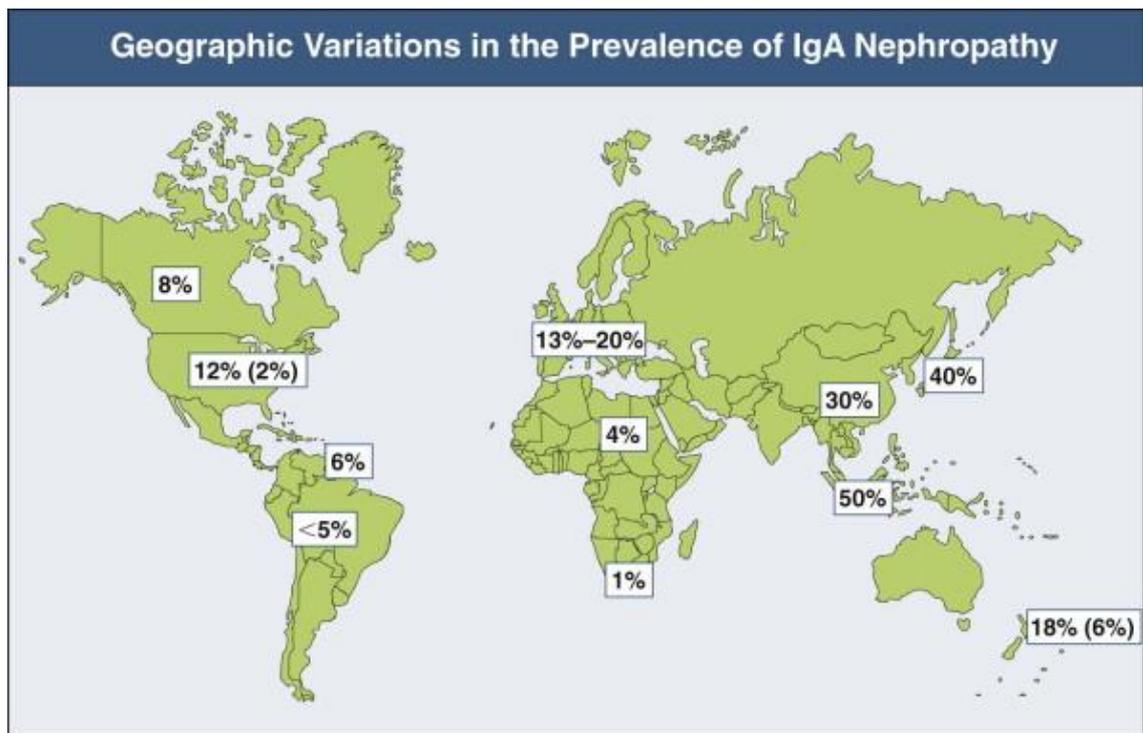


Figure 1.2: Geographical variations in the prevalence of IgAN: Percentages represent the proportion of cases of IgAN compared to all patients with glomerular disease. The numbers in brackets represent minority racial groups; African Americans in the United States of America and Polynesians in New Zealand. From (Feehally and Floege, 2010).

1.1.3.2 – Multi-hit hypothesis

A multi-step hypothesis has been presented to describe the pathogenesis of IgAN (Figure 1.3). This first “hit” in this process involves the production of poorly O-galactosylated IgA1, which combines with the production of autoantibodies that bind this aberrant, galactose-deficient IgA1 (hit two) to form immune complexes (hit three) which are then deposited in the renal mesangium (hit four) (H. Suzuki *et al*, 2011).

1.1.3.3 – Renal damage

The binding of IgA1 and IgA-containing immune complexes to mesangial cells and their deposition in the renal mesangium leads to mesangial cell proliferation, extracellular matrix production, release of pro-inflammatory cytokines and growth factors, podocyte damage, disruption of the glomerular filtration barrier, glomerulosclerosis and tubulointerstitial scarring (Figure 1.4) (Boyd *et al*, 2012).

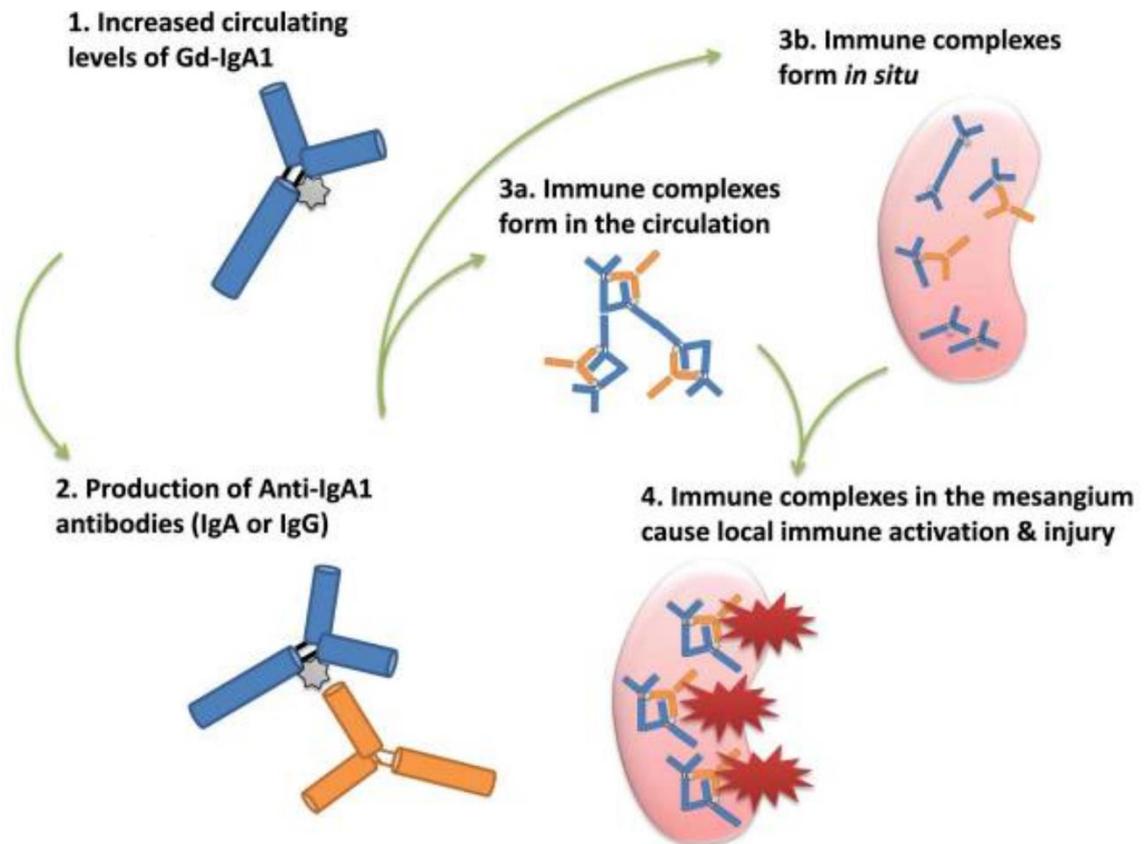


Figure 1.3: Depiction of the multi-hit hypothesis of the pathogenesis of IgAN: Hit 1: Elevated levels of circulating galactose-deficient IgA1 (Gd-IgA1) are produced. Hit 2: IgA or IgG autoantibodies for the undergalactosylated Gd-IgA1 hinge region are produced. Hit 3: Immune complexes are formed from Gd-IgA1 and IgA or IgG autoantibodies. Hit 4: Immune complexes are deposited in the renal mesangium, leading to mesangial cell proliferation, extracellular matrix production, release of pro-inflammatory cytokines and growth factors, podocyte damage, disruption of the glomerular filtration barrier, glomerulosclerosis and tubulointerstitial scarring. From (Canetta, Kiryluk and Appel, 2014).

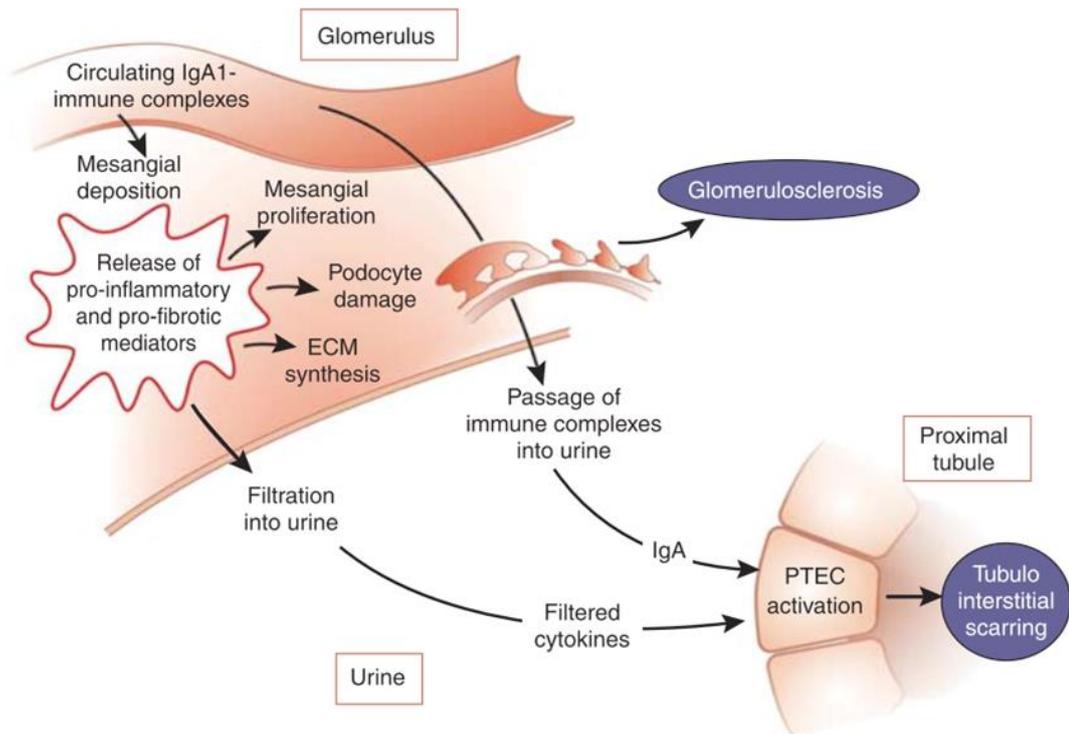


Figure 1.4: Deposition of IgA immune complexes and triggering of renal injury: Deposition of IgA1 immune complexes in the renal mesangium leads to release of pro-inflammatory and pro-fibrotic mediators, causing mesangial cell proliferation, podocyte damage, synthesis of extracellular matrix components, and filtration of mediators into the proximal tubule. Glomerular filtration barrier damage leads to an increase in proteinuria and also filtration of mesangial-derived mediators and IgA1 immune complexes into the proximal tubule, causing the release of pro-inflammatory and pro-fibrotic cytokines from proximal tubular epithelial cells into the interstitial space resulting in tubulointerstitial fibrosis. From (Boyd *et al*, 2012).

1.1.4 – Genetics

Genetic factors are very likely to have a contributory effect on the development of IgAN. The large variance in incidence of IgAN across different ethnicities suggests the presence of susceptibility genes at different frequencies. Familial aggregation of IgAN is also well documented across the world.

1.1.4.1 – Heritability

Cases of IgAN in multiple members of the same family have been reported in Europe, North America and Asia (Scolari *et al*, 1999; Karnib *et al*, 2007; Lavigne *et al*, 2010; Tam *et al*, 2009). Inheritance of IgAN occurs in an autosomal dominant pattern in most reported families (Kiryluk and Novak, 2014; Fennelly *et al*, 2018). Clinically patients with familial IgAN will present similarly to those with sporadic IgAN, and no differences can be seen in the histopathology of renal biopsies between these. Familial IgAN is thought to account for around 5% of all IgAN cases (Mestecky *et al*, 2013); however, due to the necessity of a renal biopsy to diagnose IgAN, the true prevalence of familial IgAN is unknown. This also means familial IgAN has important clinical implications, for instance in the selection of appropriate donor kidneys from family members for transplantation.

Studies have also shown that elevated levels of galactose-deficient IgA1 (Gd-IgA1) in circulation, thought to be one of the main pathogenic features of IgAN, is a heritable trait in IgAN in multiple populations worldwide. This has been found to be consistent regardless of what age diagnosis of IgAN is made at, in both sporadic and familial IgAN and also in secondary forms of IgAN (Gharavi *et al*, 2008; Hastings *et al*, 2010; Kiryluk, Moldoveanu, John *et al*, 2011; Lomax-Browne *et al*, 2016). However, blood relatives of IgAN patients with high circulating Gd-IgA1 levels who themselves had high levels of Gd-IgA1 have been shown to have no evidence of kidney disease, suggesting that although they represent a risk factor for the disease, increased Gd-IgA1 levels are alone insufficient to cause IgAN.

1.1.4.2 – Candidate genes and susceptibility loci

Several genome-wide linkage studies in familial IgAN have been reported, identifying several candidate gene loci in families with at least two cases of IgAN.

The earliest of these identified a locus on chromosome 6q22-23 in 60% of the families studied, with the remainder showing linkage to chromosome 3p24-23 (Gharavi *et al*, 2000). Subsequent studies replicated the linkage to chromosome 6q22-23, but also detected signals for linkage to chromosomes 4q26-31, 17q11-22 and 2q36 (Bisceglia *et al*, 2006; Paterson *et al*, 2007). Genetic association studies in sporadic IgAN have also been carried out, leading to conflicting data on association of genes involved in glycosylation pathways with IgAN. In Chinese populations, single nucleotide polymorphisms (SNPs) of the gene for the galactosyltransferase enzyme C1GalT1 (C1GALT1, chromosome 7p14-13) and the gene for the chaperone protein of C1GalT1, Cosmc (C1GALT1C1, chromosome Xp24), have been found to associate with IgAN (G. - Li *et al*, 2007; W. L. Li and Lu, 2015; Eleonora Bertinetto *et al*, 2012). However, in European populations associations with IgAN have been found in C1GALT1 but not C1GALT1C1 (Pirulli *et al*, 2009; Malycha *et al*, 2009). Candidate gene studies of this nature select genes for analysis based on any known or suspected disease mechanism. Along with relatively small sample sizes, this means they often lack power. The inconsistency seen in these also holds to underpin the genetic heterogeneity of IgAN.

1.1.4.3 – Genome-wide association studies

Genome-wide association studies (GWAS) utilise high-throughput genotyping technologies to analyse large numbers of genetic variants across the entire genome. Typically examining SNPs, a GWAS involves analysis of thousands of samples in a case-control or family-based nature and have a greater power to detect novel disease susceptibility loci than candidate gene studies.

The first IgAN GWAS was performed in 2010 in a cohort of IgAN patients of European origin, identifying associations at the major histocompatibility complex (MHC) region (Feehally *et al*, 2010). A subsequent GWAS involved a discovery cohort of Chinese ancestry with targeted follow-up in Chinese and European cohorts. This study showed associations with IgAN in three HLA loci at the MHC region at chromosome 6p21, a locus at chromosome 22q12 (HORMAD2 locus) and with a common deletion of CFHR1 and CFHR3

(chromosome 1q32) (Gharavi *et al*, 2011). *HORMAD2* encodes HORMA domain-containing protein 2 which is involved in regulating meiosis, and *CFHR1* and *CFHR2* encode complement factor H-related proteins. The next reported GWAS was in a Chinese cohort and replicated the five associations found in the previous study, while also identifying two new loci; one at chromosome 17p23 (*TNFSF13* locus) and one at chromosome 8p23 (*DEFA* locus) (Xue-Qing Yu *et al*, 2011). *TNFSF13* (tumor necrosis factor superfamily 13) encodes *APRIL* which is important for B cell development, and *DEFA* encodes the antimicrobial protein defensin alpha 1.

In 2014, a fourth IgAN GWAS was performed using populations from all three previous studies and including further populations from Asia and Europe (Kiryluk *et al*, 2014). This study replicated the previous findings and reported several new loci associated with IgAN: at chromosome 9q34 (*CARD9* locus), chromosome 1p13 (*VAV3* locus) and four at chromosome 16p11 (*ITGAM-ITGAX* locus). *CARD9* encodes caspase recruitment domain-containing protein 9, an adapter protein that promotes activation of NF- κ B in macrophages. *VAV3* encodes vav guanine nucleotide exchange factor 3 which is involved in chemokine signalling. *ITGAM-ITGAX* encodes integrins α M and α X. A further study in a Chinese cohort replicated the *ITGAM-ITGAX* and *DEFA* loci associations and also reported new associations at chromosomes 3q27, 11p11 and 8q22 (*ST6GAL1*, *ACCS* and *ODF1-KLF10* loci respectively) (M. Li *et al*, 2015); these novel loci however require validation in a European cohort. *ST6GAL1* encodes the sialyltransferase enzyme ST6 beta-galactosamide alpha-2,6-sialyltransferase. *ACCS* encodes 1-aminocyclopropane-1-carboxylate synthase-like protein 1 that belongs to the class-I pyridoxal-phosphate-dependent aminotransferase family. *ODF1* encodes outer dense fiber protein 1 which forms cytoskeletal structures in sperm tails. *KLF10* encodes Kruppel-like factor 10 which is a transcriptional repressor of TGF- β signalling.

All five IgAN GWAS to date have identified associations of IgAN with multiple genes at the MHC; a region encoding cell surface proteins essential for the acquired immune system to recognise foreign molecules. *TNFSF13* encodes *APRIL* (a proliferation inducing ligand), a cytokine involved in the development of IgA producing B cells. Many of the other reported loci have functions involving

maintenance of the intestinal epithelial barrier and defence against mucosal pathogens. Geographic differences in the prevalence and association of many of these loci have been identified, paralleling the geographic differences in the incidence of IgAN itself (Kiryluk *et al*, 2012).

1.1.5 – Prognosis

The prognosis of IgAN is very variable but is associated with a significant risk of progressive kidney disease. In Caucasian populations, 20-40% of IgAN patients will progress to end-stage renal disease (ESRD) within 20 years of diagnosis (Geddes *et al*, 2003), and there is currently no fully validated clinically relevant method of predicting the prognosis of the disease at diagnosis.

There have been several markers suggested to be useful in predicting the progression of IgAN (Maixnerova *et al*, 2016). A higher combined MEST score, as well as a higher score of each factor individually, have been proposed and validated as markers for progression; however, this still requires the initial risk to the patient of a renal biopsy. In recent years, there has been more focus on identifying markers less invasively, such as those found in serum or urine. Serum levels of Gd-IgA1 and Gd-IgA1-specific IgA and IgG autoantibodies have been proposed thusly (Zhao *et al*, 2012; Berthoux *et al*, 2012; Yanagawa *et al*, 2014). Other reported serum markers include cytokines such as TNF receptors 1 and 2, and IL-17A (Oh *et al*, 2015; Watorek and Klinger, 2015). With regards to urinary markers of progression in IgAN, urinary levels of EGF, IL-6 and MCP-1 have been reported (Stangou *et al*, 2009). These candidate markers do however require validation in larger cohorts of patients to determine their actual usefulness in predicting progression of IgAN

1.1.6 – Treatment

At present there is no cure for IgAN, and treatment options for patients are quite limited and rarely a long-term success, with no IgAN-specific treatments currently available. Standard treatments for glomerulonephritis that are used with in IgAN include renin-angiotensin blockade with angiotensin converting enzyme inhibitors or angiotensin receptor blockers. Immunosuppressive therapy by the use of systemic corticosteroids is a common treatment option for patients who have not responded to the above treatments, but comes with many side effects

after long-term use and is usually only prescribed in six-month courses because of this (Buchman, 2001; Coppo, 2017). A formulation of the corticosteroid budesonide targeted for release at Peyers's patches in the ileum of the gut has recently been shown to be a promising treatment for IgAN and avoids the many side effects of systemic treatments with the same (Smerud *et al*, 2011; Fellström *et al*, 2017). However, for those in whom the disease progresses, renal replacement therapy either by dialysis or renal transplantation are currently the main options for treatment.

1.1.7 – Secondary forms of IgAN

The most common form of secondary IgAN is called IgA vasculitis (IgAV), formerly known as Henoch-Schönlein purpura (HSP). IgAV is caused by IgA deposition in the walls of small blood vessels and can affect areas such as the skin, joints, gut and kidneys leading to symptoms in these areas such as rash, arthralgia, abdominal pain and nephritis. IgAV has been described as a systemic form of IgAN, as the renal biopsies from IgAV patients with nephritis are histologically indistinguishable from IgAN (Waldo, 1988). Patients with IgAV have also been found to have elevated serum levels of Gd-IgA1 similarly to IgAN patients (Kiryluk, Moldoveanu, Sanders *et al*, 2011).

IgAN has also been documented in patients with a variety of other diseases but is referred to as being secondary to the underlying disorder. These disorders are not necessarily renal diseases, and can include chronic liver disease, inflammatory diseases, autoimmune diseases, chronic infections and neoplasms (Pouria and Barratt, 2008; Saha *et al*, 2018). Due to the fact that mesangial IgA deposition can be found in healthy subjects without any prior clinical manifestation of IgAN, associations between IgAN and other diseases could be an exacerbation of underlying asymptomatic IgAN caused by the disease in question.

1.2 – IgA and the immune system

1.2.1 – Adaptive immunity

The adaptive immune system, otherwise known as the acquired immune system, utilises clonal selection of lymphocytes to provide immune responses to

specific pathogens. It holds the function of immunological memory, in that the recognition of specific pathogen molecules is “remembered” by specific memory B and T lymphocytes in order to provide a faster immune response upon subsequent exposure to these same pathogens.

1.2.2 – B cell development

All B cells develop from hematopoietic stem cells found in bone marrow, beginning with functional rearrangement of the immunoglobulin gene segments (Pieper, Grimbacher and Eibel, 2013). These immature B cells will then migrate via the blood to secondary lymphoid tissues such as the spleen, lymph nodes or Peyer’s patches whereupon they differentiate into naïve, follicular or marginal zone B cells as the final stage of their early development. These cells, once activated by recognition of specific antigens, begin proliferating, differentiate further and undergo antibody class switching to produce immunoglobulins of different isotypes or subtypes for secretion in two forms; soluble for circulation in blood plasma and membrane-bound as B cell receptors to modulate immune responses. Marginal zone B cells and follicular B cells can develop into short-lived plasma cells for immediate production of protective antibodies to acute infections (Martin and Kearney, 2002). Follicular B cells can also migrate to germinal centres in lymph nodes and, once activated by antigen binding and supported by T-helper cells, differentiate into memory B cells or through plasmablasts into long-lived plasma cells (Perez-Andres *et al*, 2010). Long-lived plasma cells are utilised for a longer-term antibody production, while memory B cells present a single class and subtype of immunoglobulin on their surface, in effect “remembering” the antigen that activated their development, and are thus able to differentiate into antibody-producing plasma cells upon any future stimulation with their respective antigen (Calame and Shapiro-Shelef, 2005). A diagram of B cell development from marginal zone and follicular B cells is included as Figure 1.5.

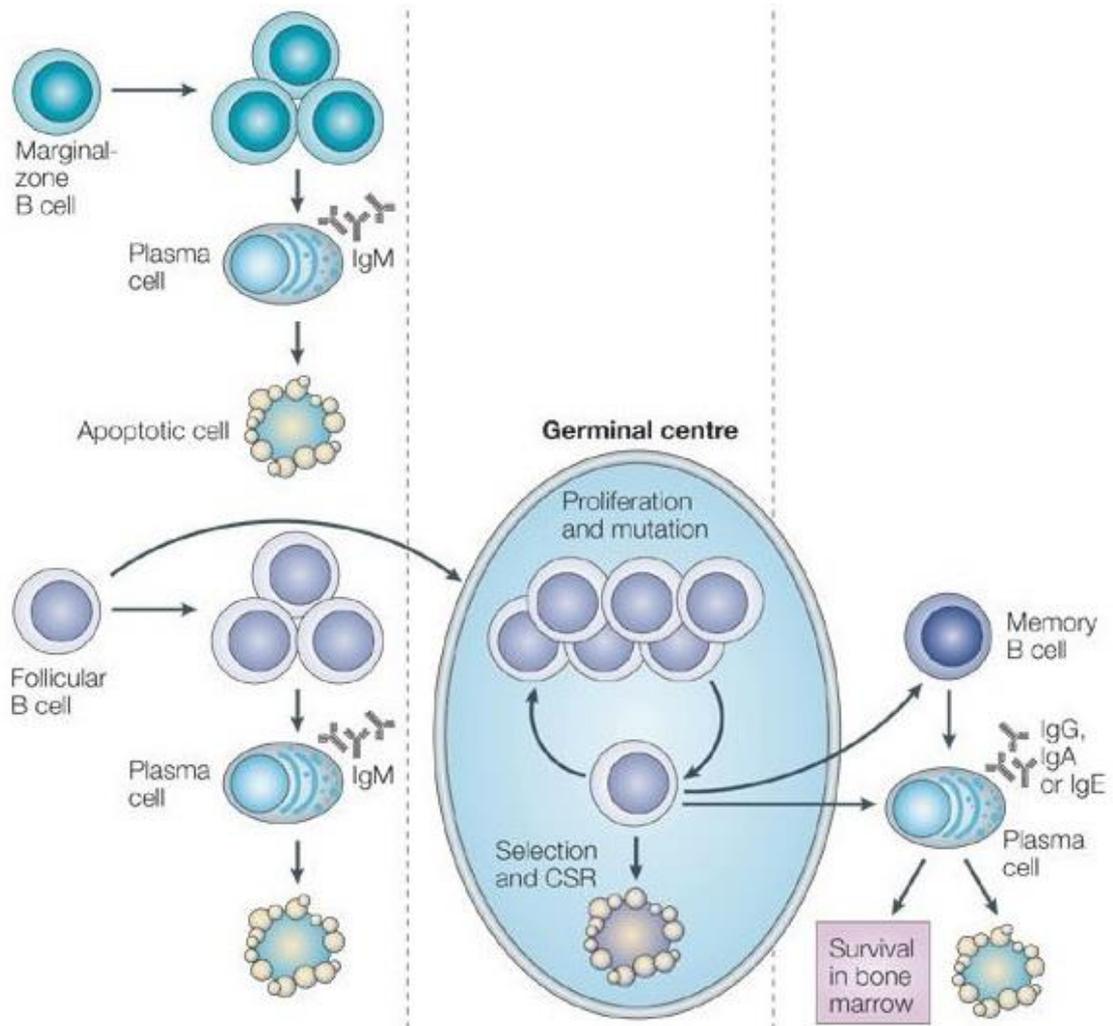


Figure 1.5: Diagram of B cell development from marginal zone B cells and follicular B cells to antibody secreting plasma cells: Marginal zone B cells and follicular B cells in secondary lymphoid tissue such as in the spleen, lymph nodes or Peyer's patches can develop into short-lived plasma cells for immediate production of protective antibodies to acute infections. Follicular B cells migrate to germinal centres in lymph nodes and, once activated by antigen binding and supported by T-helper cells, undergo class-switch recombination (CSR, see section 1.2.5) and differentiate into memory B cells or through plasmablasts into long-lived plasma cells. From (Calame and Shapiro-Shelef, 2005).

1.2.3 – The mucosal immune system

Mucosal surfaces are internal areas of the body in contact with the external environment; these surfaces being the epithelia of the gastrointestinal tract, the genitourinary tract and the respiratory system. The mucosa-associated lymphoid tissues (MALT) consist of a large amount of secondary lymphoid tissue found at mucosal membranes; this forms the mucosal immune system which provides a primary line of defence against pathogens from the environment. It also serves as a barrier to prevent systemic immune responses caused by the body's normal commensal flora. The main isotype of immunoglobulin produced in the MALT in mucosal secretions is IgA.

1.2.4 – B cell homing

Homing is the action of cells migrating to different sites in the body, either for maturation purposes or for functional reasons; a process that involves various receptors and chemokines. Naïve B cells are undifferentiated, and therefore express few homing receptors as they are freely able to move through the lymphatic system to the spleen, lymph nodes or Peyer's patches where early differentiation can occur (Kantele, Kantele and Arvilommi, 1996). Upon exposure to antigens and differentiation into mature cells, B cells begin to express homing receptors at a higher level. This allows the cells to traffic through circulation to effector sites such as mucosal surfaces upon the action of organ-specific chemokines. These sites have large amounts of cellular adhesion molecules, which allows the undifferentiated cells to bind. For example; $\alpha 4\beta 7$ integrin is the receptor for the ligand MAdCAM-1 (mucosal vascular addressin cellular adhesion molecule 1) in the mucosal surfaces of the gut (Mora and von Andrian, 2008).

1.2.5 – Immunoglobulins

Immunoglobulins, interchangeably called antibodies, are glycoproteins synthesised by B cells as a critical part of adaptive immune responses (humoral immunity). They are utilised to recognise and bind specific, normally foreign, pathogenic antigens and either directly neutralise or aid in the removal of these potentially damaging antigens from the body by marking them as foreign.

All immunoglobulins, regardless of specificity or isotype share the structure of two identical heavy chains and two identical light chains forming variable and constant regions. There are two types of light chain named kappa (κ) and lambda (λ), and five types of heavy chain named mu (μ), delta (δ), alpha (α), gamma (γ) and epsilon (ϵ). These heavy chains give rise to the five immunoglobulin isotypes: IgM, IgD, IgA, IgG and IgE respectively. The two heavy and two light chains are joined by disulphide bonds into an overall structure comprised of two Fab (fragment, antigen binding) regions and one Fc (fragment, crystallisable) region. The Fab regions are known together as the variable domain, and this highly heterogenous region is responsible for the multitude of different antigen-recognising antibodies that can be synthesised. The Fc region is responsible for the binding of immunoglobulins to cell surface receptors, thus allowing activation of immune responses. The presence of hinge regions between the Fab and Fc regions allows for an increased flexibility of the immunoglobulin structure and therefore an increase in the capacity for antigen binding (Adlersberg, 1976).

Immature, or naïve, B cells will initially only produce IgM and IgD before being exposed to antigens. Antibody class switching to the different immunoglobulin isotypes is caused by specific signalling cascades begun by antigen detection inducing activation and maturation of B cells. This causes deletions and functional rearrangements in the heavy chain gene locus, known as V(D)J recombination (Figure 1.6), leading to the production of different immunoglobulin isotypes specific to the type of presented antigen (Borghesi and Milcarek, 2006). Class switching is known to be highly regulated by a range of cytokines; some of which can also modify or interrupt this process. Once a B cell reaches a certain stage of differentiation, it will lose the ability for further class switches and be set producing a single immunoglobulin isotype.

Alternative splicing of the individual heavy chain genes during normal B cell differentiation regulates the production of membrane-bound and secreted immunoglobulins, with initial production of membrane-bound forms shifting to secreted forms as the cells mature. It has also been observed that changes in the synthesis ratio of membrane-bound to secreted form, and thus B cell differentiation, is strongly influenced by the presence of cytokines. The translated

membrane-bound forms contain an extended hydrophobic Fc region which allows for anchoring in the cell surface membrane; and in combination with accessory proteins, the membrane-bound immunoglobulins form B cell antigen receptor complexes, allowing for signal transduction of the recognition of antigen binding and causing the cell to proliferate and convert to synthesising the secreted form of the antibody for immune response. Different arrangements of each heavy chain locus also form the diversity of the variable region that is the key to the recognition of the vast number of antigens.

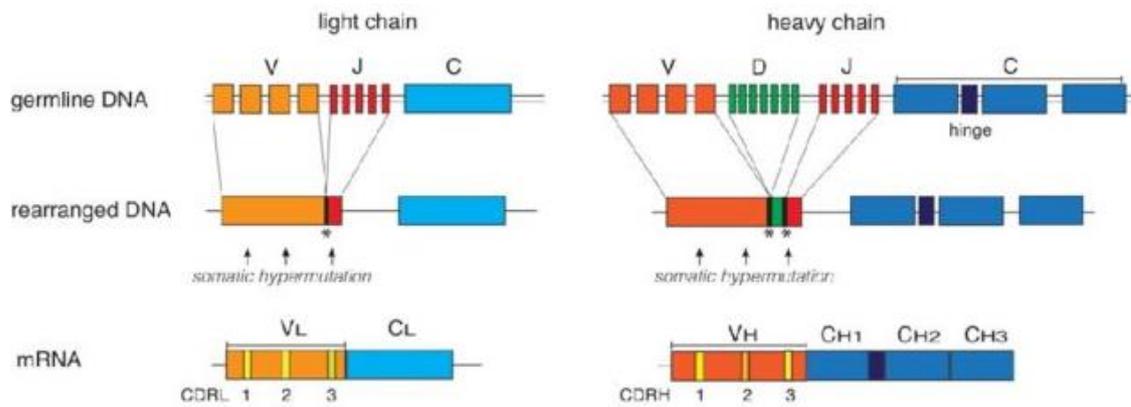


Figure 1.6: V(D)J recombination of the immunoglobulin heavy chain gene: Different germline gene segments coding for the variable Ig heavy and light chains are joined by somatic V(D)J gene rearrangement. Addition or removal of nucleotides during recombination at the junctions (*) and somatic hypermutation (arrows) in the complementary-determining regions of the VL and VH genes results in a high diversity of immunoglobulins. The constant regions of the heavy chain are joined by RNA splicing to the variable regions. Adapted from (Feederle and Schepers, 2017).

1.2.6 – Immunoglobulin A

Immunoglobulin A (IgA) is the most heavily synthesised antibody in humans, with around 66mg/kg of body weight being produced per day (Pabst, 2012), which is more than twice the production of IgG, and comprises around 15% of the immunoglobulin content in serum. IgA is also the main antibody produced at mucosal surfaces such as the epithelia of the gastrointestinal tract, the genitourinary tract and the respiratory system. This means that IgA provides a front-line defence at the bodily surfaces most in contact with the external environment.

1.2.6.1 – Structure of IgA

As with all immunoglobulins, human IgA consists of two heavy chains and two light chains, with the heavy chains being of the α class and either of the light chains κ and λ . Unlike the other immunoglobulin classes, IgA can exist in both monomeric or polymeric forms; with IgM only existing in polymeric form and IgD, IgG and IgE only existing as monomers. The monomeric form of IgA comprises more than 90% of the IgA in serum (systemic IgA) and is produced in the bone marrow, while polymeric IgA (mostly dimeric but some tetramers) is mainly produced in the MALT (mucosal IgA).

Polymeric IgA, and IgM, is joined together at the end of the Fc regions by a small protein called the immunoglobulin joining chain (J chain) (Johansen, Braathen and Brandtzaeg, 2000). Polymeric IgA produced at mucosal surfaces is also associated with secretory component (SC), and as it is also then a component of mucous secretions it is termed secretory IgA (sIgA). SC is bound to the Fc regions of the IgA subunits in polymeric IgA only through the presence of J chain and is thought to provide resistance to degradation by proteases.

1.2.6.2 – Function of IgA

As stated above, the main function of IgA is at mucosal surfaces in the form of sIgA, where it forms a front-line defence in order to neutralise biologically active pathogens such as microorganisms, toxins and enzymes and prevent entry into the body at the mucosa; this process is known as immune exclusion (Pabst, 2012). The methods by which sIgA functions in this manner include

entrapping targets in mucous secretions and preventing any binding to cell surface receptors, aggregating bacteria to reduce their mobility and limit their invasiveness, and by assisting in the uptake of antigens through the epithelium to lymphoid tissue for degradation.

1.2.6.3 – Isoforms of IgA

Human IgA exists in two isoforms; IgA1 and IgA2 (Figure 1.7). Most mammalian species that possess IgA will produce isoforms of IgA more closely related to IgA2, while only humans and higher primates produce IgA1. These different human isoforms arise through the composition of the heavy chain, with separate α genes on chromosome 14 for IgA1 and IgA2 ($\alpha 1$ and $\alpha 2$ respectively). The two isoforms of IgA differ in the hinge region, with IgA1 possessing an extended hinge region of 26 amino acids, while the IgA2 hinge region is 13 amino acids long. This extended hinge region allows for greater flexibility in the structure of IgA, which in turn allows for greater capacity for antigen binding (Adlersberg, 1976). Unlike the shorter IgA2 hinge region, the IgA1 hinge region has several sites for O-glycosylation. The IgA1 hinge region is also highly susceptible to degradation by a number of bacterial proteases (Chintalacharuvu *et al*, 2003).

The proportion of IgA1 and IgA2 varies in humans depending on whether the IgA is produced systemically or at different mucosal surfaces. Systemically in serum, around 90% of the IgA content is IgA1. In the respiratory system, IgA1 predominates. In the MALT of the gastrointestinal tract (gut-associated lymphoid tissue, GALT), this varies further; with equal amounts of IgA1 and IgA2 produced in the small intestine and more IgA2 than IgA1 produced in the large intestine (Delacroix *et al*, 1982).

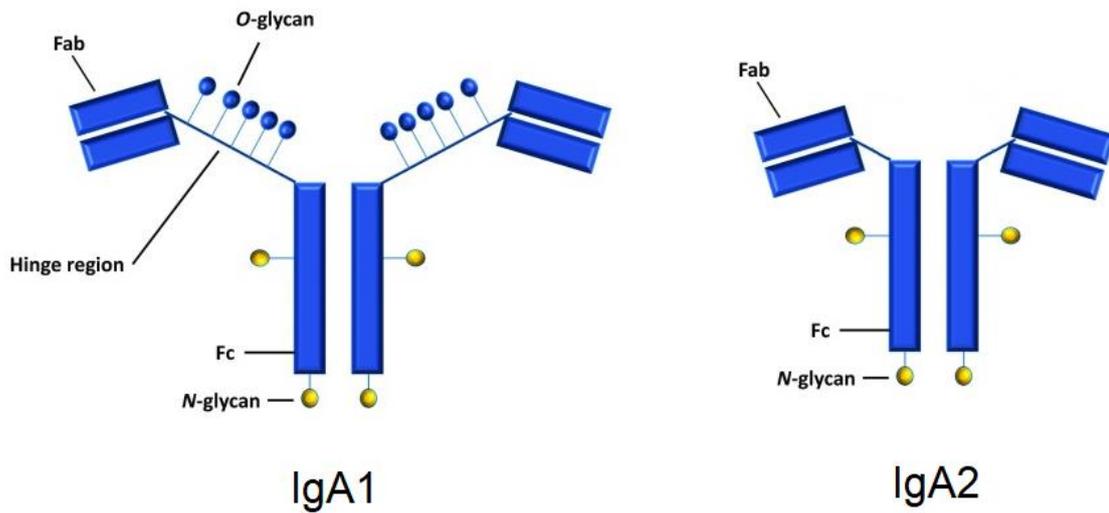


Figure 1.7: Comparison the structure of IgA1 and IgA2: Both IgA1 and IgA2, like all immunoglobulins, comprise two heavy and two light chains. The heavy chain genes for IgA1 and IgA2 are named $\alpha 1$ and $\alpha 2$ respectively. Unlike IgA2, IgA1 contains an extended hinge region between the Fab and Fc regions. This extended hinge region is rich in serine and threonine residues, three to six of which can be sites for O-glycosylation.

1.2.6.4 – IgA1 hinge region O-glycosylation

One of the most important post-translational modifications made to proteins is glycosylation, defined as the enzymatic attachment of carbohydrate moieties to translated proteins. This process provides a greater proteomic diversity than other types of post-translational modifications; and is critical to many biological processes such as cell signalling and protein-ligand interactions in the cell, cell differentiation and adhesion, and can also influence the correct folding of some proteins. The O-linked glycosylation of secretory or membrane-bound glycoproteins occurs specifically in the Golgi apparatus at serine or threonine residues of peptide chains and is a major category of glycosylation. O-glycosylation is essential to the synthesis of mucins, a family of high molecular weight proteins that form mucous secretions, and crucial in the formation of the core proteins in extracellular matrix components (Bergstrom and Xia, 2013). Due to the importance of glycosylation to biological processes, acquired and inherited mutations in glycosylation pathways have been found to be associated with the pathology of a number of diseases.

Commonly, immunoglobulins are heavily glycosylated, with human IgA1 being one of the few circulating glycoproteins which can be O-glycosylated as well as N-glycosylated, along with IgD (Mellis and Baenziger, 1983). The IgA1 hinge region, as described above, is 26 amino acids long and is rich in serine and threonine residues; up to six of which can be sites for O-glycosylation (Tarelli *et al*, 2004).

The common core 1 O-glycan structure Gal- β 1,3-GalNAc-Ser/Thr is a precursor for many extended mucin-type O-glycan structures such as those found in the IgA1 hinge region. The synthesis of core 1 is first initiated by the transfer of N-acetylgalactosamine (GalNAc) from UDP-GalNAc to serine or threonine residues, catalysed by the GalNAc transferase enzyme GalNT2 (polypeptide N-acetylgalactosaminyltransferase 2) (Iwasaki *et al*, 2003a). This initial structure is known as the Tn-antigen. Galactosylation of the Tn-antigen can then occur through the transfer of galactose from UDP-Gal to GalNAc by the enzyme core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 (C1GalT1), forming the Gal- β 1,3-GalNAc-Ser/Thr

structure more commonly known as the T-antigen (T. Ju *et al*, 2002). C1GalT1, upon translation, requires a molecular chaperone protein called Cosmc for correct folding and activation of its enzymatic activity, or is otherwise signalled for degradation by the ubiquitin-proteasome system (T. Z. Ju and Cummings, 2002; Y. Wang *et al*, 2010). Both the Tn- and T-antigens can be sialylated by the addition of N-acetylneuraminic acid (sialic acid). This is catalysed by N-acetylgalactosaminide- α -2,6-sialyltransferase (ST6GalNAc) enzymes for an α 2,6 linkage of sialic acid to GalNAc and by β -galactoside- α -2,3-sialyltransferase enzymes (ST3Gal) for an α 2,3 linkage of sialic acid to galactose (Harduin-Lepers *et al*, 2001). Figure 1.8 gives a depiction of O-glycosylation at the IgA1 hinge region.

1.2.6.5 – Cytokine and chemokine regulation of IgA synthesis

The activation of B cells and their regulation as they mature is influenced by the action of many cytokines and chemokines. These can also have an action in preferentially causing B cells to class-switch to specific immunoglobulin production. With regards to IgA production, TGF- β (transforming growth factor β) is heavily involved in inducing class-switch recombination of α 1 and α 2 germline transcripts from the immunoglobulin heavy chain gene locus. In effect, TGF- β induces the IgA response (Sonoda *et al*, 1992), and cooperates with CD40L on the surface of T cells to initially activate naïve B cells to this end (Dullaers *et al*, 2009). Independently of T-cells, APRIL (a proliferation inducing ligand) and BAFF (B cell activating factor) also function to activate naïve B cells by engaging B-cell maturation antigen (BCMA), BAFF receptor (BAFFR) and TACI (transmembrane activator and CAML interactor) (Litinskiy *et al*, 2002). It has also been documented that APRIL can preferentially induce IgA2 transcripts and BAFF IgA1 (He *et al*, 2007). IL-10 plays a significant role in inducing differentiation of B cells to IgA secreting plasma cells (Marconi *et al*, 1998; Hirano *et al*, 2003; Lafarge *et al*, 2011).

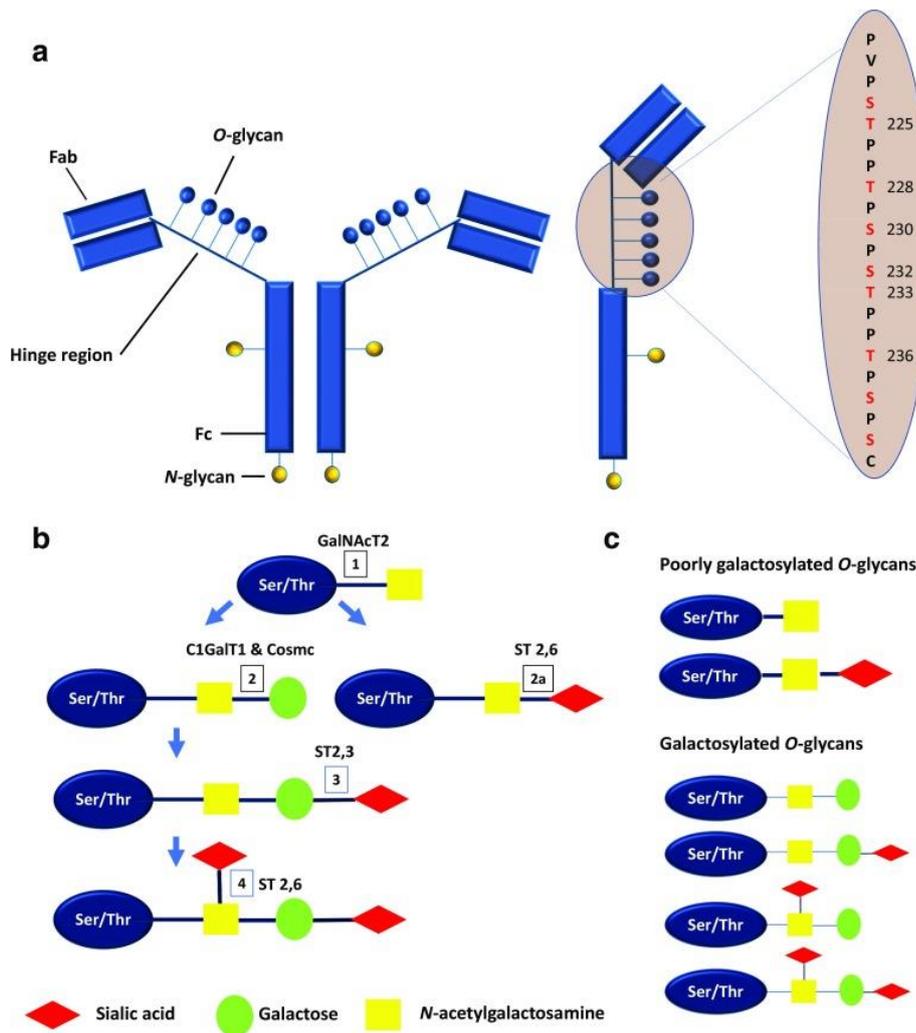


Figure 1.8: IgA1 hinge region O-glycosylation: From (Yeo, Cheung and Barratt, 2018).

a: Structure of the IgA1 molecule and its hinge region between the Fab and Fc regions. This hinge region is 26 amino acids long and is rich in serine and threonine residues, three to six of which can be sites for O-glycosylation.

b: Mechanism of O-glycosylation at the IgA1 hinge region. First, GalNAc2 enzyme catalyses the addition of N-acetylgalactosamine (GalNAc) to Ser/Thr residues. Second, C1GalT1 and its chaperone protein Cosmc catalyse the addition of galactose to GalNAc. Thirdly, sialic acid can be added to galactose by ST3Gal enzymes, or to GalNAc by ST6GalNAc enzymes before or after galactose addition.

c: Depiction of galactosylated and non-galactosylated O-glycan structures that can be found at the IgA1 hinge region.

It has been shown that the pattern of O-glycosylation at the IgA1 hinge region can be altered by the action of a variety of cytokines on IgA producing cells; namely by altering the regulation of C1GalT1 and ST6GalNAc enzymes causing a reduction in the amount of galactose present. This has been demonstrated in an immortalised IgA1 producing cell line isolated from a carcinoma by stimulation with a major virulence factor of *Helicobacter pylori*, CagA, and with the cytokines IL-4, TGF- β and IL-17 (Yamada *et al*, 2010; Xiao *et al*, 2017; Lin *et al*, 2018). This same effect has been seen in immortalised IgA1 producing cells isolated from healthy subjects and IgA nephropathy patients that were stimulated with IL-4 and IL-6 (H. Suzuki *et al*, 2014).

1.2.6.6 – IgA in IgAN

1.2.6.6.1 – IgA1 O-glycosylation in IgAN

Serum from IgAN patients is known to contain higher levels of IgA1 that is hypogalactosylated at the hinge region (galactose-deficient IgA1, Gd-IgA1) compared to serum from healthy subjects (Coppo and Amore, 2004; Moldoveanu *et al*, 2007; Mestecky *et al*, 2008; Barratt, Smith and Feehally, 2012; Novak *et al*, 2012). In IgAN patients, the levels of circulating Gd-IgA1 have been shown to correlate with levels of Gd-IgA1 isolated from deposits in the renal mesangium (Allen *et al*, 2001). These increased levels of Gd-IgA1 are thought to be due to under-expression or low activity of C1GalT1 or Cosmc decreasing the ability to add galactose, or due to high expression or activity of sialyltransferases blocking the addition of galactose by over-sialylation of terminal GalNAc (Allen *et al*, 1997; Qin *et al*, 2005; Buck *et al*, 2008; Raska *et al*, 2008; Smith *et al*, 2008). In recent years, the presence of higher levels of Gd-IgA1 in serum has been linked with a faster decline in renal function in IgAN (Hiki, 2009; Zhao *et al*, 2012). This pattern has been described in multiple ethnicities (Shimozato *et al*, 2008; Hastings *et al*, 2010); but a direct comparison of Gd-IgA1 levels in IgAN across ethnic groups has yet to be made.

Measurements of the extent of galactose-deficiency of IgA1 in serum are most commonly made by way of an ELISA based method (Allen, Harper and Feehally, 1995; Gomes *et al*, 2010). This method utilises lectins that preferentially bind to terminal O-linked GalNAc residues; the most common lectin in use

worldwide being from the *Helix aspersa* (HA) garden snail. However, there are up to six sites for O-glycosylation on the IgA1 hinge region, which leads to a vast number of potential combinations of glycoforms; many of which will lack galactose at some sites and thus contain an exposed terminal or sialylated GalNAc. In health and in IgAN, serum contains a mixture of IgA1 glycoforms which bind the HA lectin to differing extents, leading to an overlap in the levels of circulating Gd-IgA1 between IgAN patients and healthy subjects and making its use as a diagnostic tool more difficult. Glycobiology techniques such as mass spectrometry can be used to measure more accurately glycosylation of the IgA1 hinge region, giving site-specific data of the aberrancy of IgA1 O-glycosylation in IgAN. However, this can be quite expensive and labour intensive and while several proof of concept studies analysing the IgA1 hinge region by mass spectrometry have been published, only one small study has compared the IgA1 hinge region from IgAN patients with that of healthy subjects; finding three site-specific galactose-deficient O-glycan structures that were specific to IgAN (Odani *et al*, 2010).

As with IgA1, IgD can also be O-glycosylated at its hinge region. However, no aberrancy in IgD O-glycosylation has been seen in IgAN (Smith, De Wolff *et al*, 2006). It is inferred from this that any defect in IgAN that causes reduced galactosylation of the IgA1 hinge region is not a generic feature of B cells, but rather a defect in an upstream regulatory pathway as they class switch from naïve B cells to IgA1 producing B cells.

1.2.6.6.2 – Pathogenic IgA origins

It has been theorised that the higher levels of circulating Gd-IgA1 seen in IgAN is produced by B cells that have been miss-trafficked from the mucosa, due to the fact that IgA1 produced in the mucosa is known to be less glycosylated than that derived from the bone marrow (Royle *et al*, 2003; Smith, Molyneux *et al*, 2006). This is supported by the commonly reported flare-up of IgAN alongside mucosal infections, potentially causing an increase in cytokines which stimulate B cells to produce more Gd-IgA1 (Barratt *et al*, 1999; Floege and Feehally, 2016).

1.2.6.6.3 – Immune complex formation

The undergalactosylated hinge region of IgA1 is thought to be antigenic for IgA and IgG autoantibodies. These autoantibodies are produced systemically and are thought to be targeted to GalNAc epitopes, which are expressed in structures on the surface of Gram-positive bacteria and on some viruses (Freymond *et al*, 2006; Lei *et al*, 2015). When these autoantibodies bind Gd-IgA1, they form immune complexes which are then deposited in the kidneys leading to renal injury (Kokubo *et al*, 1997; Tomana *et al*, 1999; Novak *et al*, 2008; H. Suzuki *et al*, 2009; Placzek *et al*, 2018).

1.2.6.6.4 – IgA receptors and renal mesangium deposition

The deposition of IgA and IgA-containing immune complexes in the renal mesangium is the hallmark of IgAN, however the mechanism of deposition is at present not well understood. Renal clearance of IgA normally occurs through receptor mediated endocytosis and catabolism, this process being able to clear IgA up to a point. Human mesangial cells have been well documented to express the transferrin receptor (TfR, CD71), which itself can be a receptor for IgA1 but not IgA2. TfR has been found to be overexpressed in mesangial cells and glomeruli from IgAN patients and also to preferentially bind Gd-IgA1 (Moura *et al*, 2001; Moura *et al*, 2004). It has been shown that binding of IgA to TfR on mesangial cells results in cellular proliferation and release of the cytokines IL-6 and TGF- β ; two factors that can lead to renal damage seen in glomerulonephritides such as IgAN. Other receptors for IgA on mesangial cells have recently been identified, such as the integrins α 1 β 1 and α 2 β 1 (Kaneko *et al*, 2012), and β 1,4-galactosyltransferase 1 (Molyneux *et al*, 2017).

Evidence of a positive feedback loop in the deposition of IgA in the renal mesangium has been provided by studies identifying functional cooperation of TfR on human mesangial cells and IgA. These showed that deposition of IgA in the renal mesangium caused increased expression of TfR which in turn allowed more binding of IgA and thus more IgA deposition (Moura *et al*, 2005; Tamouza *et al*, 2007).

It has been found that healthy subjects can have deposition of IgA in the renal mesangium, without any clinical manifestation of IgAN (Varis *et al*, 1993; K. Suzuki *et al*, 2003). Interestingly, a kidney containing IgA deposits that is transplanted into a patient with a renal disease other than IgAN has the capacity to clear the deposited IgA (Sanfilippo, Croker and Bollinger, 1982; Sofue *et al*, 2013). Deposits of IgA have also been shown to recur in up to 50% of IgAN patients that have been transplanted with a kidney showing no IgA deposition (Berger *et al*, 1975; Odum *et al*, 1994; Ponticelli and Glasscock, 2010), and recurrence in transplanted tissue can potentially be predicted by factors including Gd-IgA1 and Gd-IgA1-specific autoantibody levels in serum (Berthelot *et al*, 2015).

Several studies have also shown that both IgA and IgA containing immune complexes isolated from IgAN patients has a stronger stimulatory effect on mesangial cells in culture than that isolated from healthy subjects (Novak *et al*, 2002; Novak *et al*, 2003; Novak *et al*, 2005; Novak *et al*, 2011). This together provides strong evidence that defects in IgA itself are fundamental to the pathogenesis of IgAN. However, in conjunction with this it has also been found that mesangial cells from IgAN patients are more susceptible to Gd-IgA1 than mesangial cells from healthy subjects, strengthening the idea of the multi-factorial nature of the disease (Ebefors *et al*, 2016).

1.3 – Hypothesis and aims of this work

The hypothesis for this project is that genetic variation can modulate O-glycosylation of the IgA1 hinge region, the mechanism behind which can be altered during B cell development and can also be regulated by the action of cytokines and chemokines. Individually or together, these factors have the potential to be a major influence on the severity and risk of progression of IgAN.

The specific aims of this thesis are as follows:

1. To determine and compare the levels of circulating Gd-IgA1 across different disease states and ethnicities and assess any genetic components therein.
2. To investigate the genetic control of IgA1 O-glycosylation in circulating B cells at distinct development stages.
3. To study the effect of cytokines and chemokines on IgA1 O-glycosylation in IgA1 producing B cells.

Chapter 2 – Methods and materials

2.1 – Ethical approval

Ethical approval for blood collection from healthy subjects with no history of renal or systemic disease in the University of Leicester was granted by the University of Leicester Department of Infection, Immunity and Inflammation Ethics Committee (see Appendix I).

2.2 – Blood sampling

Blood samples were collected from volunteers with informed consent by a trained phlebotomist. Blood was collected either into EDTA monovettes (Sarstedt 01.1605), empty 15ml tubes or 15ml tubes with 10iU sterile heparin per ml of blood. Samples previously collected as part of GWASs were collected in accordance with the Declaration of Helsinki and approved by a UK multicentre research ethics committee (MREC) and local research ethics committees.

2.3 – Genotyping

Genotyping of single nucleotide polymorphisms (SNPs) was performed using TaqMan SNP Genotyping Assays (Applied Biosystems 4351379) according to the manufacturer's instructions.

2.4 – Serum generation

After incubating at room temperature for 45 minutes, whole blood was centrifuged at 1000g for 10 minutes at room temperature. The serum was removed using a Pastette and stored at -20°C in aliquots of 500µl.

2.5 – Enzyme-linked immunosorbent assay (ELISA)

2.5.1 – *Helix aspersa* (HA) lectin binding ELISA

Helix Aspersa agglutinin is a lectin which recognises terminal GalNAc which is O-linked to a polypeptide. Serum levels of Gd-IgA1 were measured using an in-house HA lectin binding ELISA-based method. Nunc Maxisorp polystyrene 96 well immunoplates were coated with mouse anti-human IgA α heavy chains (Dako A0260), diluted 1:1000 in 0.05M carbonate/bicarbonate coating buffer and incubated overnight at 4°C. The plates were washed four times

with 300µl wash buffer per well (PBS/0.3M NaCl/0.1% TWEEN20) using an automated plate washer, then excess protein-binding sites were blocked with 100µl 2% bovine serum albumin (BSA) in PBS per well for 1 hour at room temperature. The plates were washed as above, samples and standards loaded in 50µl duplicates onto the plate and incubated at 4°C overnight. Serum samples were diluted 1:100 to ensure equal well saturation with IgA1 between samples (as confirmed by IgA1 ELISA, see section 2.5.3) in PBS before loading. Three standard serum samples of known high, medium and low HA lectin binding were included for plate to plate normalisation purposes. For culture supernatants where not enough IgA was present in the samples for well saturation, samples were loaded onto the plate undiluted; along with a standard curve constructed from a serial dilution of 50ng/ml degalactosylated and desialylated IgA1 (Immuno-Biological Laboratories). The plates were washed again and desialylation of the captured IgA was performed by treating the plate with 5 units per well neuraminidase enzyme (New England Biolabs P0720) overnight at 37°C in sodium acetate buffer with 1% PenStrep (penicillin-streptomycin solution, 100U/mL penicillin and 100µg/mL streptomycin). Replicate plates without neuraminidase treatment were carried out where necessary for comparison of desialylated and native measures of Gd-IgA1. The plates were washed again and sequential 90 minute incubations at room temperature of first biotinylated HA lectin (Sigma L8764), diluted 1:1000, then HRP-conjugated streptavidin (R&D DY998), diluted at 1:500, were performed; washing the plates between and after. The HRP was quantitated using 50µl OPD substrate (see Appendix VII) stopping the reaction once sufficient colour was evident with 75µl 1N sulphuric acid and the plate was read at 492nm. Results for serum samples were expressed Gd-IgA1 levels (normalised mean OD 492nm) relative to each other with equal IgA1 well saturation. Results for culture supernatants were first interpolated from the standard curve then divided by IgA concentration (µg/ml) (see section 2.5.2) and presented as Gd-IgA1/µg IgA. Intra- and inter-assay variations were less than 10%.

Due to a shortage of HA lectin for use in the ELISA method of Gd-IgA1 measurement, there has been a shift to another lectin from *Vicia villosa* (VV). This lectin directly replaces HA in the ELISA and has been used as such in

studies of IgAN, as it binds the same terminal O-linked GalNAc epitope (Tollefsen and Kornfeld, 1983). The Gd-IgA1 measurement made using VV lectin was found to correlate extremely strongly with the same measurement made with HA lectin in serum (Supplementary figure S2.1)

2.5.2 – IgA ELISA

Immunoplates were coated, washed and blocked as described in the HA lectin binding ELISA described previously. Serum samples were diluted 1:20,000 and culture supernatants were undiluted. Samples were loaded along with a standard curve constructed by serial dilution of an international reference standard (NIBSC 67/086, containing IgG, IgA and IgM with previously determined IgA content) starting at 1µg/ml IgA and incubated at 4°C overnight. Detection was performed with HRP-conjugated mouse anti-human IgA α heavy chains (Dako P0216) diluted 1:2000 followed by incubation at room temperature for 90 minutes. After a final wash, the plates were developed as described in the HA lectin binding ELISA method. IgA concentrations were interpolated from the standard curve and results presented as IgA concentration in µg/ml.

2.5.3 – IgA1 ELISA

Standard immunoplates were coated, washed and blocked as described in the HA lectin binding ELISA above. Serum samples were diluted 1:20,000 and loaded alongside a standard curve constructed by serial dilution of an international reference standard (NIBSC 67/086, containing IgG, IgA and IgM with previously determined IgA1 content) starting at 0.83µg/ml IgA1 and incubated at 4°C overnight. For confirmation of equal IgA1 well saturation in conjunction with the HA lectin binding ELISA (2.5.1), the same 1:100 diluted serum was applied and no standard curve was necessary. The plates were washed again, after which sheep anti-human IgA1 (Binding Site RK015) was applied, diluted 1:2000. The plates were then incubated at room temperature for 90 minutes before a further wash. Detection was performed with peroxidase-conjugated donkey anti-sheep/goat immunoglobulins (Binding Site AP360) diluted 1:2000 and incubated at room temperature for 90 minutes. After a final wash, the plates were developed and read in the same way as the HA lectin binding ELISA. IgA1 concentrations were interpolated from the standard curve

and results presented as IgA1 concentration in $\mu\text{g/ml}$. For confirmation of equal IgA1 well saturation in conjunction with the HA lectin binding ELISA, the OD readings were read and observed to be consistent in each well.

2.5.4 – C1GalT1 ELISA

To detect low levels of C1GalT1, an ELISA method was developed. Several experiments were performed with serially diluted purified recombinant C1GalT1 (Prospec ENZ-721) to optimise the dilution and combination of monoclonal and polyclonal C1GalT1 antibodies for capture and detection of C1GalT1 in cell lysates, and the following protocol was devised. Monoclonal mouse anti-human C1GalT1 (Santa Cruz sc-100745) was diluted 1:1000 in coating buffer and applied 100 μl per well on standard immunoplates, after which the plates were stored overnight at 4°C. After the plates were washed and blocked with 2% BSA, 50 μl of sample was added to the plate in duplicate; along with a standard curve constructed from serially diluted purified C1GalT1, the top standard being 5 $\mu\text{g/ml}$. The plates were incubated at 4°C overnight and washed again before sequential 90 minute incubations of 50 μl per well 1:1000 polyclonal rabbit anti-human C1GalT1 (Santa Cruz sc-98433) and 50 μl per well 1:1000 HRP-conjugated anti-rabbit immunoglobulins (Dako P0448) for detection. After a final wash, the plates were developed, read and the results processed in the same way as the above ELISA methods, with the results presented as C1GalT1 concentration in ng/ml.

2.6 – Isolation of B cell-enriched peripheral blood mononuclear cells

2.6.1 – RosetteSep B cell enrichment

RosetteSep Human B Cell Enrichment Cocktail (Stemcell Technologies 15024) is a tetrameric antibody mix that binds non-B cells together, allowing pelleting of non-B cells upon centrifugation. To 40ml heparinised blood collected as in section 2.2, 200 μl of the RosetteSep antibody mix were added, the blood divided into 10ml aliquots and gently mixed by rotation for 20 minutes.

2.6.2 – Density gradient centrifugation

Ten millilitres of rosetted blood were diluted 1:2 with HBSS (Gibco 14170138) before carefully layering onto 15ml Histopaque-1077 (Sigma 10771)

pre-equilibrated to room temperature in sterile 50ml centrifuge tubes. The tubes were centrifuged at 1200g for 20 minutes at room temperature, without the centrifuge brakes. The interphase layer formed between the Histopaque and plasma, containing the B cell-enriched PBMCs, was aspirated with a pastette and transferred to a new 50ml centrifuge tube. The tubes were topped up to 20ml with sterile PBS and a 10 μ l sample assessed for cell numbers using a haemocytometer, before being centrifuged at 1000g for 5 minutes at room temperature. Cells were then resuspended in 20ml HBSS with 2% foetal calf serum for overnight storage at room temperature prior to staining. A sample of 1ml of the cell suspension was removed to a 2ml tube, pelleted, lysed in 1ml TRIzol (Thermo 15596018) and stored at -20°C for later extraction of whole B cell RNA and protein.

2.7 – Fluorescence activated cell sorting (FACS)

2.7.1 – Cell surface staining

All parts of the staining protocol were performed using sterile plasticware. The B cell enriched PBMCs were washed by centrifuging at 1000g, removing the supernatant and resuspending with 20ml sterile PBS, then centrifugation was repeated, the final supernatant removed, and cells resuspended in a set volume of sterile PBS depending on the number of FACS controls needed (200 μ l per control plus 3ml, see below). The cell suspension was split into the required number of 200 μ l aliquots for FACS controls and three 1ml aliquots for complete staining, in separate 2ml Eppendorf tubes. Pre-optimised volumes of fluorescently-conjugated antibodies (see Supplementary table S2.1 for working details of antibodies and working concentrations) and 0.5 μ l cell viability stain (SYTOX Red, Thermo S34859) were added to each relevant cell suspension and incubated on ice in the dark for 1 hour, with gentle mixing at regular intervals. Cells were washed twice and resuspended in 500 μ l sterile PBS before being transferred through a 50 μ m filter into 5ml polypropylene FACS tubes and stored on ice in the dark for transport to the flow cytometer for sorting.

2.7.2 – Intracellular staining

For intracellular staining, 40 μ l Protein Transport Inhibitor Cocktail (Brefeldin A and Monensin, eBiosciences 00-4980-93) was added to the cells in

overnight storage media (see section 2.6.2) to block extracellular transport of proteins. After the overnight storage, cells were surface stained as above. Cells were then fixed and permeabilised using BD Cytofix/Cytoperm Kit (BD 554714). First, cells were resuspended in 200µl Fixation/Permeabilisation solution and incubated on ice for 20 minutes. Cells were then washed twice in 200µl Perm/Wash buffer and resuspended in 200µl Perm/Wash buffer. Fluorescently-conjugated antibodies were added in the same optimised volume as for surface staining and incubated on ice for 30 minutes in the dark. Cells were washed twice in Perm/Wash buffer then resuspended, transferred to FACS tubes and stored on ice in the dark for transport to the flow cytometer for sorting.

2.7.3 – FACS

All cell sorting was performed using a 5-laser BD FACS Aria II. Operation of the machine and analysis of the measurements was performed using FACSDiva software Version 6.1.3; overseen by the FACS operator. Controls for FACS included cells stained with a single marker and fluorescence minus one staining for accurate compensation (Maecker and Trotter, 2006). Dot-plot diagrams were used for analysis and to identify regions of positive and negative fluorescence, the results of which were then used to assign cell collection gates. Sorted cells were collected into 5ml polypropylene FACS tubes containing 500µl sterile PBS and kept on ice before RNA and protein isolation.

2.8 – RNA and protein isolation

RNA, DNA and protein were isolated using several methods, all according to the manufacturer's instructions. Firstly, using TRIzol (Invitrogen 15596018) for phase separation of RNA, DNA and protein. Secondly, using Qiagen's AllPrep RNA/Protein column-based kit for isolation of RNA and native-form protein (Qiagen 80404). Thirdly, using Direct-zol RNA microprep columns for direct isolation of RNA from samples lysed in TRIzol (Zymo R2061), followed by acetone precipitation of the initial flow-through for protein extraction. All RNA was stored at -80°C, protein from TRIzol or acetone precipitation at -20°C and protein from AllPrep RNA/Protein kit at -80°C.

2.9 – Gene expression analysis

2.9.1 – Quantitative real-time PCR (qPCR)

2.9.1.1 – Reverse transcription of RNA

cDNA synthesis was carried out using Promega's ImProm-II Reverse Transcription System (Promega A3800). RNA was quantified using a NanoDrop spectrophotometer and up to 1µg in a total volume of 8.5µl with nuclease-free water was incubated with 0.5µl each of random and Oligo dT primers at 70°C then chilled at 4°C, both for 5 minutes before briefly centrifuging. A 10.5µl reaction mixture was prepared containing 4µl 25mM MgCl₂, 4µl 5x reverse transcriptase buffer, 1µl deoxyribose nucleoside triphosphate (dNTP) mix, 0.5µl RNasin ribonuclease inhibitor and 1µl ImProm-II Reverse Transcriptase. The reaction mixture was added to the RNA-primer mixture and placed in a thermocycler with the following cycles of incubation: 25°C for 5 minutes, then 42°C for 1 hour, then 70°C for 15 minutes and finally held at 4°C for at least 5 minutes. The synthesised cDNA was stored at -20°C.

2.9.1.2 – Preamplification of cDNA

Preamplification of cDNA was performed using TaqMan PreAmp Master Mix (Applied Biosystems) according to the manufacturer's instructions. Briefly, pooled TaqMan gene expression assays were diluted to a 0.2x concentration in 1x TE buffer. Preamplification reactions were set up with 12.5µl PreAmp Mastermix, 6.25µl pooled assay mix and 6.25µl cDNA solution. Reactions were then run in a thermocycler for an initial 10 minute 95°C incubation followed by 14 cycles of 95°C for 15 seconds and 60°C for 4 minutes. Reactions were then chilled on ice for 5 minutes before being diluted 1:20 with 1x TE buffer and stored at -20°C.

2.9.1.3 – qPCR

Gene expression analysis was carried out on an Applied Biosystems QuantStudio 6 Flex instrument using TaqMan gene expression assays (Applied Biosystems 4331182) on 96 well PCR plates. Each assay was carried out in duplicate; and for each reaction, 10µl TaqMan gene expression Master Mix was combined with 1µl TaqMan gene expression assay mix and either 7µl nuclease-

free water and 2µl cDNA (see section 2.9.1.1) or 9µl preamplified cDNA (see section 2.9.1.2). The reactions were carried out with the following qPCR cycling conditions: 1 cycle of 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Human proteasome subunit 6 (PSMB6) (García-Vallejo *et al*, 2004) or the IgA1 heavy chain (IGHA1) were used as the endogenous reference genes (ERG) in the analysis, and data analysed by comparing normalised ΔCt values or by the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001).

2.9.2 – Allele-specific qPCR

Allele-specific qPCR was performed on cDNA using TaqMan SNP Genotyping Assays (Applied Biosystems 4351379) under normal qPCR gene expression conditions (2.9.1.3). These assays contain two allele-specific TaqMan probes with distinct fluorescent dyes and a PCR primer pair to detect specific SNP targets to allow SNP genotyping. Under gene expression conditions, these assays can be utilised to quantify the expression of each allele simultaneously. Results were analysed by looking for differences in the expression of each allele by comparing normalised ΔCt values.

2.9.3 – NanoString nCounter multiplex gene expression

NanoString nCounter XT Gene Expression assays (see Figure 2.1 for a schematic diagram of the principle behind the technology) were performed according to the manufacturer's instructions. Briefly, 70µl of hybridisation buffer was added to the reporter codeset once thawed to form the Master Mix. RNA samples were diluted to 20ng/µl in RNase-free water, 5µl aliquots of which were then combined with 8µl of Master Mix and incubated at 65°C for at least 16 hours and no more than 24 hours to hybridise the RNA to the reporter probes. Cartridges were then equilibrated to room temperature, sample volume brought to 30µl with RNase-free water and loaded into the cartridge. The cartridges were sealed and run in the NanoString nCounter Sprint Profiler instrument. Data were analysed using NanoString's nSolver software, normalising the data to the internal housekeeping genes, positive and negative controls before pooling replicate data and assessing the fold change to control samples.

2.10 – Protein analysis

2.10.1 – Protein quantification

Total protein was quantified either by Bio-Rad's DC protein assay (Bio-Rad 5000111) according to the manufacturer's instructions or using a NanoDrop spectrophotometer.

2.10.2 – Western blotting

Protein samples isolated by any method from any experiment were diluted 1:2 in standard reducing or non-reducing buffers (see Appendix IV). Under reducing conditions, samples were boiled for 5 minutes to denature tertiary structures and centrifuged briefly to remove condensation before loading onto SDS-polyacrylamide stacking and resolving gels (see Appendix IV). Under non-reducing conditions, samples were loaded directly onto the gels without boiling. The gels were run at 200 volts for ~20 minutes. Transfer of the proteins from the gels to nitrocellulose membranes was performed at 100 volts for 1 hour with a cooling block. The membranes were then blocked for 1 hour with 5% BSA in TTBS, then were washed 3 times for 5 minutes with TTBS. Immunoblotting took place overnight at 4°C or 2 hours at room temperature. If the primary antibody was unconjugated, secondary detection antibodies were incubated for 2 hours at room temperature after 3 washes with TTBS for 5 minutes (see Supplementary table S2.2 for specific primary and secondary antibody concentrations). Three 10 minute washes with TTBS were then performed, after which the membrane was developed with enhanced chemiluminescence (ECL) reagents (Thermo) for 5 minutes. The membrane was then imaged using BioRad's ChemiDoc Touch Imaging System and the image was processed and analysed using BioRad's Image Lab software.

2.11 – DAKIKI cell stimulation

The DAKIKI cell line (ATCC TIB206) is a surface IgA+ lymphoblast cell line immortalised by transformation with EBV. These non-adherent cells were grown in suspension in RPMI 1640 with 10% FBS and 1% PenStrep (100U/mL penicillin and 100µg/mL streptomycin) at 37°C with 5% CO₂ and were maintained between 1x10⁵ and 1x10⁶ cells/ml. Stimulation experiments took place at

passages 6 to 12; the cells were seeded at 2×10^5 cells/ml media per well on 48 or 24 well culture plates for 48 hours after the addition of cytokines. Cells and supernatants were removed together from the plates, centrifuged at 250g for five minutes and culture supernatants removed and stored at -20°C . Cells were washed twice with sterile PBS and lysed with TRIzol, and RNA and protein isolated using Direct-zol columns and acetone precipitation respectively (section 2.8).

2.12 – Statistical analysis

Results are expressed as the mean \pm the standard deviation of the mean (SD). Statistical analysis was performed using GraphPad Prism 7. Distribution of data was determined by the D'Agostino-Pearson omnibus normality test. Unpaired t-tests were performed for individual comparisons. One-way ANOVA with post-hoc Tukey tests were used for multiple group analysis, reporting multiplicity adjusted p values. Correlations between relevant data were made using the Pearson or Spearman methods depending on the distribution of the data. Results were considered statistically significant with a p value less than 0.05.

Chapter 3 – Systematic analysis of aberrant IgA1 O-glycosylation in IgAN

3.1 – Introduction and aims

Aberrant IgA1 O-glycosylation, specifically hypogalactosylation at the IgA1 hinge region, is a key finding in IgAN (Coppo and Amore, 2004; Moldoveanu *et al*, 2007; Mestecky *et al*, 2008; Barratt, Smith and Feehally, 2012; Novak *et al*, 2012). Thought to be the first of four pathogenic hits for the disease, it is referred to as galactose-deficient IgA1 (Gd-IgA1). This Gd-IgA1 forms immune complexes with IgA or IgG autoantibodies which can then be deposited in the renal mesangium, leading to renal injury. The presence of higher levels of Gd-IgA1 in IgAN is thought to be due to under-expression or low activity of the galactose-adding enzyme C1GalT1 or its chaperone protein Cosmc decreasing the ability to add galactose, or due to high expression or activity of sialyltransferases preventing the addition of galactose by over-sialylation of terminal GalNAc (Raska *et al*, 2008; Smith *et al*, 2008; H. Suzuki *et al*, 2014).

The mechanism behind the synthesis of aberrant O-glycosylation at the IgA1 hinge region in IgAN is still largely not well understood, along with any genetic control that may be involved. Studies have identified higher levels of Gd-IgA1 in serum from IgAN patients in a number of ethnicities, but no direct comparison between ethnicities has yet been made. The incidence of IgAN in Caucasian males is twice that of Caucasian females, a discrepancy that is not seen in populations of Asian ancestry (Barratt *et al*, 2012; Wyatt and Julian, 2013). The ethnic differences seen in the prevalence and incidence between genders in IgAN suggests that genetic factors are very likely to have a contributory effect on the development of IgAN. This genetic influence may involve variability in the levels of Gd-IgA1.

Serum Gd-IgA1 levels have also been found to be stable over long periods of time. As part of an MSc project in our lab in 2011 (Cheshire, 2011), serum samples from healthy subjects and IgAN patients attending the University Hospitals of Leicester renal clinics collected at multiple times over 20 years were analysed for serum Gd-IgA1 levels. In serum from healthy subjects, the mean coefficient of variation (CV) of Gd-IgA1 levels between earliest and latest

collected was 11.8 across a mean time of 9.5 years. In IgAN patients, the CV was 17.6 across a mean of 6.5 years. There was also a significant correlation between Gd-IgA1 levels at the earliest and latest time points in both groups (Supplementary figure S3.1). This indicates that Gd-IgA1 levels are stable in serum over time, and therefore are potentially regulated by genetic factors.

The aim of the work in this chapter was to determine and compare the levels of circulating Gd-IgA1 across different disease states and ethnicities and assess any genetic components therein. To this end, this chapter was split into three smaller aims:

1. To find out if genetic factors have an impact on levels of circulating Gd-IgA1 in IgAN. This was performed by comparing serum Gd-IgA1 levels between Caucasian IgAN, a Caucasian disease control (membranous nephropathy, MN) and Chinese IgAN patients. This data was also used for linear regression with genome-wide association study (GWAS) data to identify single nucleotide polymorphisms (SNPs) that may be associated with levels of circulating Gd-IgA1
2. To assess any relationship between Gd-IgA1 levels in circulation and the progression of renal injury in IgAN in Caucasian and Chinese IgAN, and whether this relationship was specific to IgAN by comparing to Caucasian membranous nephropathy (MN) patients.
3. To investigate any differences in levels of circulating Gd-IgA1 between genders in the various groups studied, and subsequently whether this causes any differences in the progression of renal injury in IgAN.

3.2 – Methods

The UK Glomerulonephritis DNA Bank (UKGDB) IgAN cohort was established in 2000. Participants of European ancestry were recruited through four UK centres (Glasgow, Leicester, London, and Oxford), selecting patients who were younger than 50 years of age at the time of diagnosis and older than 18 years of age at the time of recruitment. Individuals with evidence of liver disease or IgAV were excluded. Diagnosis of IgAN was confirmed in all cases by direct review of renal biopsy histopathology reports and clinical case records. Where available, samples were also collected from the parents of affected

individuals to enable a study of family-based association. The UKGNDB IgAN cohort comprises 791 serum samples; which include 480 patients, 136 complete parent-child trios and 39 parent-child duos. Ten year follow-up data is now available on this cohort, and before any samples were analysed this follow-up data was used to define patients with non-progressive IgAN (<10% change in serum creatinine over a minimum of 10 years follow-up) and progressive IgAN (>100% increase in serum creatinine or ESRD during follow-up). Out of the 480 patients in the cohort; 160 (33.3%) had developed progressive IgAN, 194 (40.4%) were non-progressors, 54 (11.25%) were indeterminate at 10 years' follow-up and 72 (15%) had no follow-up data available. Clinical characteristics of these patients at the time of sample collection are shown in Table 3.1. No differences were seen in clinical characteristics between different ages of the patients.

The UKGDB also contains a cohort of patients with MN, another immune complex-mediated glomerulonephritis that has no IgA involvement. This cohort consists of serum from 353 MN-confirmed patients, to be used as a disease control. Data on the progression of MN in these patients was unavailable. Clinical characteristics of these patients at the time of sample collection are shown in Table 3.1.

The Caucasian healthy subject group were 519 serum samples from unrelated adults from the GRAPHIC (Genetic Regulation of Arterial Pressure of Humans in the Community) cohort from the Leicester Research Archive. In this group, all individuals had normal urinalysis and plasma urea and creatinine at time of recruitment, thus were suitable as healthy controls for this study.

Serum from a cohort of 1000 Chinese IgAN patients was obtained from the Nephrology Division at the First Affiliated Hospital of Sun Yat-Sen University, China; along with serum from a group of 110 healthy Chinese volunteers. Ten year follow-up data was not available for this Chinese IgAN cohort, so a similar definition of progressive and non-progressive IgAN to the Caucasian cohort could not be made. The stage of chronic kidney disease (CKD) of each patient at the time of sample collection was known in the Chinese cohort, and an analysis was performed based on this. Clinical characteristics of these patients at the time of sample collection are shown in Table 3.1.

	Number of subjects	Age	eGFR at serum collection
Caucasian IgAN - UKGNDB	480 (296 male, 140 female)	35.3 ± 11.3	87.7 ± 53.9
Progressive Caucasian IgAN	160 (124 male, 33 female)	36.0 ± 11.2	49.8 ± 24.4
Non-progressive Caucasian IgAN	194 (108 male, 81 female)	32.7 ± 10.3	103.1 ± 31.7
Chinese IgAN - Sun Yat-Sen cohort	1000 (479 male, 499 female)	34.6 ± 11.4	73.4 ± 106.7
Chinese IgAN CKD 1	298 (101 male, 197 female)	29.7 ± 9.4	114.4 ± 21.1
Chinese IgAN CKD 2	190 (97 male, 93 female)	34.9 ± 9.4	75.1 ± 8.5
Chinese IgAN CKD 3	195 (110 male, 85 female)	39.9 ± 11.4	44.1 ± 8.8
Chinese IgAN CKD 4	97 (58 male, 39 female)	38.7 ± 12.1	21.9 ± 4.6
Chinese IgAN CKD 5	107 (67 male, 40 female)	34.7 ± 11.2	8.3 ± 3.0
Caucasian MN - UKGNDB	353 (236 male, 111 female)	53.7 ± 13.6	70.1 ± 35.3

Table 3.1: Clinical characteristics of selected Caucasian and Chinese IgAN patients and Caucasian MN patients: data shown as mean ± standard deviation.

All serum samples were assessed for serum Gd-IgA1 levels using the HA lectin binding method described in 2.5.1 and statistical analysis performed as appropriate. Narrow sense heritability (h^2) is defined as the proportion of trait variance that is due to genetic, and therefore inherited, factors rather than environmental factors. Heritability estimates can range between zero and one; with a value close to zero indicating trait variability is due to environmental factors with very little genetic influence, and a value close to one indicating almost all the trait variability is due to genetic differences. To estimate heritability of serum Gd-IgA1 levels in the Caucasian IgAN cohort, only the results from the 136 parent-child trios were utilised. First, the mean value for each set of both parents was determined (mid-parental value), after which all the data was standardised to the population mean and standard deviation using the formula $z=(x-m)/s$ (z =standardised value, x =raw value, m =mean, s =standard deviation). Next, correlation of patients (y) with the corresponding mid-parental values (x) was performed on the standardised values, and the h^2 value taken as the slope of the linear regression of this correlation.

3.3 – Results 1 – Genetic control of Gd-IgA1 levels in circulation

3.3.1 – Serum Gd-IgA1 levels are a heritable trait in Caucasians

Using the serum samples from the 136 UKGNDB IgAN cohort with both parent samples available, an analysis of the narrow-sense heritability of serum Gd-IgA1 levels was performed (Figure 3.1). This resulted in a h^2 value of 0.387, strongly indicating that genetic factors play a role in determining the serum levels of Gd-IgA1 in Caucasian IgAN. This is consistent with previously published studies (Gharavi *et al*, 2008; Kiryluk, Moldoveanu, John *et al*, 2011; Lomax-Browne *et al*, 2016).

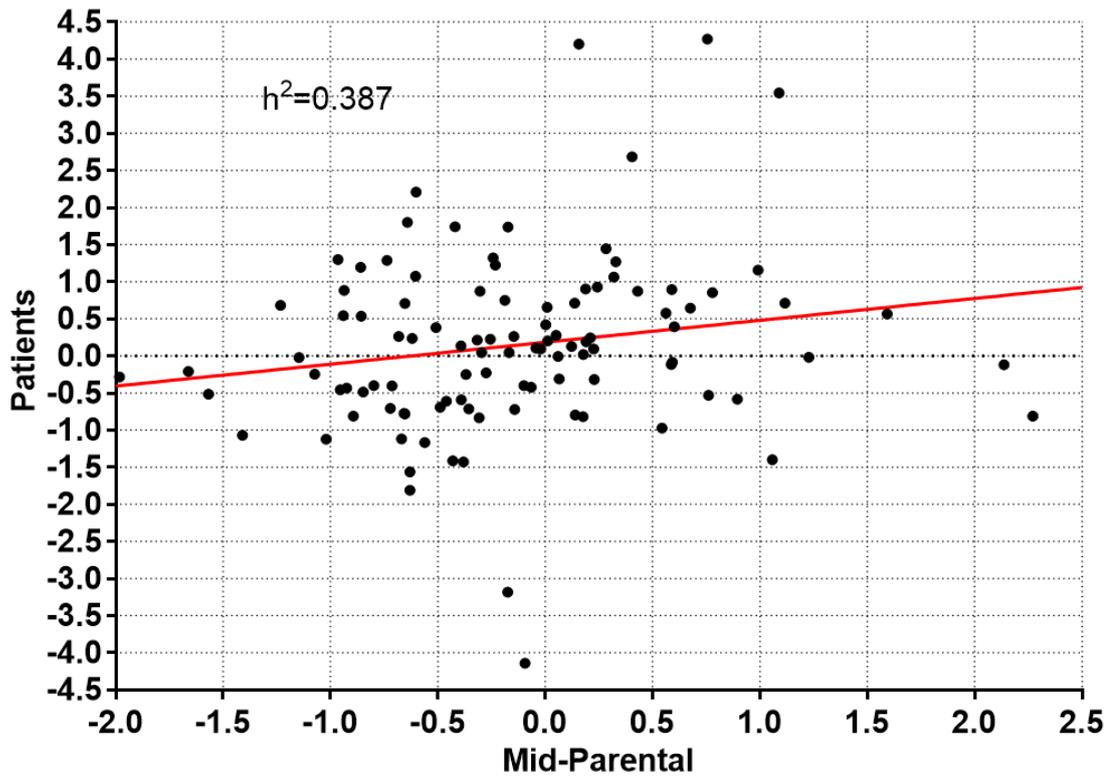


Figure 3.1: Heritability of Gd-IgA1 levels in Caucasian IgAN: Narrow-sense heritability (h^2) of serum Gd-IgA1 levels in 136 parent-child trios in the UKGNDB IgAN cohort estimated at 0.387, indicating that genetic factors play an important role in determining the serum levels of Gd-IgA1 in Caucasian IgAN. Data analysed by plotting standardised Gd-IgA1 data between patients and average parental and calculating the linear regression slope of the correlation.

3.3.2 – Serum Gd-IgA1 levels are elevated in IgAN, but not in MN

Again consistent with the literature, the UKGNDB IgAN cohort displayed significantly higher serum Gd-IgA1 levels than healthy subjects ($p < 0.0001$, Figure 3.2) (Coppo and Amore, 2004; Moldoveanu *et al*, 2007; Mestecky *et al*, 2008; Barratt, Smith and Feehally, 2012; Novak *et al*, 2012). The levels of Gd-IgA1 in serum from the UKGNDB IgAN cohort were also elevated compared to the UKGNDB MN cohort ($p < 0.0001$; Figure 3.2), with the UKGNDB MN cohort showing no difference to Caucasian healthy subjects (MN vs HS data not shown). Serum Gd-IgA1 levels in Chinese IgAN patients were found to be elevated compared to Chinese healthy subjects, similar to that seen in Caucasians ($p = 0.0016$; Figure 3.3). These data reinforce the IgAN-specificity of elevated serum Gd-IgA1 levels across Caucasian and Chinese populations.

3.3.3 – Chinese populations show reduced serum Gd-IgA1 levels compared to Caucasians

Upon direct comparison of serum Gd-IgA1 levels between the Caucasian and Chinese cohorts studied here (Figure 3.4), both Chinese IgAN patients and Chinese healthy subjects showed significantly lower levels of Gd-IgA1 in serum than their Caucasian counterparts ($p < 0.0001$ for both). The Chinese IgAN patients also showed reduced levels of serum Gd-IgA1 compared to Caucasian healthy subjects ($p < 0.0001$), highlighting an ethnic difference in serum Gd-IgA1 levels in general; whilst elevated levels are still present in IgAN compared to healthy subjects regardless of ethnicity. This ethnic difference could potentially be due to environmental or dietary differences in the two populations.

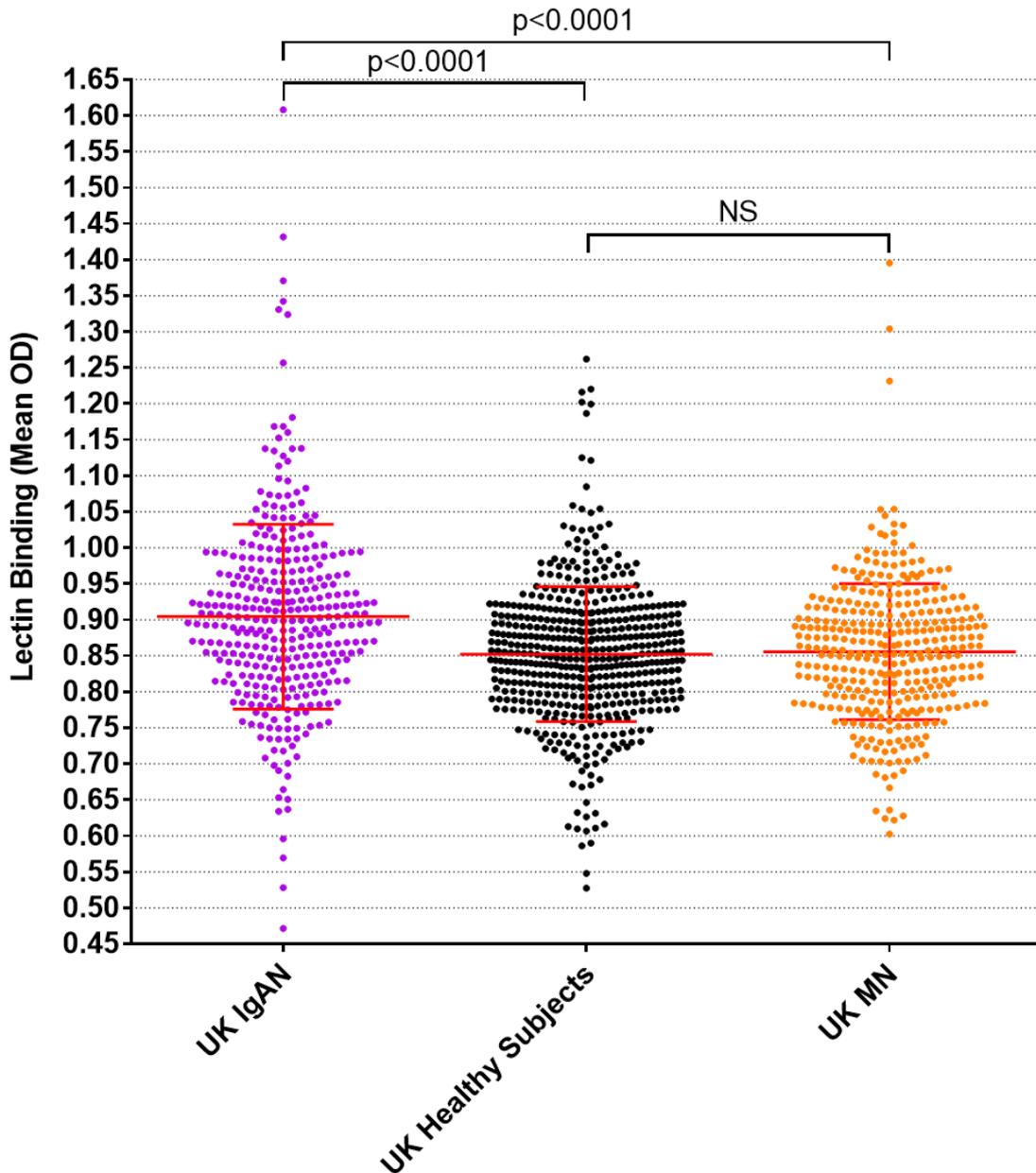


Figure 3.2: Comparison of Gd-IgA1 levels in Caucasian IgAN, healthy subjects and MN: Significantly elevated serum levels of Gd-IgA1 seen in patients from the UKGNDB IgAN cohort compared to Caucasian healthy subjects from the GRAPHIC cohort and compared to Caucasian MN patients from the UKGNDB cohort as measured by HA lectin binding ELISA. Data from 480 IgAN patients, 519 healthy subjects and 353 MN patients. Graph shows mean \pm SD. Data analysed by one-way ANOVA with post-hoc Tukey test.

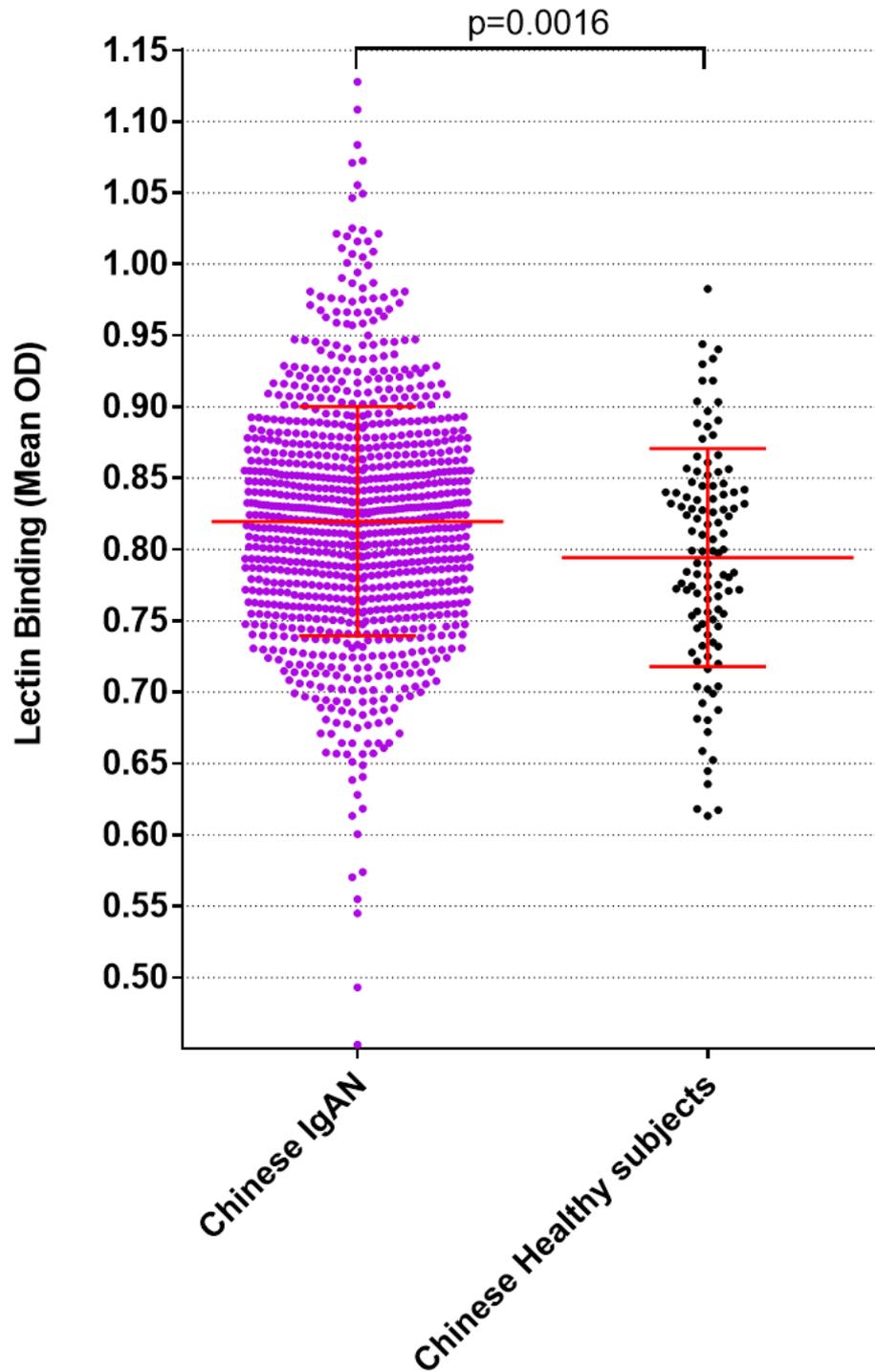


Figure 3.3: Comparison of Gd-IgA1 levels in Chinese IgAN and healthy subjects: Significantly elevated levels of Gd-IgA1 seen in Chinese IgAN compared to Chinese healthy subjects as measured by HA lectin binding ELISA. Data from 1000 IgAN patients and 110 healthy subjects. Graph shows mean \pm SD. Data analysed by unpaired t-test.

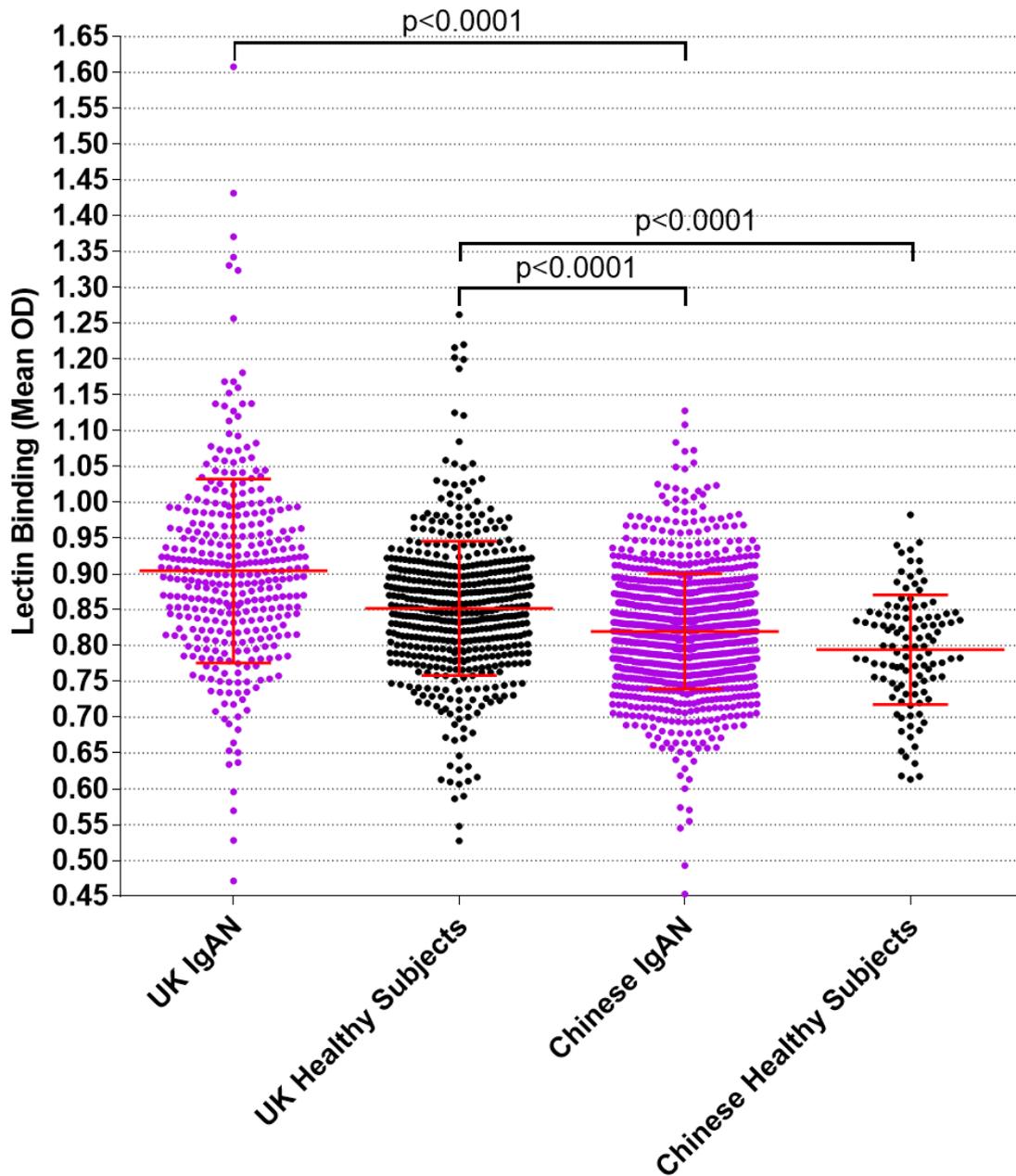


Figure 3.4: Comparison of Caucasian and Chinese Gd-IgA1 levels: Both Chinese IgAN patients and healthy subjects show significantly lower levels of Gd-IgA1 in serum than their Caucasian counterparts as measured by HA lectin binding ELISA. Chinese IgAN patients also show significantly lower levels of Gd-IgA1 than Caucasian healthy subjects. Data from 480 Caucasian IgAN patients, 519 Caucasian healthy subjects, 1000 Chinese IgAN patients and 110 Chinese healthy subjects. Graph shows mean \pm SD. Data analysed by one-way ANOVA with post-hoc Tukey test.

3.3.4 – A haplotype of C1GALT1 shows strong association with higher levels of serum Gd-IgA1

The UKGNDB IgAN cohort, UKGNDB MN cohort, Caucasian healthy subjects, Chinese IgAN patients and Chinese healthy subjects all have genetic data available from previous genome-wide association studies (GWAS) (Tobin *et al*, 2005; Feehally *et al*, 2010; Stanescu *et al*, 2012). Standardised data on serum Gd-IgA1 levels was utilised in genome-wide linear regression analysis to identify genetic factors that could influence serum Gd-IgA1 levels. This analysis was performed by collaborators at University College London.

Using the Caucasian IgAN patient's data as the discovery cohort, a significant association was found between higher Gd-IgA1 levels in serum and a single locus on Chromosome 7 spanning the C1GALT1 gene (Figure 3.5). This association with C1GALT1 was robust to correction for age, gender and renal function, and no other significant associations were seen elsewhere in the genome. Haplotype analysis of this association confirmed it was attributable to a single haplotype of C1GALT1, termed H1, which was found to have a frequency of 0.32 in the UK population (Figure 3.6). This finding was replicated in both the Caucasian MN patient and Caucasian healthy subject cohorts, despite these cohorts averaging significantly lower levels of serum Gd-IgA1 than the UKGNDB IgAN cohort.

In the Chinese IgAN patients and Chinese healthy subjects assessed for serum Gd-IgA1 levels here, the same haplotype of C1GALT1 identified in Caucasians was found to be present but rare in the Chinese population, with a frequency of 0.035 (Figure 3.7). However, whilst the haplotype is ten times less frequent in Chinese individuals than in Caucasians, it still showed a strong association with higher serum Gd-IgA1 levels.

As a confirmation of the association of the H1 haplotype of C1GALT1, serum levels of Gd-IgA1 in the combined Caucasian cohort and the combined Chinese cohort were stratified by the number of copies of the H1 haplotype present in each individual. Figure 3.8 shows that in both Caucasians and Chinese populations, serum Gd-IgA1 levels show a tendency to increase as copies of the H1 haplotype increase.

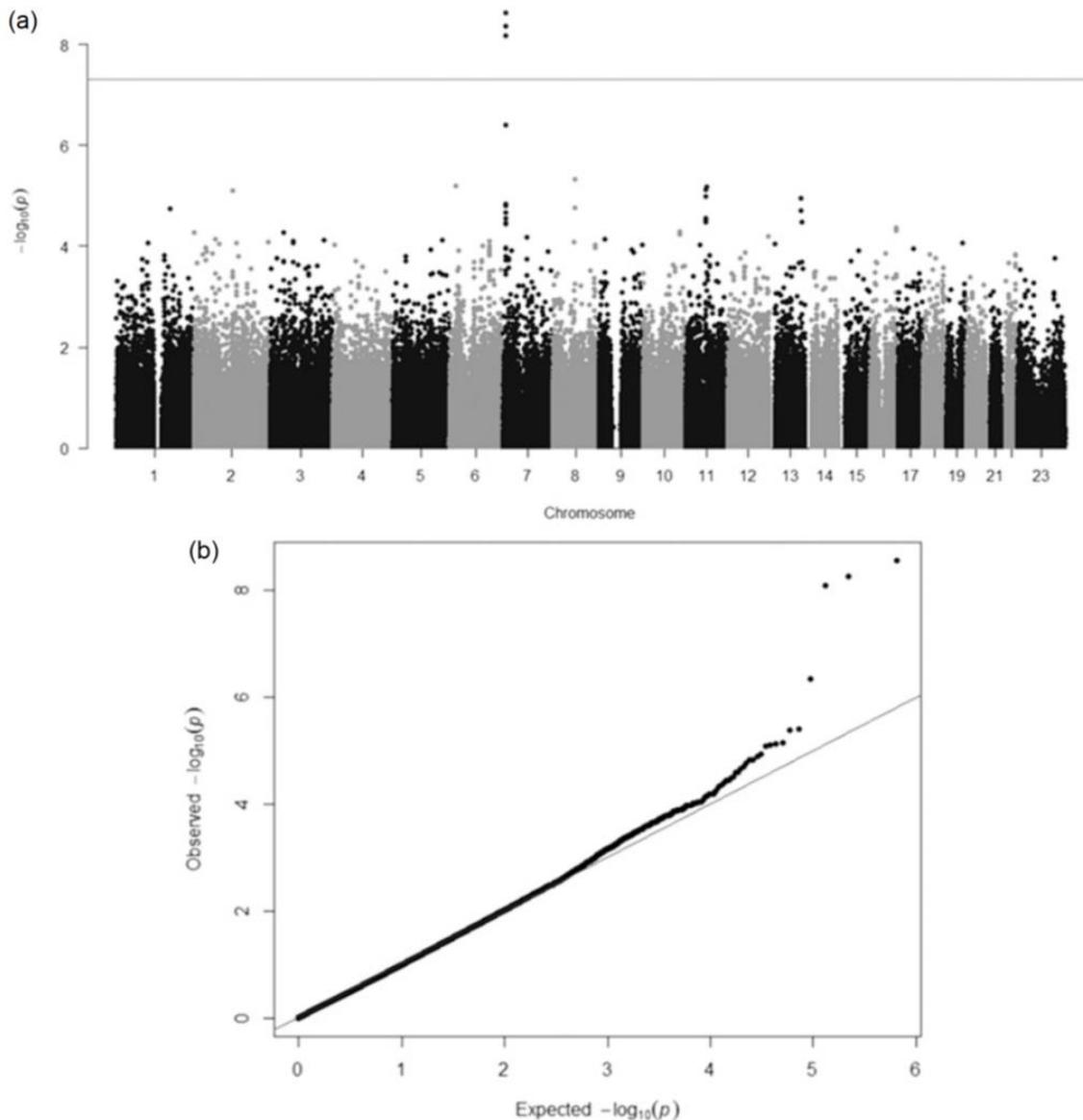


Figure 3.5: GWAS of Gd-IgA1 levels in Caucasian individuals:

Association is shown between Gd-IgA1 levels in serum and a single locus on Chromosome 7 at C1GALT1 ($\beta=0.26$, $p=2.353 \times 10^{29}$). Manhattan plot (a) showing significance of the association with Gd-IgA1 level by plotting the negative logarithm to the base 10 of the p value against the genomic position. Horizontal line indicates conventional genome-wide significance ($p=5 \times 10^{-8}$). Quantile-quantile plot (b) is a plot of the observed $-\log_{10}(p)$ against the $-\log_{10}(p)$ values that would be expected under the null hypothesis of no association. Deviation above the $y=x$ line indicates lower p values than would be expected to occur by chance and implies statistically significant association. The genomic inflation factor was 1.00196. Results courtesy of D. Gale (Gale *et al*, 2017).

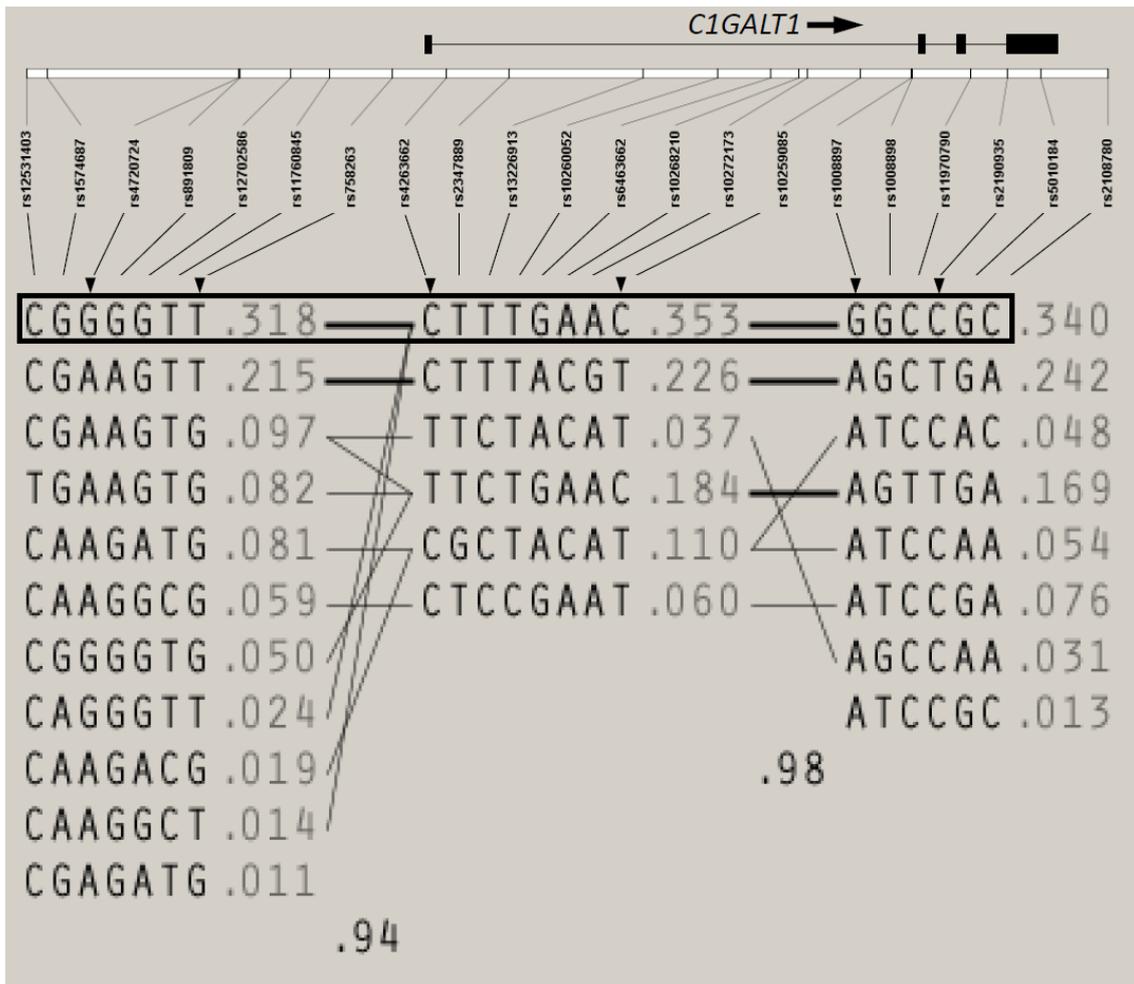


Figure 3.6: Depiction of the H1 haplotype of C1GALT1 in Caucasian individuals: Alleles of neighbouring SNPs along a chromosome tend to be inherited together in haplotype blocks. The blocks occurring across the C1GALT1 locus (position of the gene is shown at the top of the figure) in the Caucasian population are shown. The three blocks that span the gene and its upstream region are almost always inherited together as an extended haplotype. The box indicates the ‘H1’ haplotype occurs at a frequency of >0.3 in the Caucasian population. SNP IDs of the H1 haplotype are indicated vertically at the top. Inverted triangles show the SNPs that tag the H1 haplotype. Results courtesy of D. Gale (Gale *et al*, 2017)

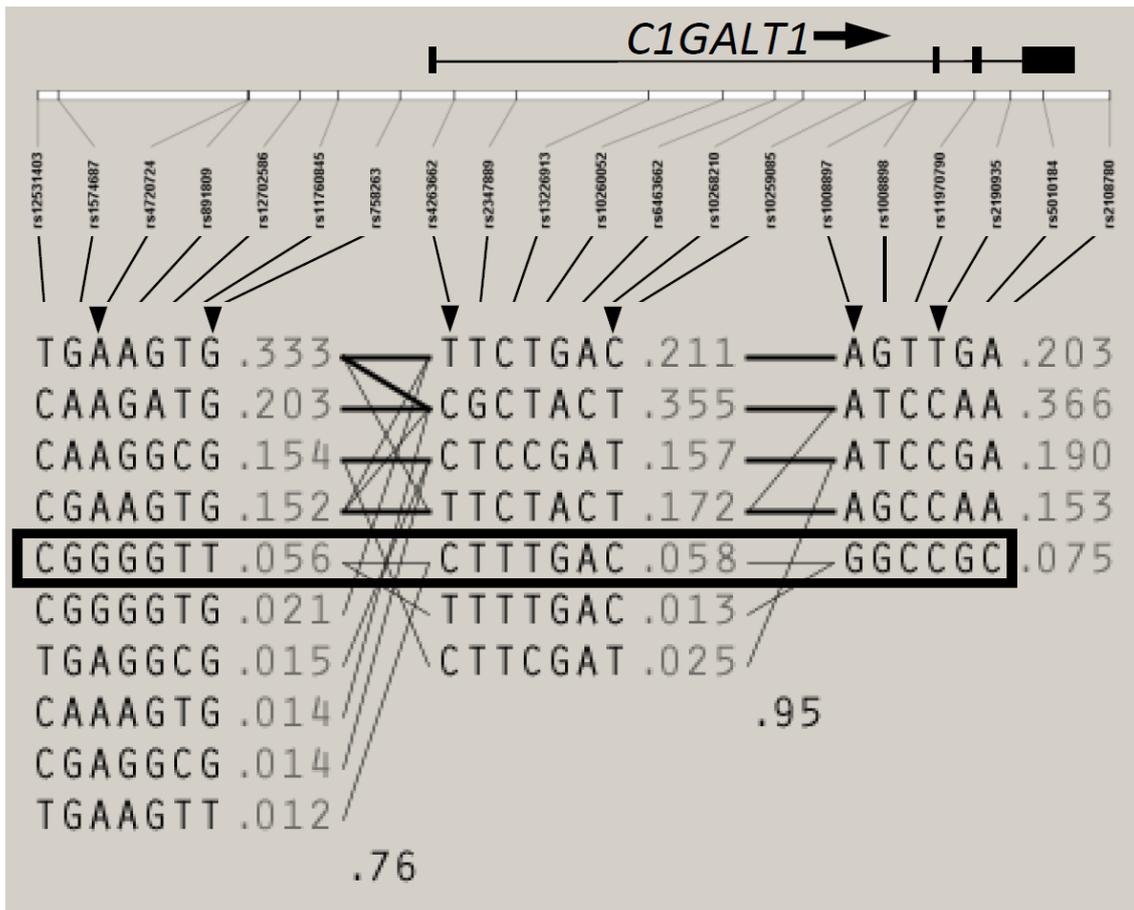


Figure 3.7: Depiction of the H1 haplotype of C1GALT1 in Chinese individuals: Alleles of neighbouring SNPs along a chromosome tend to be inherited together in haplotype blocks. The blocks occurring across the C1GALT1 locus (position of the gene is shown at the top of the figure) in the Chinese population are shown. The three blocks that span the gene and its upstream region are almost always inherited together as an extended haplotype. The H1 haplotype (boxed) is present but at substantially lower frequency than in Caucasians. SNP IDs of the H1 haplotype are indicated vertically at the top. SNPs tagging the H1 haplotype are marked with inverted triangles. Results courtesy of D. Gale (Gale *et al*, 2017).

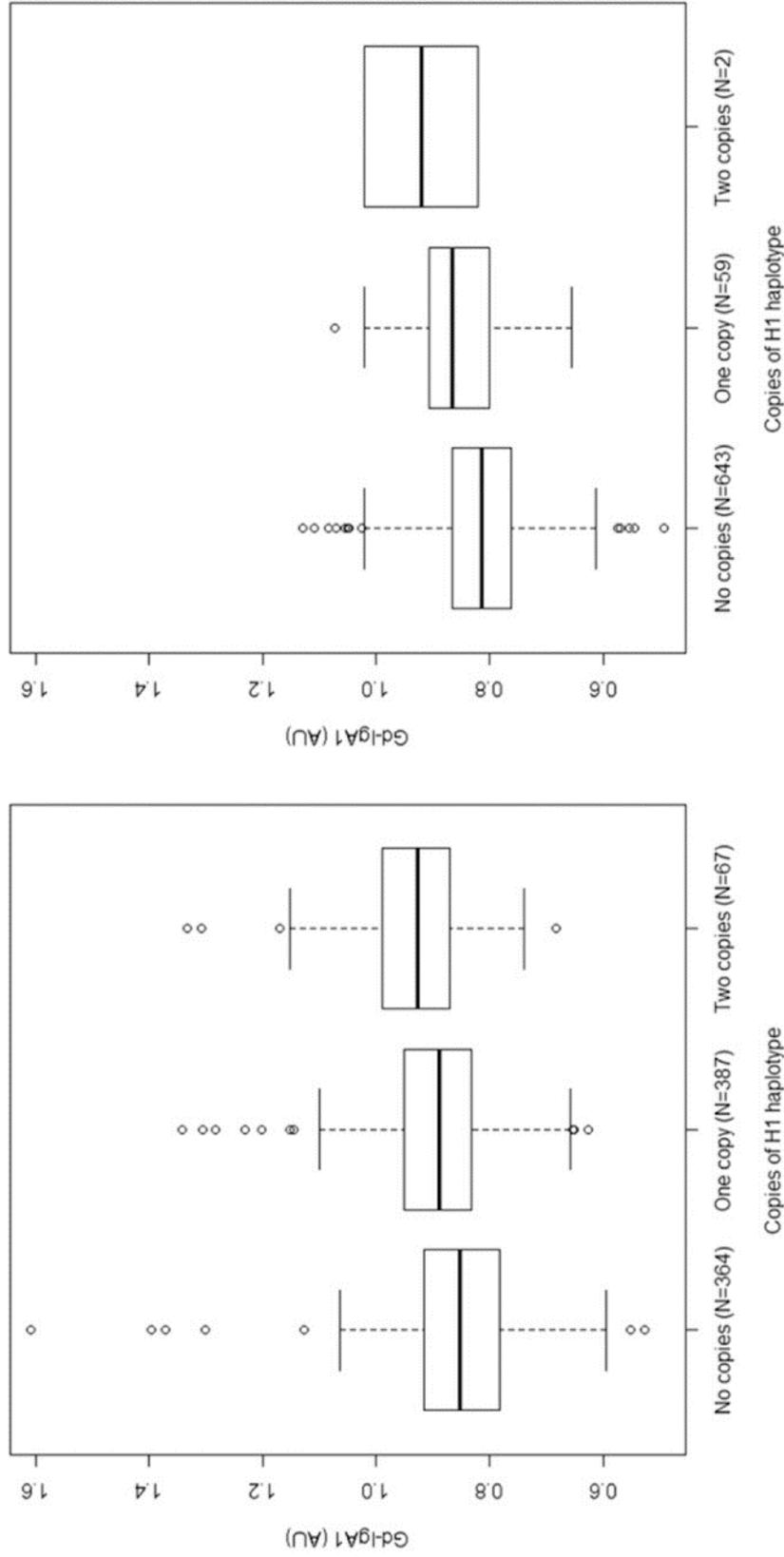


Figure 3.8: Gd-IgA1 levels split by H1 haplotype status: Serum Gd-IgA1 levels increase with copies of the H1 haplotype in both Caucasian (left) and Chinese (right) populations as measured by HA lectin binding ELISA. No statistical significance seen. Figure courtesy of D. Gale (Gale *et al*, 2017).

3.4 – Results 2 – Relationship of Gd-IgA1 levels in circulation with renal function and the progression of IgAN

3.4.1 – Higher serum Gd-IgA1 levels in Caucasian IgAN are associated with worse renal function, but not in MN

To assess any association between serum Gd-IgA1 levels in IgAN with renal function, first the data from the UKGNDB IgAN cohort was correlated with the estimated glomerular filtration rate (eGFR) at the time serum was collected. A significant negative correlation between the levels of serum Gd-IgA1 and eGFR, indicating that those in the UKGNDB IgAN cohort with worse renal function have higher levels of Gd-IgA1 in serum ($r=-0.1361$, $p=0.0262$; Figure 3.9). When the same analysis was made with serum Gd-IgA1 levels and eGFR in the UKGNDB MN cohort, no significant correlation was seen (Figure 3.10). However; as the trend lines are similarly downward, this difference could be due to the lower numbers of MN patients providing less statistical power.

3.4.2 – Caucasian progressive IgAN patients show higher serum Gd-IgA1 levels than non-progressive

As the data was available from ten years' follow-up in the Caucasian IgAN cohort, an analysis of serum Gd-IgA1 levels was performed based on whether the patients were defined as having progressive or non-progressive forms of IgAN (a doubling of serum creatinine or ESRD over ten years for progressive IgAN, and a less than 10% change in serum creatinine for non-progressive IgAN) (Figure 3.11). Caucasian patients with progressive or non-progressive IgAN both were found to have elevated serum Gd-IgA1 levels compared to those from Caucasian healthy subjects ($p<0.0001$ for progressive IgAN, $p=0.0003$ for non-progressive). The patients with progressive IgAN also showed significantly higher levels of serum Gd-IgA1 than non-progressive IgAN patients ($p=0.0002$). This, together with the apparent IgAN-specific correlation with eGFR, reinforces the idea of increased Gd-IgA1 levels in serum being specific and important to the pathogenesis of IgAN.

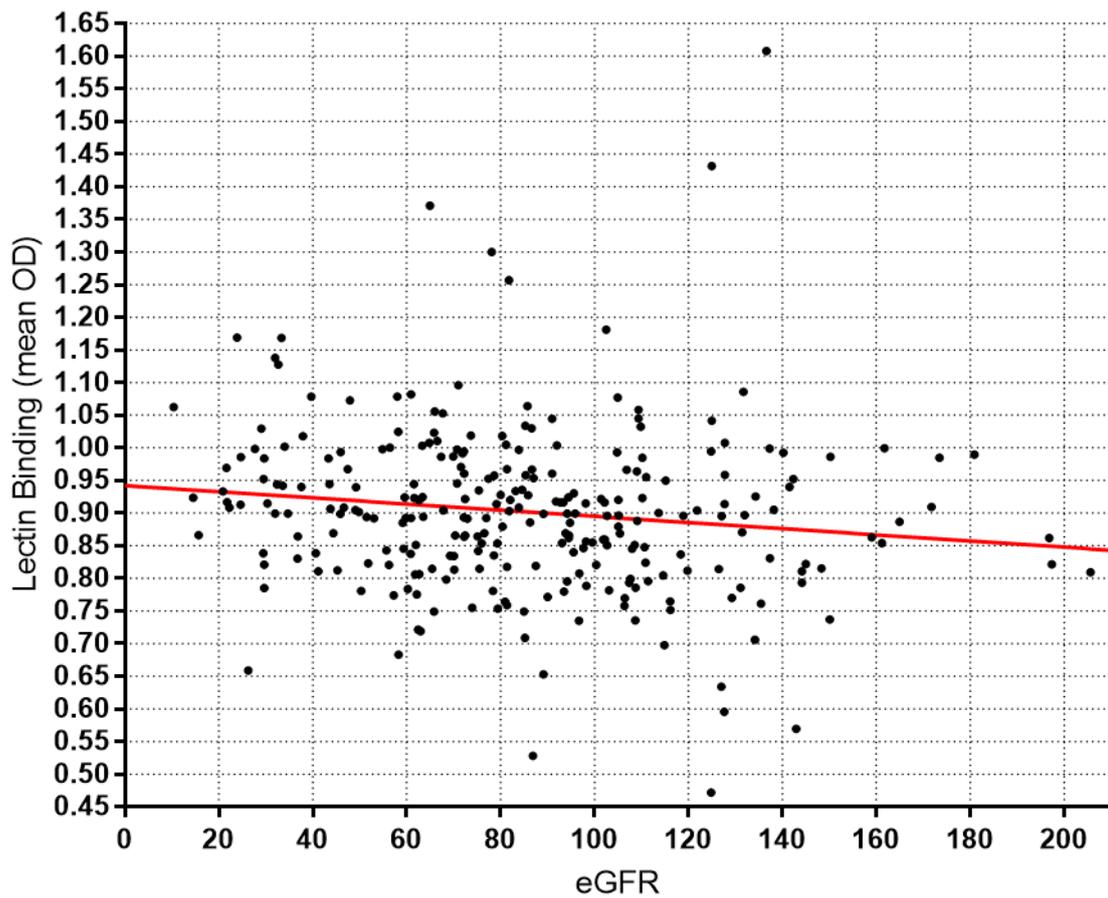


Figure 3.9: Correlation of Gd-IgA1 levels and eGFR in Caucasian IgAN: Negative correlation seen between serum Gd-IgA1 levels as measured by HA lectin binding ELISA and eGFR in 480 patients from the UKGNDB IgAN cohort. Data was correlated using the Pearson method; $r=-0.1361$, $p=0.0262$.

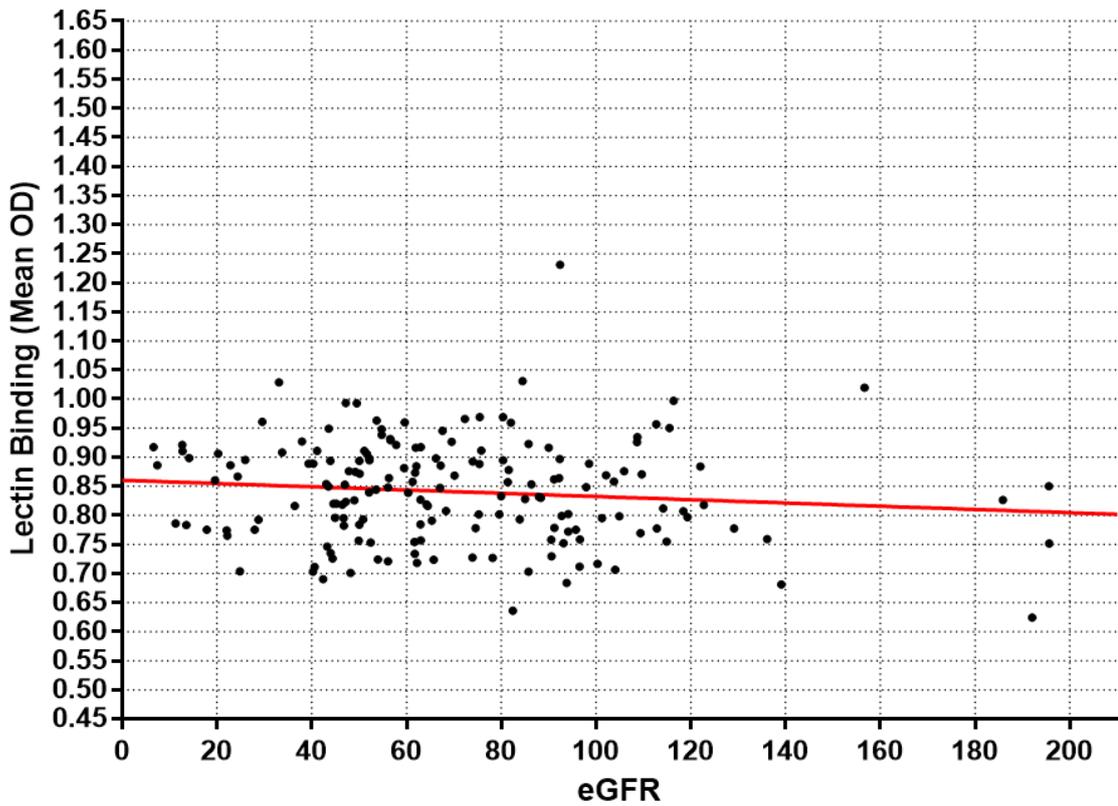


Figure 3.10: Correlation of Gd-IgA1 levels and eGFR in Caucasian MN:

No significant correlation seen between serum Gd-IgA1 levels as measured by HA lectin binding ELISA and eGFR in 353 patients from the UKGNDB MN cohort. Data was correlated using the Pearson method; $r=-0.1116$, $p=0.1485$.

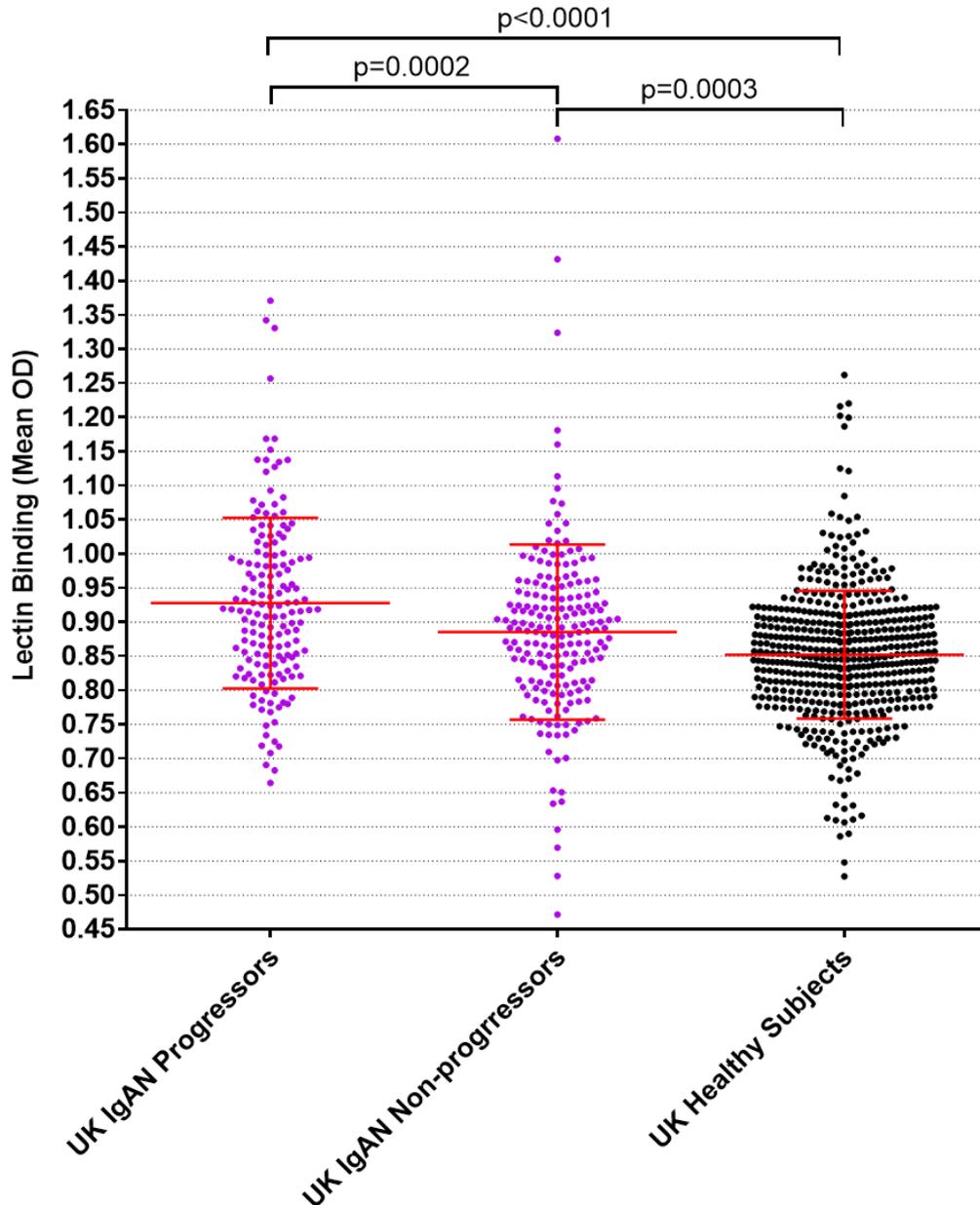


Figure 3.11: Comparison of Gd-IgA1 levels in Caucasian progressive and non-progressive IgAN and Caucasian healthy subjects: Both progressive and non-progressive IgAN patients from the UKGNDB IgAN cohort (160 and 194 patients respectively) show significantly elevated serum Gd-IgA1 levels compared to 519 Caucasians healthy subjects from the GRAPHIC cohort as measured by HA lectin binding ELISA. Gd-IgA1 levels in progressive IgAN are also significantly elevated over non-progressive. Graph shows mean \pm SD. Patients whose progression status was indeterminate or lacked follow-up data were excluded (126 patients). Data analysed by one-way ANOVA with post-hoc Tukey test.

3.4.3 – Higher serum Gd-IgA1 levels in Chinese IgAN are associated with worse renal function

The same correlation analysis made in the UKGNDB IgAN cohort between serum Gd-IgA1 levels and eGFR at the time of serum collection was made in Chinese IgAN patients. This demonstrated a significant negative correlation, indicating that Chinese IgAN patients with worse renal function have higher levels of Gd-IgA1 in serum ($r=-0.2189$, $p<0.0001$; Figure 3.12). This correlation was found to be stronger than that seen in Caucasian IgAN, despite the lower overall levels of serum Gd-IgA1 seen in the Chinese cohort and shows a further ethnic difference in IgAN. However, this effect could simply be due to the Chinese cohort being more powered to show this association, having more than twice as many patients as the Caucasian cohort.

3.4.4 – Chinese IgAN at CKD stages 4 and 5 show elevated serum Gd-IgA1 levels

Unfortunately, data from ten years follow-up was not available for the Chinese IgAN cohort, so a similar stratification of progressive and non-progressive IgAN could not be made. However, the stage of chronic kidney disease (CKD stage) of each patient at the time of serum collection was known, so an analysis was made based on this (Figure 3.13). Only those Chinese IgAN patients with advanced kidney damage or ESRD (those at CKD stages 4 and 5 respectively) showed elevated serum Gd-IgA1 levels compared to Chinese healthy subjects ($p<0.0001$ for both). Interestingly, the Chinese IgAN patients at CKD stages 4 and 5 both also showed significantly higher serum Gd-IgA1 levels than at any other CKD stage ($p<0.001$ for all).

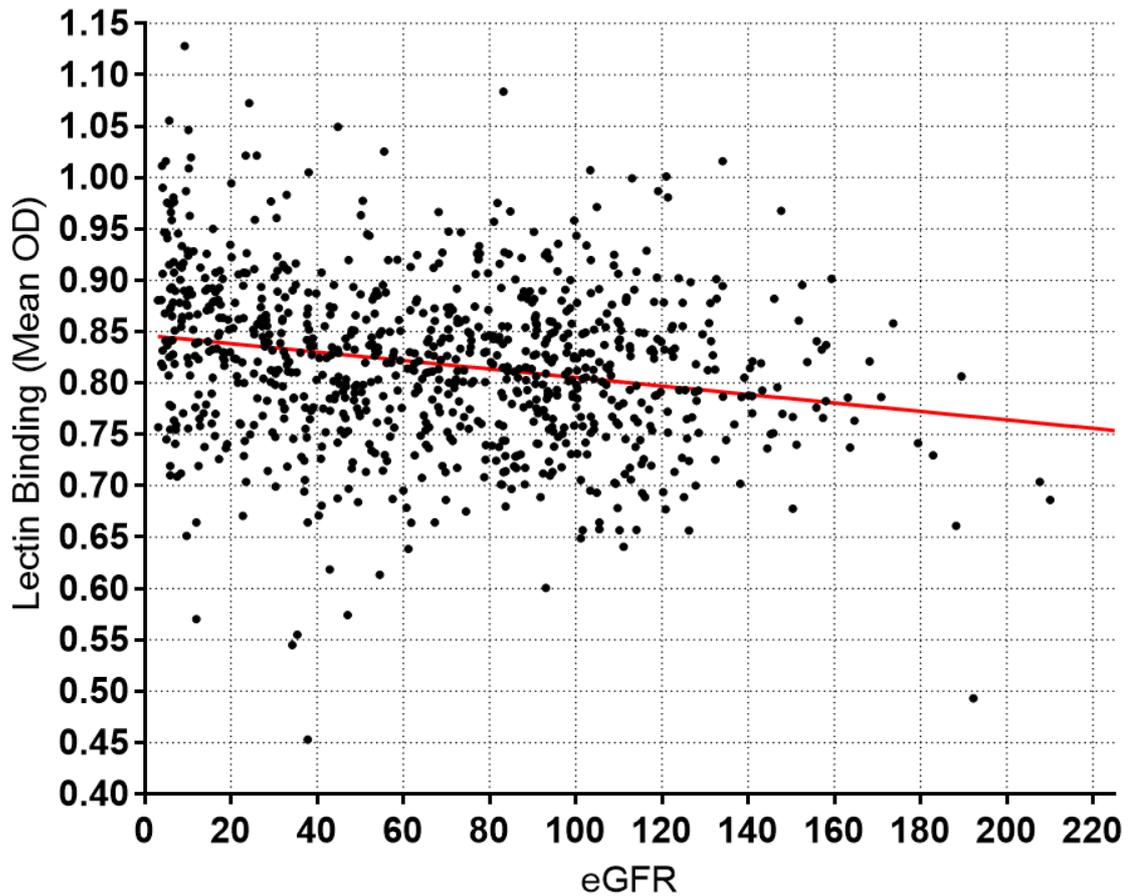


Figure 3.12: Correlation of Gd-IgA1 levels and eGFR levels in Chinese IgAN: Negative correlation seen between serum Gd-IgA1 levels as measured by HA lectin binding ELISA and eGFR in 1000 Chinese IgAN patients. Data was correlated using the Pearson method; $r=-0.2189$, $p<0.0001$.

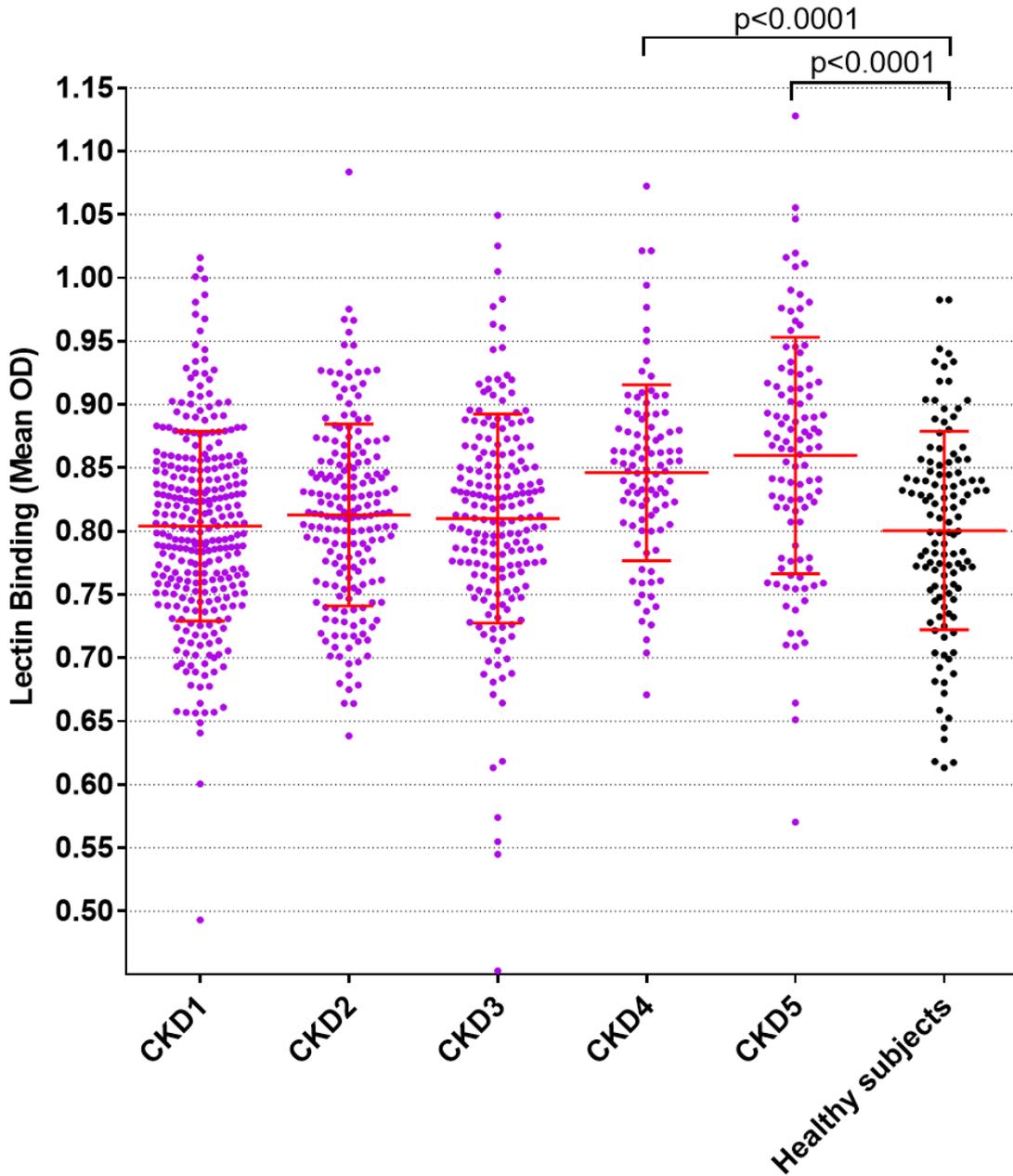


Figure 3.13: Comparison of Gd-IgA1 levels in Chinese IgAN split by CKD stage and Chinese healthy subjects: Chinese IgAN patients at CKD stages 1-3 show no significant difference in serum Gd-IgA1 levels compared to Chinese healthy subjects as measured by HA lectin binding ELISA. Those patients at CKD stages 4 and 5 show significant elevation in serum Gd-IgA1 levels compared to Chinese healthy subjects ($p < 0.0001$ for both). Graph shows mean \pm SD. Data analysed by one-way ANOVA with post-hoc Tukey test.

3.5 – Results 3 – Gender differences in circulating Gd-IgA1 levels in IgAN

3.5.1 – Serum Gd-IgA1 levels are greatly more heritable in females than in males

To assess differences in the heritability of serum Gd-IgA1 levels in the UKGNDB IgAN cohort based on gender, the data was standardised separately for male and female IgAN patients with their mid-parental values and the standardised data correlated as in 3.3.2 to determine narrow-sense heritability (Figure 3.14). This data showed that serum Gd-IgA1 levels are greatly more heritable in females than in males, with a h^2 value of 0.831 for females and 0.036 for males. This difference is striking; however, with data for only 62 male and 39 female patients in the UKGNDB IgAN cohort with data from both parents, this analysis lacks power.

3.5.2 – Gender differences in serum Gd-IgA1 levels in Caucasian IgAN

When data on serum Gd-IgA1 levels from the Caucasian IgAN cohort was split by gender; overall, females showed slightly higher levels than males; but without statistical significance (Figure 3.15). However, greater gender differences became apparent when looking at the relationship to worsening renal function in IgAN (see below). Sex data was not available for the Caucasian healthy subjects, and no difference was seen in Gd-IgA1 levels between sexes in the Caucasian MN cohort.

3.5.3 – Higher serum Gd-IgA1 levels in Caucasian IgAN are associated with worse renal function in females, but not in males

Analysing the relationship between serum Gd-IgA1 levels and renal function as measured by eGFR showed a significant negative correlation only in female IgAN patients ($r=-0.2604$, $p=0.0108$), with no significant correlation evident in male IgAN patients (Figure 3.16). This could indicate differences in the pathogenicity of Gd-IgA1 between genders in Caucasian IgAN; potentially being more so in Caucasian females. However; as the same downward trend is evident in males to a lesser extent than females, this could also be due to the larger scatter of the male data causing it to fall short of statistical significance.

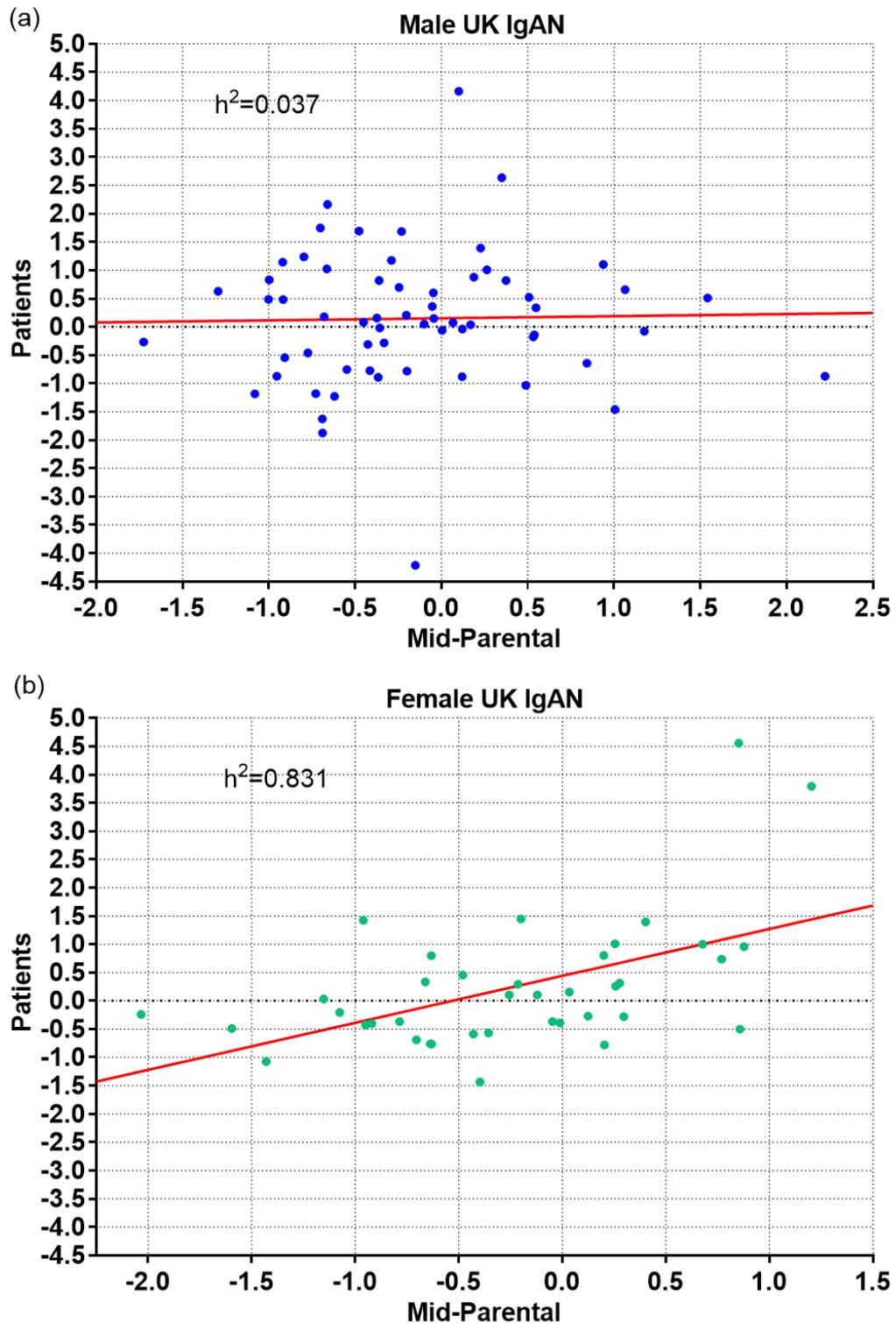


Figure 3.14: Heritability of Gd-IgA1 levels in male and female Caucasian IgAN: Differences are seen in the heritability of serum Gd-IgA1 levels in the UKGNDB IgAN cohort when split by sex as measured by HA lectin binding ELISA; greatly more heritable in females than males: (a) Male $h^2=0.036$ in 62 parent-child trios; (b) Female $h^2=0.831$ in 39 parent-child trios. Sex data was not available for 35 patients. Data was correlated using the Pearson method.

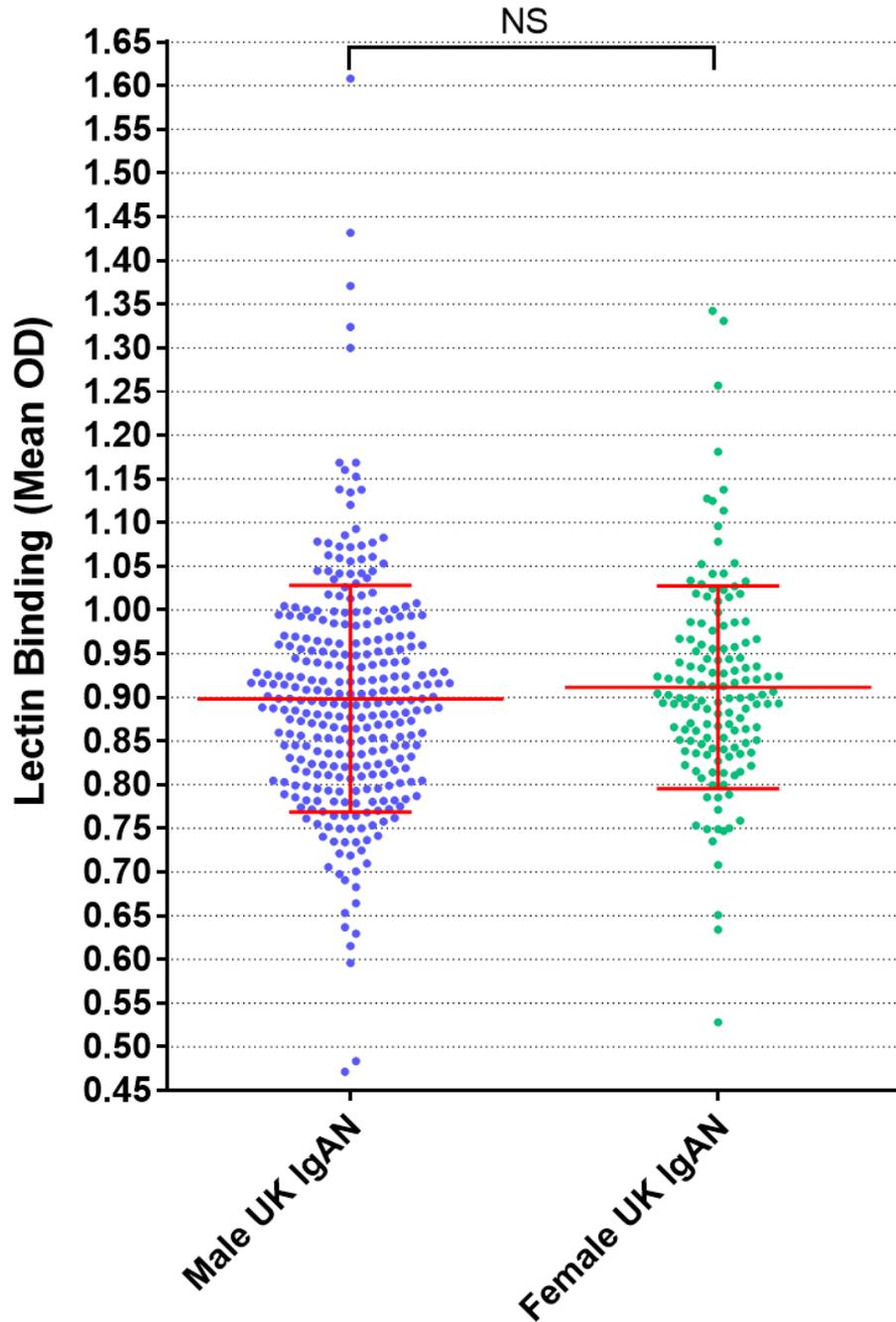


Figure 3.15: Comparison of Gd-IgA1 levels in male and female Caucasian IgAN: No significant difference was seen in serum Gd-IgA1 levels between sexes in patients from the UKGNDB IgAN cohort as measured by HA lectin binding ELISA. Graph shows mean \pm SD. Data analysed by unpaired t-test.

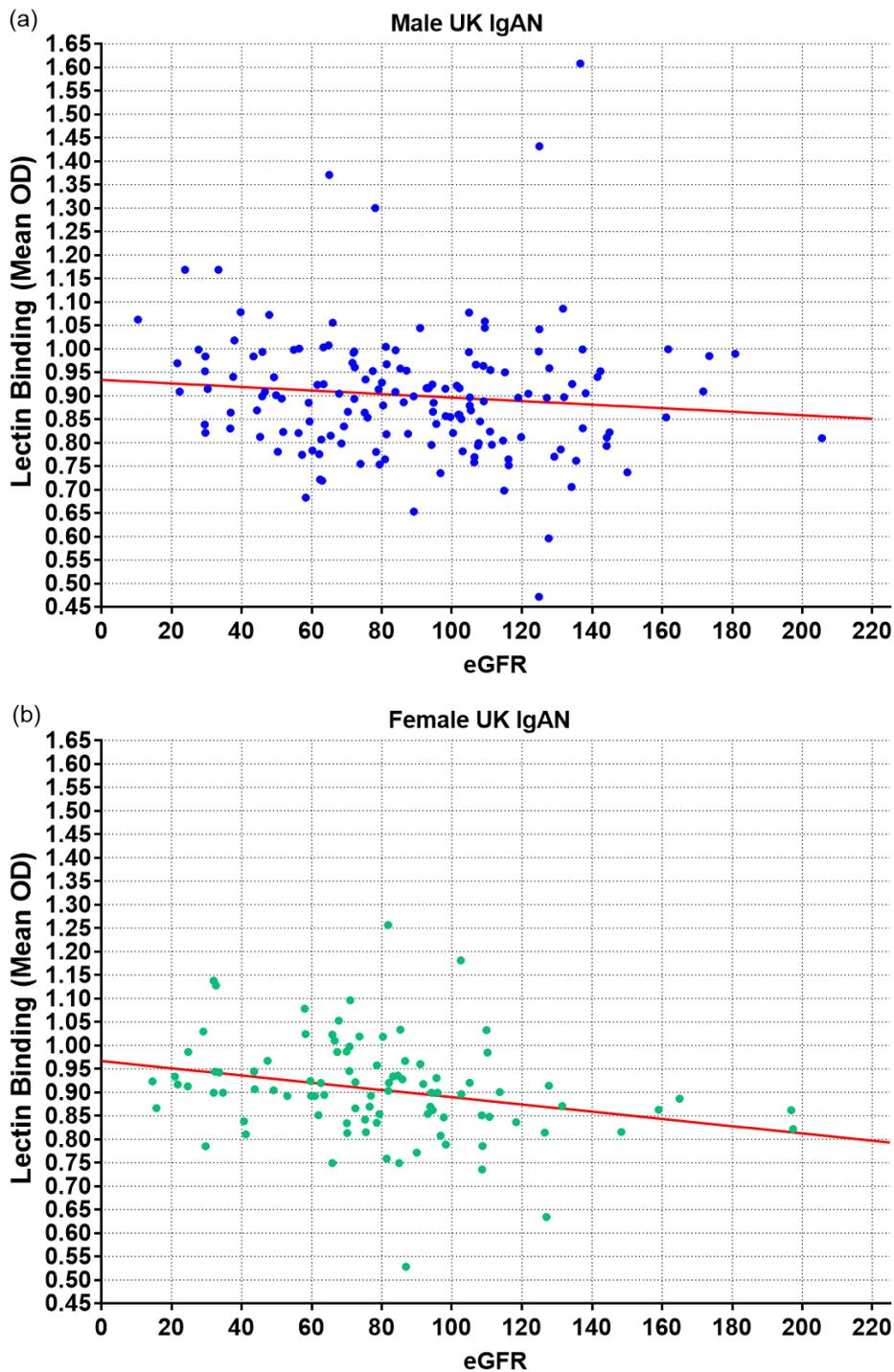


Figure 3.16: Correlations of Gd-IgA1 levels and eGFR in Caucasian male and female IgAN: Negative correlation seen between serum Gd-IgA1 levels as measured by HA lectin binding ELISA and eGFR in female patients from the UKGNDB IgAN cohort ($r=-0.2604$, $p=0.0108$). No significant correlation between Gd-IgA1 levels and eGFR in male patients was observed ($r=-0.1013$, $p=0.2111$). (a) Male; (b) Female. Data was correlated using the Pearson method.

3.5.4 – Caucasian females with progressive IgAN have higher serum Gd-IgA1 levels than male progressors

In both male and female IgAN patients, the differences in those defined as having progressive and non-progressive IgAN compared to healthy subjects were the same as in the cohort overall (both significantly elevated compared to healthy subjects and progressive IgAN was higher than non-progressive; Figures 3.17 & 3.18). When directly comparing serum Gd-IgA1 levels between male and female Caucasian patients with progressive and non-progressive IgAN (Figure 3.19), no gender difference was seen in non-progressors. However, female progressors displayed significantly higher levels than male progressors ($p=0.0323$), potentially strengthening the idea of pathogenic differences in Gd-IgA1 between genders in Caucasian IgAN.

3.5.5 – Gender differences in serum Gd-IgA1 levels in Chinese IgAN

In the Chinese IgAN cohort, females displayed significantly elevated serum Gd-IgA1 compared to males ($p=0.0004$; Figure 3.20); a greater difference than that seen in the Caucasian cohort. Data on sex in the Chinese healthy subjects cohort was not available.

3.5.6 – Higher serum Gd-IgA1 levels in Chinese IgAN are associated with worse renal function in both males and females

A further ethnic difference was identified when looking at gender differences in the association of serum Gd-IgA1 levels and renal function (Figure 3.21). Unlike the Caucasian IgAN cohort, significant negative correlations were seen between serum Gd-IgA1 levels and eGFR in both male and female Chinese IgAN patients. It was also found that the correlation in male Chinese IgAN patients is stronger than that in females ($r=-0.3051$ in males, $r=-0.1676$ in females); potentially meaning, in opposition to Caucasians, that Gd-IgA1 is more pathogenic in IgAN in Chinese males than females. However; a larger cohort of Caucasian IgAN patients with sex data available would be needed to discount the ethnic gender differences simply being an effect of the larger Chinese cohort.

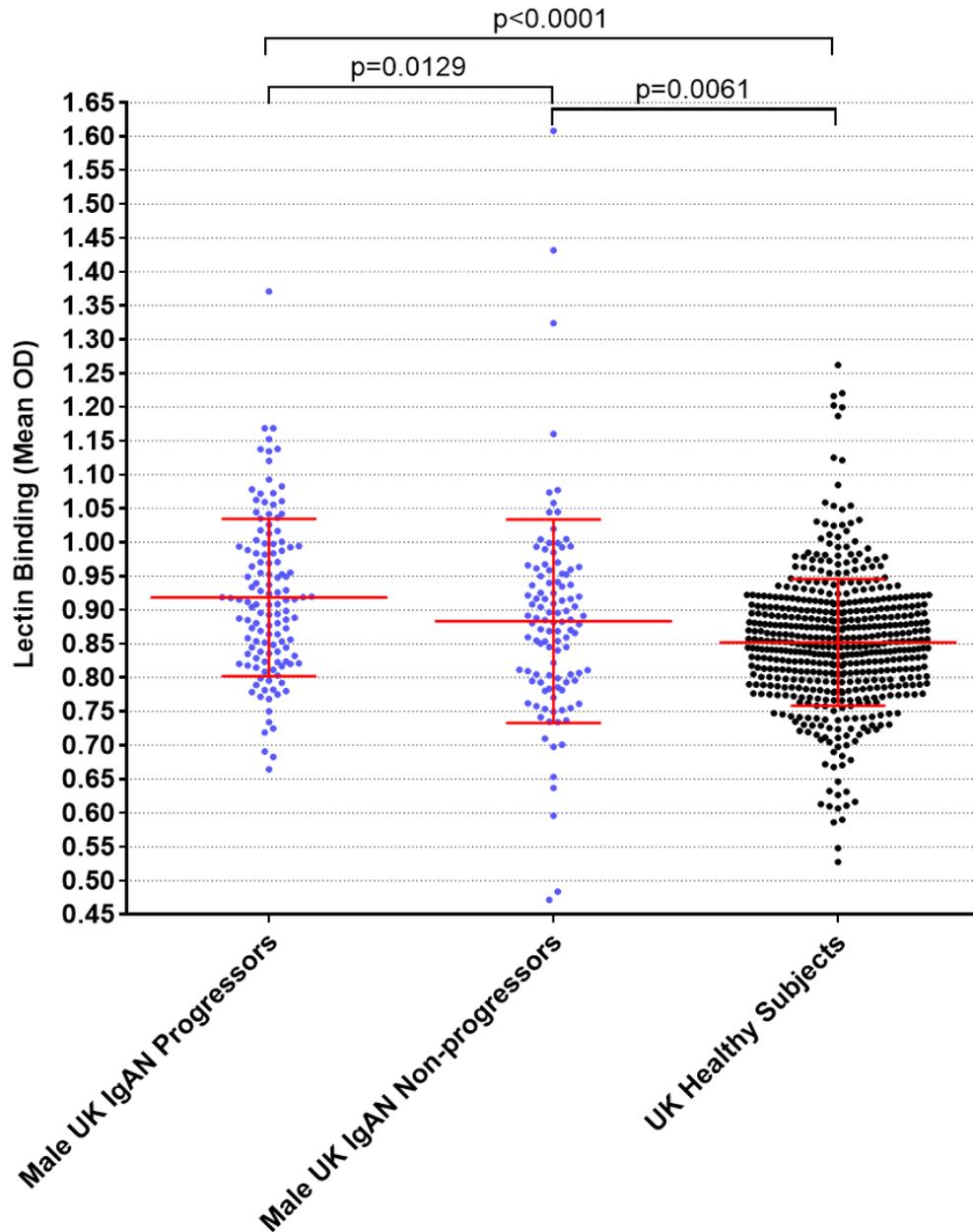


Figure 3.17: Comparison of Gd-IgA1 levels in male Caucasian progressive and non-progressive IgAN and Caucasian healthy subjects: Both progressive and non-progressive male IgAN patients from the UKGNDB IgAN cohort (124 and 108 patients respectively) show significantly elevated serum Gd-IgA1 levels compared to 519 Caucasian healthy subjects from the GRAPHIC cohort as measured by HA lectin binding ELISA. Gd-IgA1 levels in progressive male IgAN patients are also significantly elevated over non-progressive. Data on sex of the Caucasian healthy subjects was not available. Graph shows mean \pm SD. Data analysed by one-way ANOVA with post-hoc Tukey test.

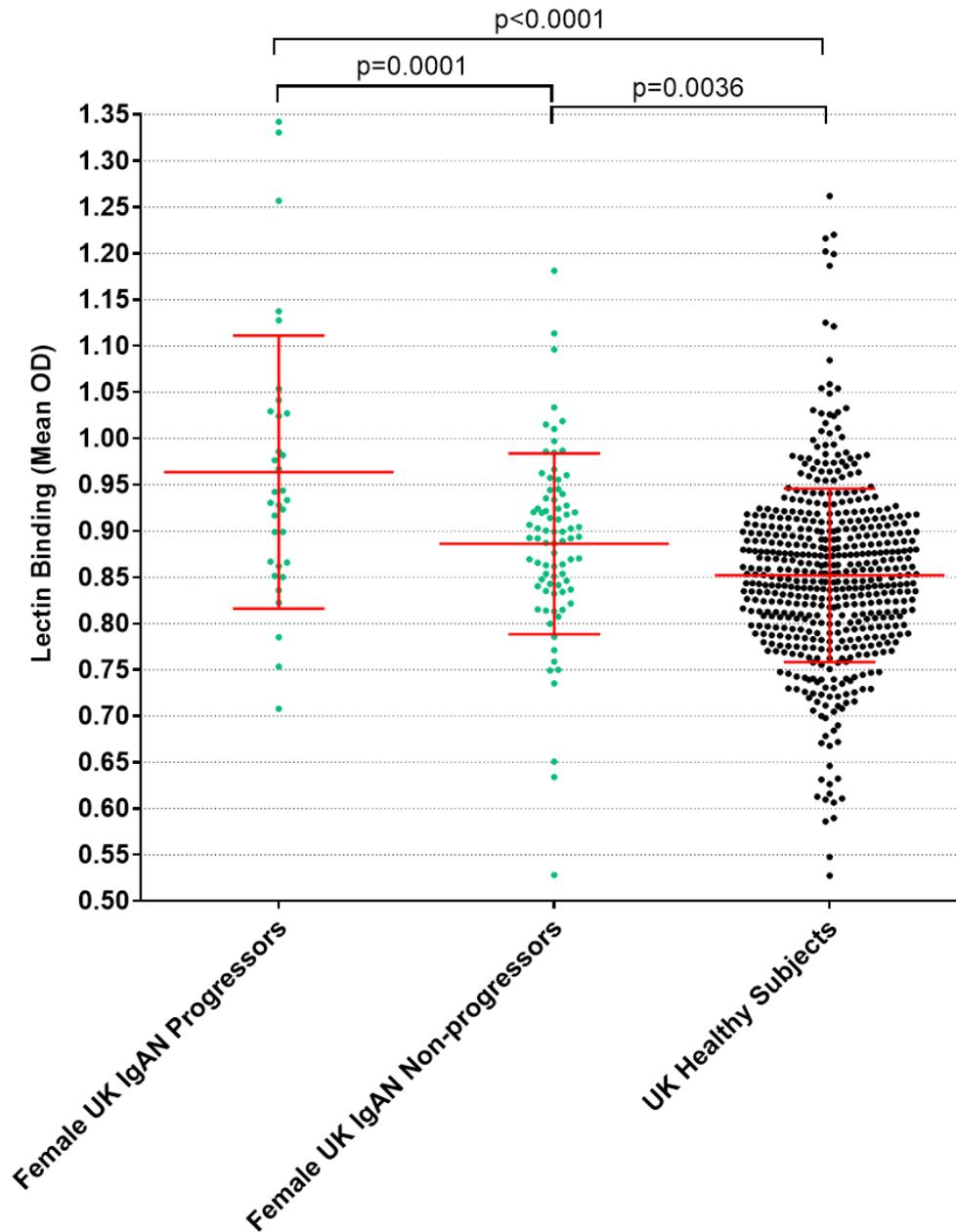


Figure 3.18: Comparison of Gd-IgA1 levels in female Caucasian progressive and non-progressive IgAN and Caucasian healthy subjects: Both progressive and non-progressive female IgAN patients from the UKGNDB IgAN cohort (33 and 81 patients respectively) show significantly elevated serum Gd-IgA1 levels compared to 519 Caucasian healthy subjects from the GRAPHIC cohort as measured by HA lectin binding ELISA. Gd-IgA1 levels in progressive female IgAN patients are also significantly elevated over non-progressive. Data on sex of the Caucasian healthy subjects was not available. Graph shows mean \pm SD. Data analysed by one-way ANOVA with post-hoc Tukey test.

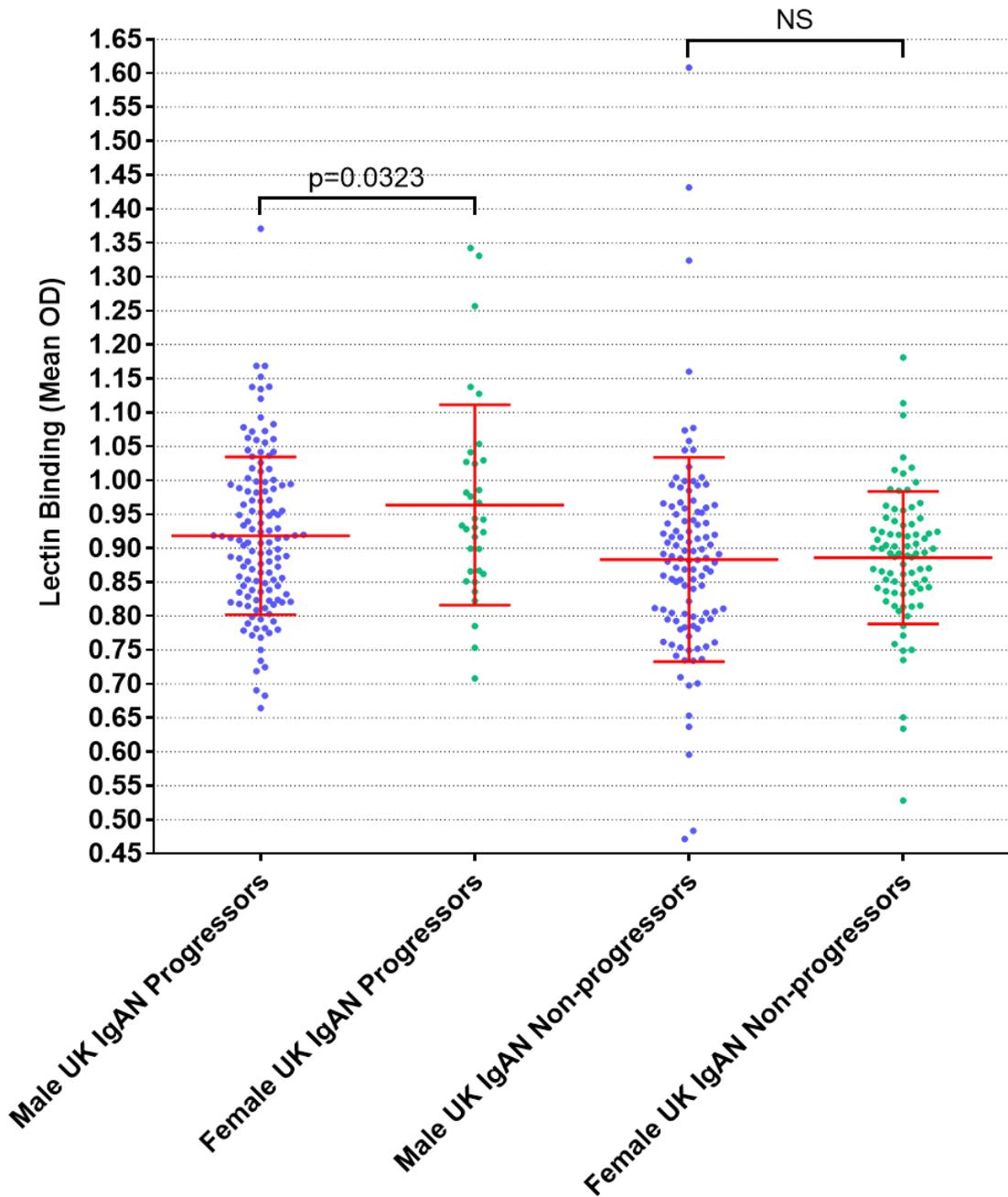


Figure 3.19: Comparison of Gd-IgA1 levels in Caucasian male and female, progressive and non-progressive IgAN: In the UKGNDB cohort, Caucasian female progressive IgAN patients show significantly elevated serum Gd-IgA1 levels compared to male progressive IgAN patients as measured by HA lectin binding ELISA. No significant difference in serum Gd-IgA1 levels was seen between male and female non-progressive IgAN patients from the same cohort. Graph shows mean \pm SD. Data analysed by unpaired t-tests between sexes at each progression stage.

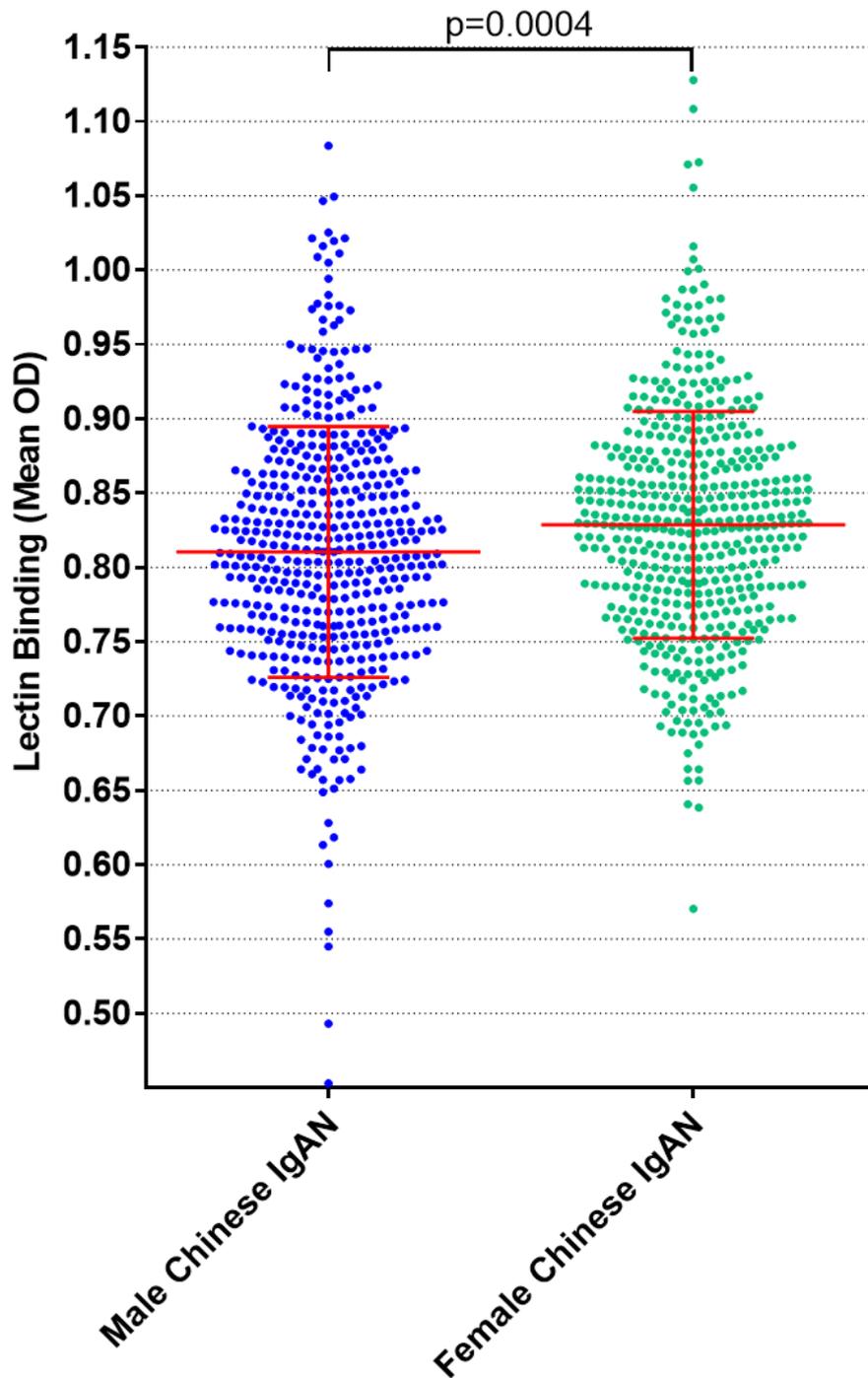


Figure 3.20: Comparison of Gd-IgA1 levels in male and female Chinese IgAN: Chinese female IgAN patients show significantly elevated serum Gd-IgA1 levels compared to male patients as measured by HA lectin binding ELISA. Graph shows mean \pm SD. Data analysed by unpaired t-test.

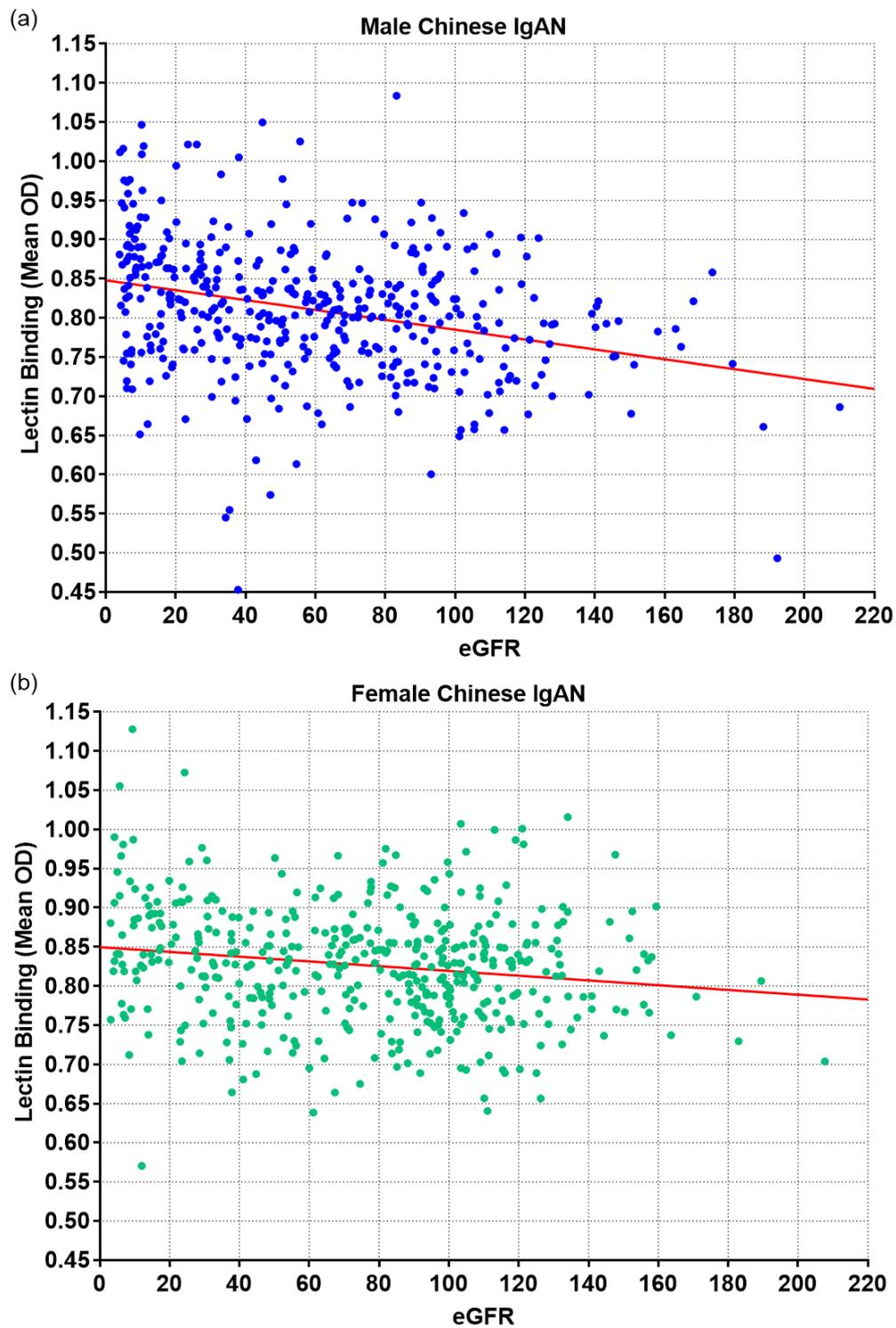


Figure 3.21: Correlations of Gd-IgA1 levels and eGFR in Chinese male and female IgAN: Negative correlation seen between Gd-IgA1 levels as measured by HA lectin binding ELISA and eGFR in both male and female patients from the Chinese IgAN cohort (a) Male, $r=-0.3051$, $p<0.0001$; (b) Female, $r=-0.1676$, $p=0.0004$. Data was correlated using the Pearson method.

3.5.7 – Chinese female IgAN patients have higher serum Gd-IgA1 levels than males at any CKD stage

As with the Chinese IgAN cohort overall, both male and female Chinese IgAN patients at CKD stages 4 and 5 showed elevated serum Gd-IgA1 levels compared to Chinese healthy subjects and CKD stages 1, 2 and 3 (Figures 3.22 & 3.23). When comparing serum Gd-IgA1 levels between Chinese male and female IgAN patients at each CKD stage, at stages 1 and 2 females displayed significantly higher levels compared to males (Figure 3.24). This difference is still visible at CKD stages 3 and 4 but does not reach statistical significance, and no gender difference at CKD stage 5 is apparent.

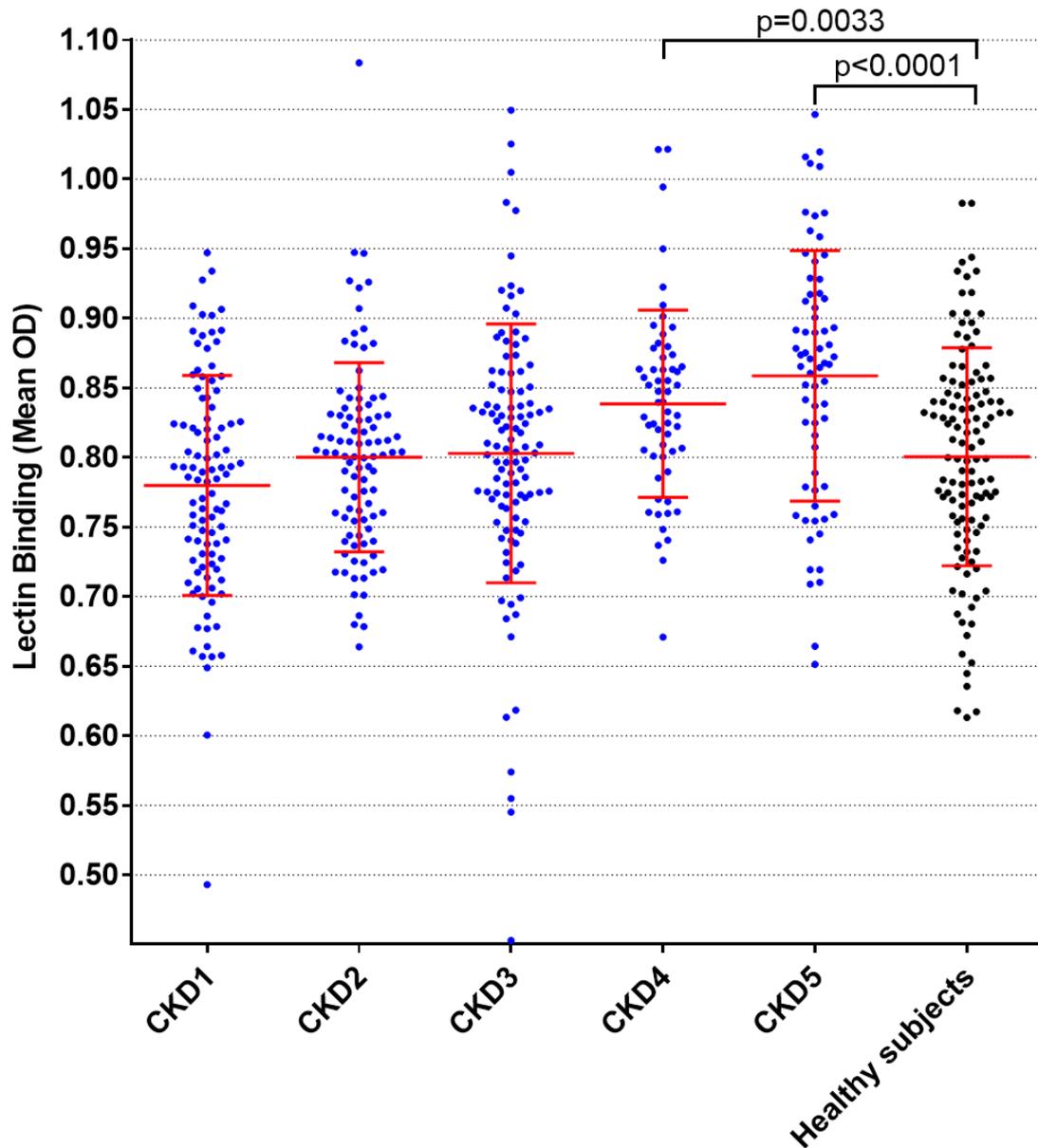


Figure 3.22: Comparison of Gd-IgA1 levels in male Chinese IgAN split by CKD stage and Chinese healthy subjects: Male Chinese IgAN patients at CKD stages 1-3 show no significant difference in serum Gd-IgA1 levels compared to Chinese healthy subjects as measured by HA lectin binding ELISA. Those male patients at CKD stages 4 and 5 show significant elevation in serum Gd-IgA1 levels compared to Chinese healthy subjects ($p=0.0033$ for CKD 4 and $p<0.0001$ for CKD 5). Data on sex of the Chinese healthy subjects was not available. Graph shows mean \pm SD. Data analysed by one-way ANOVA with post-hoc Tukey test.

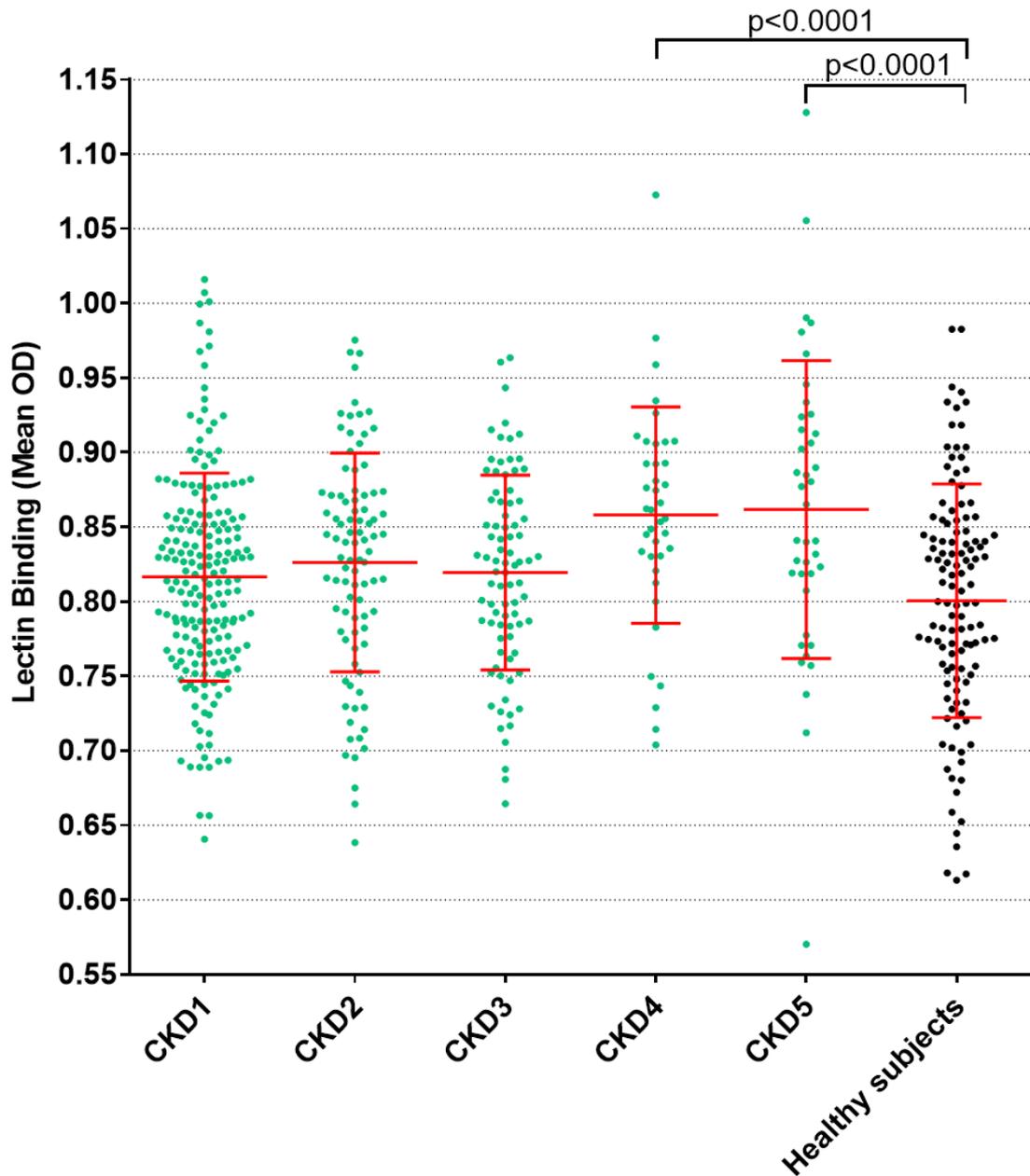


Figure 3.23: Comparison of Gd-IgA1 levels in female Chinese IgAN split by CKD stage and Chinese healthy subjects: Female Chinese IgAN patients at CKD stages 1-3 show no significant difference in serum Gd-IgA1 levels compared to Chinese healthy subjects as measured by HA lectin binding ELISA. Those female patients at CKD stages 4 and 5 show significant elevation in serum Gd-IgA1 levels compared to Chinese healthy subjects ($p < 0.0001$ for both). Data on sex of the Chinese healthy subjects was not available. Graph shows mean \pm SD. Data analysed by one-way ANOVA with post-hoc Tukey test.

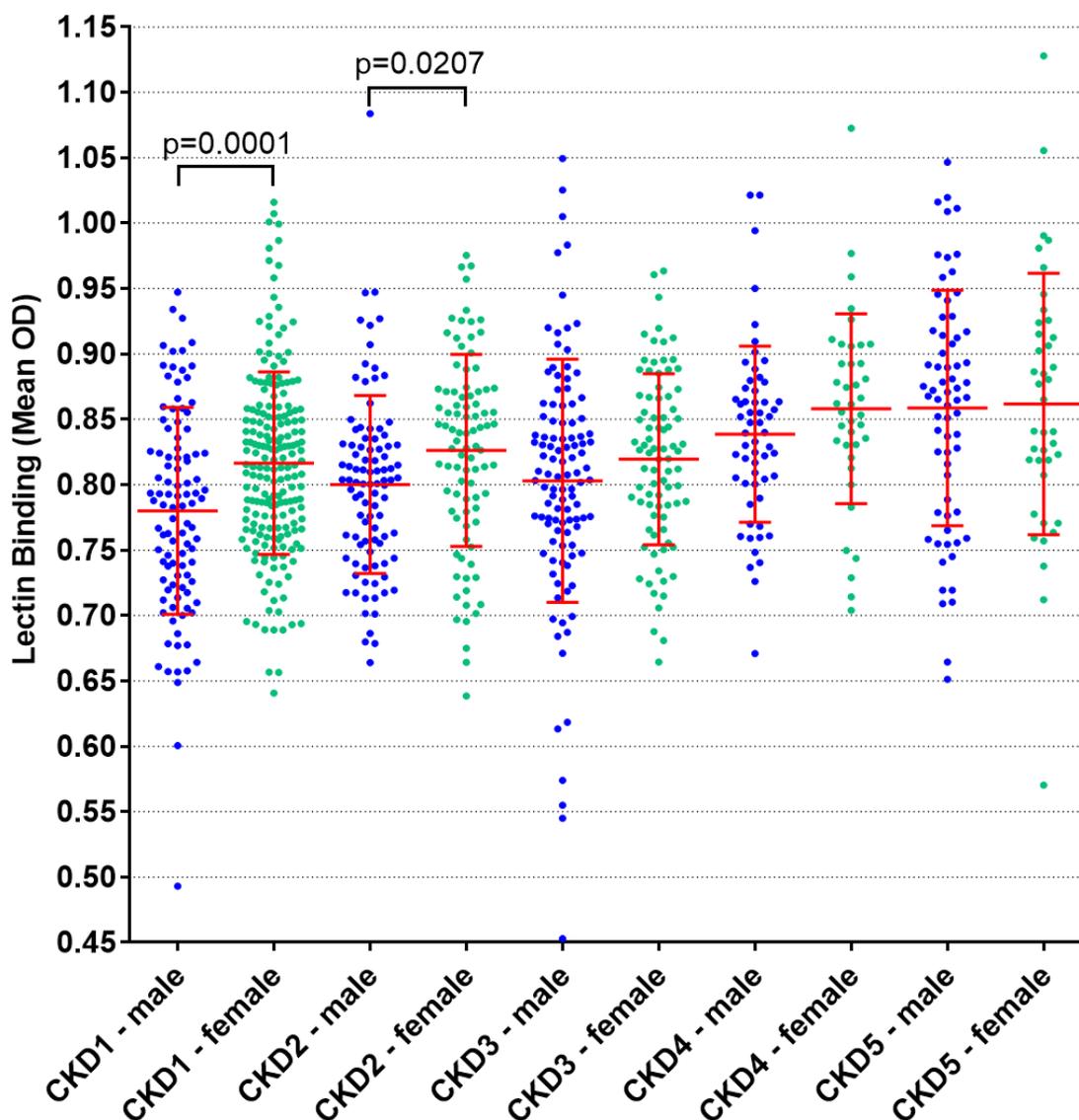


Figure 3.24: Comparison of Gd-IgA1 levels between male and female Chinese IgAN split by CKD stage: At CKD stages 1 and 2, female Chinese IgAN patients show significantly elevated serum Gd-IgA1 levels compared to male Chinese IgAN patients as measure by HA lectin binding ELISA. Graph shows mean \pm SD. Data analysed by unpaired t-tests between sexes at each CKS stage.

3.6 – Discussion

The work in this chapter has shown, firstly, that serum Gd-IgA1 levels remain stable over time; which does indicate that they are regulated by genetic factors rather than any environmental factors. Consistent with previous studies, serum levels of Gd-IgA1 were found to be heritable in the Caucasian IgAN population studied here (Gharavi *et al*, 2008; Hastings *et al*, 2010; Kiryluk, Moldoveanu, John *et al*, 2011; Lomax-Browne *et al*, 2016), which again implies the impact of genetic factors.

As has also been shown in previous studies, serum Gd-IgA1 levels were elevated in IgAN in both Caucasian and Chinese populations (Coppo and Amore, 2004; Moldoveanu *et al*, 2007; Mestecky *et al*, 2008; Barratt, Smith and Feehally, 2012; Novak *et al*, 2012); and were also found to be IgAN-specific in Caucasians as raised serum Gd-IgA1 levels were not found in MN, a similar immune complex-mediated glomerulonephritis that has no IgA involvement. A surprising finding, however, was the lower levels of serum Gd-IgA1 in Chinese subjects compared to Caucasians; in both health and IgAN. All measurement of serum Gd-IgA1 was performed under the same conditions, with the same standards run on every plate for normalisation purposes. This is the first time such a comparison has been made, and it indicates that the increased prevalence of IgAN in China cannot be attributed to differences in the serum levels of Gd-IgA1, suggesting differences in the pathogenic pathways in different ethnicities.

In both the Caucasian and Chinese populations studied here, a haplotype spanning the C1GALT1 gene was found to be associated with serum Gd-IgA1 levels; named the H1 haplotype and accounting for around 3% of the variability in serum Gd-IgA1 levels. C1GALT1 encodes the enzyme core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 (C1GalT1), which catalyses the transfer of galactose from UDP-Gal to O-linked GalNAc residues such as those found at the IgA1 hinge region. C1GalT1 also requires a molecular chaperone protein, Cosmc, for correct folding and activation which is encoded by the X-linked gene C1GALT1C1. A possible association between serum Gd-IgA1 levels and variation at C1GALT1C1 was also observed here, although this was not statistically significant at the genome-wide level. A

separate, similar study identified associations with Gd-IgA1 levels at two loci with genome-wide significance; one at C1GALT1 and another at C1GALT1C1 (Kirylyuk *et al*, 2017). The pathological importance of the H1 haplotype of C1GALT1 in IgAN requires further study, as a much larger cohort would be required to be powered enough to find any association of H1 with IgAN.

It has been shown previously that unlike in IgA1, IgD shows no aberrancy in O-galactosylation of its hinge region (Smith, De Wolff *et al*, 2006). This implies that the reduction in O-galactosylation in IgAN is a specific feature of IgA1 and therefore IgA1 producing cells; which in turn suggests that the H1 haplotype of C1GALT1 identified here leads to elevated levels of serum Gd-IgA1 through altered maturation-dependent transcriptional regulation, rather than through differences in the protein structure of C1GalT1. The idea of transcriptional control of C1GALT1 affected by the H1 haplotype is strengthened by in silico analyses. Firstly, imputation of the H1 haplotype using 1000 Genomes data indicated that there were no common coding variants that were over- or under-represented on the H1 haplotype compared with other common haplotypes. Data linking genetic variation with gene expression in lymphoblastoid cell lines has shown that SNPs found on the H1 haplotype of C1GALT1 are associated with reduced transcription of C1GALT1 (Yu, Pal and Moulton, 2016). Other in silico analysis has shown that some SNPs of the H1 haplotype are predicted to lie within transcription factor binding elements; one at a predicted SOX2-OCT4 site (J. Wang *et al*, 2012) and another within the promoter region of C1GALT1 and predicted to affect binding of RUNX3. RUNX3 is a transcription factor in B cells that is involved in class switching to IgA production (Watanabe *et al*, 2010).

Again consistent with previously published data (Zhao *et al*, 2012), higher serum Gd-IgA1 levels were found to be associated with declining renal function; showing a negative correlation with eGFR in both Caucasian and Chinese IgAN. This was also found to be specific to IgAN, as no significant correlation of eGFR and serum Gd-IgA1 levels was seen in MN. The association was strengthened by the observation of higher levels of serum Gd-IgA1 in progressive IgAN in Caucasians, and in those at CKD stages 4 and 5 in Chinese IgAN. The possibility of uraemic toxins inhibiting glycosylation of IgA1 accounting for the association of worsening renal function and higher Gd-IgA1 levels has yet to be explored;

however, the specificity of raised Gd-IgA1 levels to IgAN, the lack of significant correlation of Gd-IgA1 levels and eGFR in MN and the stability of Gd-IgA1 levels over long periods of time in both IgAN and health would seem to discount this possibility.

The incidence of IgAN in Caucasians is twice as high in males as in females, while in the Chinese population there is no difference in the incidence between genders (Barratt *et al*, 2012; Wyatt and Julian, 2013). The work in this project is the first to assess gender differences in serum Gd-IgA1 levels.

In Caucasians it was discovered that serum Gd-IgA1 levels are greatly more heritable in IgAN in females than in males (h^2 value of 0.831 for females and 0.036 for males). The numbers of samples here were quite low, making this underpowered to be conclusive; however, the gender difference in Gd-IgA1 levels is worth further investigation as a large study in Swedish twins identified a similar gender differential in the heritability of total IgA levels (61% in females and 21% in males) (Viktorin *et al*, 2014).

While no data was available on the sex of the healthy subjects assessed here, previous work in this lab on a separate cohort of healthy subjects showed no difference in Gd-IgA1 levels between males and females; highlighting the association of Gd-IgA1 and IgAN. In this project, female Caucasian IgAN patients showed slightly higher serum Gd-IgA1 levels than male Caucasian IgAN patients, and only became significant in progressive IgAN. A much greater elevation in females compared to males was seen in Chinese IgAN, and this was reflected in each stage of CKD. A correlation of serum Gd-IgA1 levels with eGFR was seen in female Caucasian IgAN patients and was not seen in male Caucasian IgAN patients, indicating differences in the pathogenicity of Gd-IgA1 between genders in Caucasian IgAN; potentially being more so in Caucasian females. Interestingly, this was found to be opposite in Chinese IgAN patients; the correlation of serum Gd-IgA1 levels with eGFR in male Chinese IgAN patients was stronger than that in females. However; the sex differences could potentially just be more evident in the Chinese IgAN cohort due to it having more than double the number of patients. A potential reason for the gender discrepancies seen in serum Gd-IgA1 levels in this project could involve the X-linked gene C1GALT1C1, encoding the

C1GalT1 chaperone protein Cosmc; however, this is speculative without further research in larger cohorts.

To summarise, the work in this chapter has observed for the first time significant ethnic and gender differences in serum levels of Gd-IgA1 in IgAN. A haplotype of C1GALT1 was also identified as being strongly associated with higher serum levels of Gd-IgA1.

Chapter 4 – Expression of glycosylation enzymes in IgA and IgD producing cells

4.1 – Introduction and aims

The O-glycan structures at the IgA1 hinge region are of the core 1 type, which are based on N-acetylgalactosamine (GalNAc) O-linked to serine or threonine residues. This can occur alone or can be further modified by the addition of galactose and N-acetylneuraminic acid (sialic acid). Core 1 O-glycans are formed in the Golgi apparatus, the initial addition of GalNAc being catalysed by the enzyme polypeptide N-acetylgalactosaminyltransferase 2 (GalNT2) (Iwasaki *et al*, 2003b). Galactose can then be added to the GalNAc residues by the enzyme core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 (C1GalT1) (T. Ju *et al*, 2002). Co-expression of a molecular chaperone protein, Cosmc, is required for correct folding and activation of newly synthesised C1GalT1, otherwise the inactive C1GalT1 is signalled for degradation (T. Z. Ju and Cummings, 2002; Y. Wang *et al*, 2010). Sialic acid can be added to either or both of the GalNAc and galactose residues on the core 1 structure; catalysed by the enzyme N-acetylgalactosaminide- α -2,6-sialyltransferase (ST6GalNAc) for an α 2,6 linkage of sialic acid to GalNAc and by a β -galactoside- α -2,3-sialyltransferase (ST3Gal) for an α 2,3 linkage to galactose (Harduin-Lepers *et al*, 2001).

In IgAN, the increased serum levels of IgA1 hypogalactosylated at the hinge region (Gd-IgA1) compared to serum from healthy subjects has been the subject of much research. It is thought to be due to under-expression or low activity of C1GalT1 or Cosmc decreasing the ability to add galactose, or due to high expression or activity of sialyltransferases blocking the addition of galactose by over-sialylation of terminal GalNAc, specifically ST6GalNAcII (Allen *et al*, 1997; Qin *et al*, 2005; Buck *et al*, 2008; Raska *et al*, 2008; Smith *et al*, 2008). However, despite much research the exact mechanisms causing the abnormality in IgAN have yet to be fully elucidated.

The hypogalactosylation of the core 1 O-glycan structure has also been found to be specific to the IgA1 hinge region. IgD is the only other immunoglobulin that can be O-glycosylated at its hinge region in a similar fashion to IgA1, but no

differences have been observed in IgD O-glycosylation between IgAN patients and healthy subjects (Smith, De Wolff *et al*, 2006). It can be inferred from this that that any defect in IgAN that causes reduced galactosylation of the IgA1 hinge region is not a generic feature of B cells in IgAN, but rather a defect in an upstream regulatory pathway as they class switch from naïve B cells to IgA1 producing B cells. This could also mean that the H1 haplotype of C1GALT1 described in Chapter 3 is differentially regulated in different B cell subtypes.

The aim of the work in this chapter was to investigate the genetic control of IgA1 O-glycosylation in circulating B cells at distinct development stages; namely naïve B cells (IgD+) and B cells that are class switched to IgA. This was performed broadly, looking at gene expression of the enzymes involved in O-glycosylation of the IgA1 hinge region, and also with a specific focus on what effect the H1 haplotype of the galactosyltransferase enzyme C1GALT1 has on the expression of C1GALT1 in these cell types.

4.2 – Methods

A total of fifty healthy volunteers were selected from the University of Leicester. A 5ml blood sample was taken for serum generation as in 2.4 and measurement of IgA, IgA1 and Gd-IgA1 levels (sections 2.5.1, 2.5.2 & 2.5.3). The cell pellet left over from serum generation was used for DNA isolation and genotyping of the H1 haplotype of C1GALT1 (section 2.3) using the SNPs described in Table 4.1.

SNP ID	Alleles	H1 haplotype
rs4720724	[A/G]	G
rs758263	[G/T]	T
rs426366	[C/T]	C
rs10259085	[C/T]	C
rs1008897	[A/G]	G
rs2190935	[C/T]	C

Table 4.1: SNPs tagging the H1 haplotype of C1GALT1: Table shows six SNPs of the H1 haplotype spanning the C1GALT1 gene. These six SNPs are inherited together with the extended haplotype and are used to tag the H1 haplotype. Volunteers were genotyped for these six SNPs to identify whether they were homozygous for the H1 haplotype, heterozygous or homozygous negative.

Volunteers selected for analysis of the O-glycosylation mechanism in naïve B cells and B cells class switched to IgA had a further 40ml blood sample taken; these subjects consisted of three homozygotes for the H1 haplotype, three heterozygotes and three that were homozygous negative for H1. B cell-enriched peripheral blood mononuclear cells (PBMCs) were isolated from this larger blood sample (section 2.6) and stained for fluorescence-activated cell sorting (FACS) of surface IgA⁺ and surface IgD⁺ B cells (section 2.7). Cell surface markers used were CD19 as the marker of B cell lineage, along with IgA and IgD to collect surface IgA⁺ and surface IgD⁺ B cells; and the fluorescently conjugated antibodies used were anti-CD19 Alexa Fluor 700, anti-IgA PE and anti-IgD FITC respectively. Concentrations of the fluorescently conjugated antibodies used were optimised in previous work from this group. Details of the antibodies and the working volumes used here are shown in Supplementary table S2.1.

RNA and protein were isolated from the sorted cells for gene and protein expression analysis (section 2.8). Targets for gene expression analysis by qPCR (section 2.9) were C1GALT1, COSMC, ST6GALNACII, and the IgA and IgD heavy chain genes (IGHA1 and IGHD respectively). PSMB6 was used as the endogenous reference gene (ERG) (García-Vallejo *et al*, 2004). Protein analysis target was C1GalT1 (section 2.10).

4.3 – Results

4.3.1 – Genotyping of the 50 healthy volunteers

Of the 50 volunteers who provided samples for genotyping of the H1 haplotype of C1GALT1, only three were found to be homozygous for the H1 haplotype; 23 were heterozygous, 19 homozygous negative and the remaining four undetermined.

4.3.2 – Gd-IgA1 and IgA levels by C1GALT1 haplotype

No differences in the levels of total serum IgA or serum IgA1 were seen across samples (data not shown). A trend of higher levels of serum Gd-IgA1 was seen with more copies of the H1 haplotype, however these differences did not reach statistical significance (Figure 4.1). This is consistent with the results from the Caucasian and Chinese populations studied in Chapter 3.

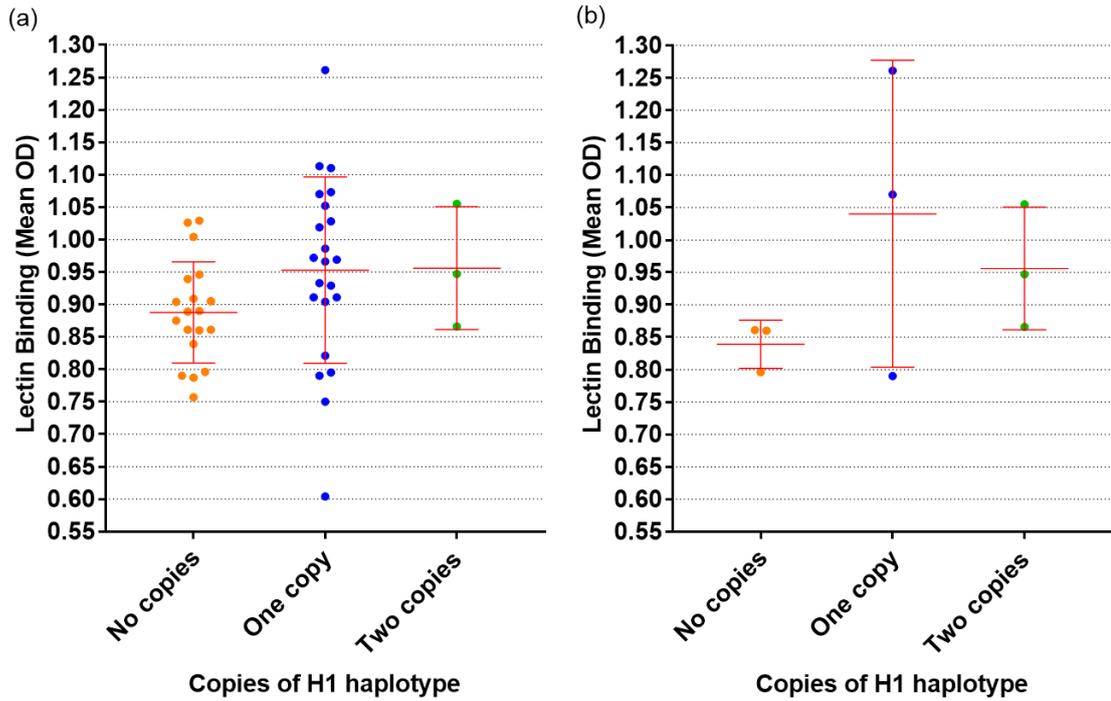


Figure 4.1: Comparison of Gd-IgA1 levels in healthy volunteers with and without the H1 haplotype of C1GALT1: Serum Gd-IgA1 levels increase with copies of the H1 haplotype in 50 healthy volunteers genotyped for H1, with no statistically significant differences. (a) Whole cohort of 50; (b) Volunteers selected for further analysis were the 3 that were homozygous for H1, 3 randomly selected heterozygotes and 3 randomly selected homozygous negatives. Graphs show mean \pm SD.

4.3.3 – FACS profile of sorted B cells

As only three of the 50 volunteers were found to be homozygous for the H1 haplotype, three heterozygotes and three homozygous negative were randomly selected to match the numbers. These nine volunteers had the larger blood sample taken for isolation of B cells for sorting using FACS. Before sorting, an aliquot of total B cells was stored for analysis. The gating strategy to collect surface IgA+ and surface IgD+ B cells from the selected volunteers is shown in Figure 4.2. This involved identifying lymphocytes by size, selecting the live cells, identifying surface CD19+ cells as those of B cell lineage and finally collecting surface IgA+ B cells (surface CD19+ and IgA+) and surface IgD+ B cells (surface CD19+ and IgA+).

The actual numbers of cells collected varied greatly from person to person. Numbers of surface IgA+ B cells ranged from 800 to 10,000 while numbers of surface IgD+ B cells ranged from 5000 to 20,000. Despite this, the proportions of surface IgA+ and surface IgD+ B cells as a percentage of total surface CD19+ cells showed no differences between any subjects (Figure 4.3).

4.3.4 – Surface IgD+ B cells express higher IGHD mRNA levels and surface IgA+ B cells express higher IGHA1 mRNA levels

Firstly, to further confirm the identity of the different populations of sorted cells, the mRNA expression of the heavy chain genes for IgA and IgD were measured; IGHA1 and IGHD respectively (Figure 4.4). It was found that the expression of IGHA1 was significantly lower in surface IgD+ B cells than both surface IgA+ B cells and total B cells, and significantly higher in surface IgA+ B cells compared to total B cells. The expression of IGHD was significantly lower in surface IgA+ B cells than both surface IgD+ B cells and total B cells, and higher but not significantly in surface IgD+ B cells compared to total B cells. This confirms that the sorted cell populations are phenotypically as expected; surface IgD+ B cells are mainly producing IgD and surface IgA+ B cells are mainly producing IgA1.

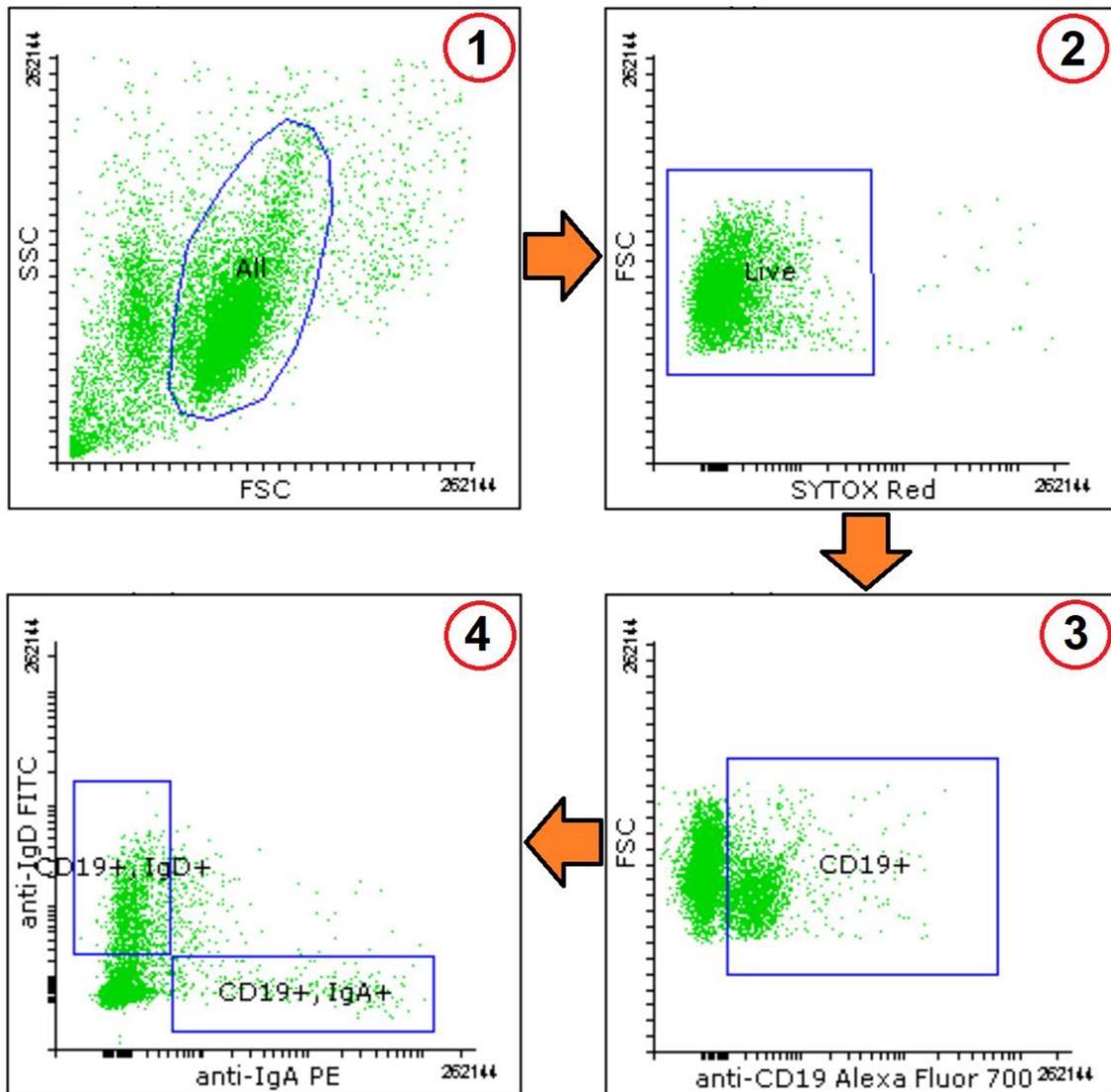


Figure 4.2: FACS profile of sorted B cells: Flow cytometric profile of the gating strategy for sorting IgA+ and IgD+ B cells from whole B cell-enriched PBMCs; using regions of positive and negative fluorescence of CD19-AlexaFluor700, IgA-PE and IgD-FITC, with SYTOX red as the live/dead cell stain. Cells sorted from 9 healthy volunteers.

Gating strategy:

- 1; Lymphocytes identified by size.
- 2; Live lymphocytes identified by low to negative SYTOX red fluorescence.
- 3; B cells, identified as surface CD19+.
- 4; Surface IgA+ and surface IgD+ B cells collected (CD19+, IgA+ and CD19+, IgD+).

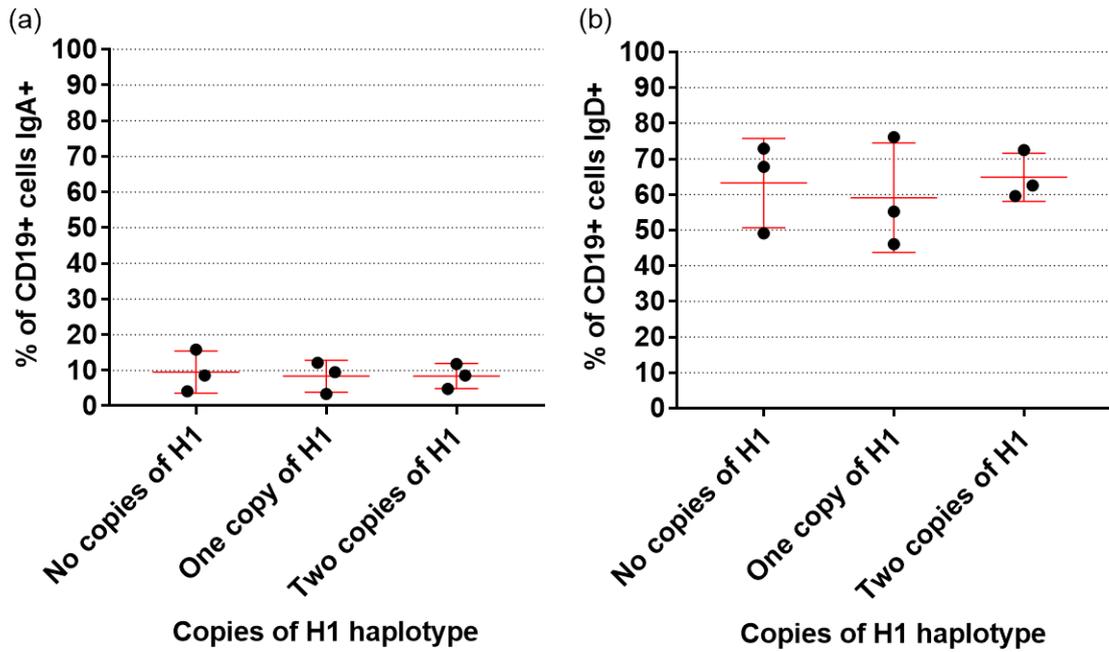


Figure 4.3: Proportions of surface IgA+ and surface IgD+ B cells sorted from B cell enriched PBMCs: Actual numbers of collected surface IgA+ and surface IgD+ B cells varied from person to person, but as a percentage of total B cells (surface CD19+) there was no difference between people with none, one or two copies of the H1 haplotype of C1GALT1. (a) % of surface CD19+ cells that are surface IgA+. (b) % of surface CD19+ cells that are surface IgD+. Graphs show mean % \pm SD. Analysis performed using one-way ANOVA with post-hoc Tukey test.

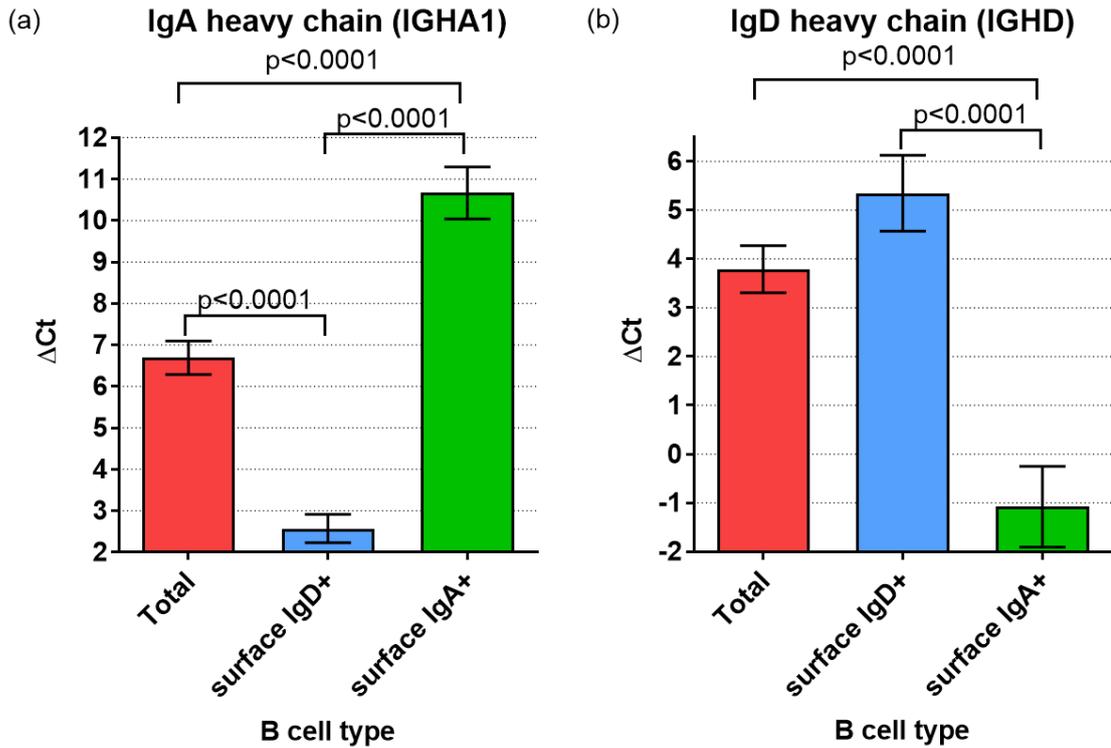


Figure 4.4: IGHA1 and IGHG mRNA expression in total, surface IgA+ and surface IgD+ B cells: mRNA expression of the IgA (a) and IgD (b) heavy chains (IGHA1 and IGHG respectively) in 9 healthy volunteers. Significantly higher IGHA1 expression in surface IgA+ B cells compared to surface IgD+ B cells, and significantly higher IGHG expression in surface IgD+ B cells compared to surface IgA+ B cells confirms phenotype of sorted cells. n=9 for each bar on the graphs. Graphs show mean \pm SD. Analysis was performed on the normalised ΔCt values with PSMB6 as the endogenous reference gene (ERG) and using unpaired t-tests.

4.3.5 – Surface IgA+ B cells express mRNA for glycosylation enzymes at different levels compared to surface IgD+ B cells

Next, mRNA expression of the enzymes involved in IgA1 O-glycosylation was measured in the three cell types sorted from the combined nine volunteers (Figure 4.5). In these, mRNA for the galactosyltransferase enzyme C1GalT1 showed no significant differences between the cell types, but a trend for lower expression in surface IgA+ B cells was observed. The C1GalT1-specific chaperone protein, Cosmc, has two transcript variants of its mRNA formed by alternative splicing, both encoding the same protein. Both transcript variants of COSMC showed significantly elevated mRNA levels in surface IgA+ B cells compared to total B cells and surface IgD+ B cells. Levels of the mRNA for the sialyltransferase ST6GalNAcII were also significantly elevated in surface IgA+ B cells compared to total B cells and surface IgD+ B cells. These results serve to confirm the action of maturation-dependent transcriptional regulation of glycosylation enzymes in B cells during class switching from naïve B cells to IgA producing B cells.

4.3.6 – Lower C1GALT1 expression in IgA producing B cells is accentuated with homozygosity for the H1 haplotype

The results of the C1GALT1 mRNA expression in B cells from the nine volunteers were next split based on the presence or absence of the H1 haplotype of C1GALT1 (three homozygous for the H1 haplotype, three heterozygotes and three homozygous negative) (Figure 4.6). From this, it was evident that the lower expression of C1GALT1 mRNA seen in surface IgA+ B cells in the whole data set was significantly accentuated by homozygosity for the H1 haplotype. This means that the H1 haplotype has a significant effect on maturation-dependent transcriptional regulation of the C1GALT1 gene.

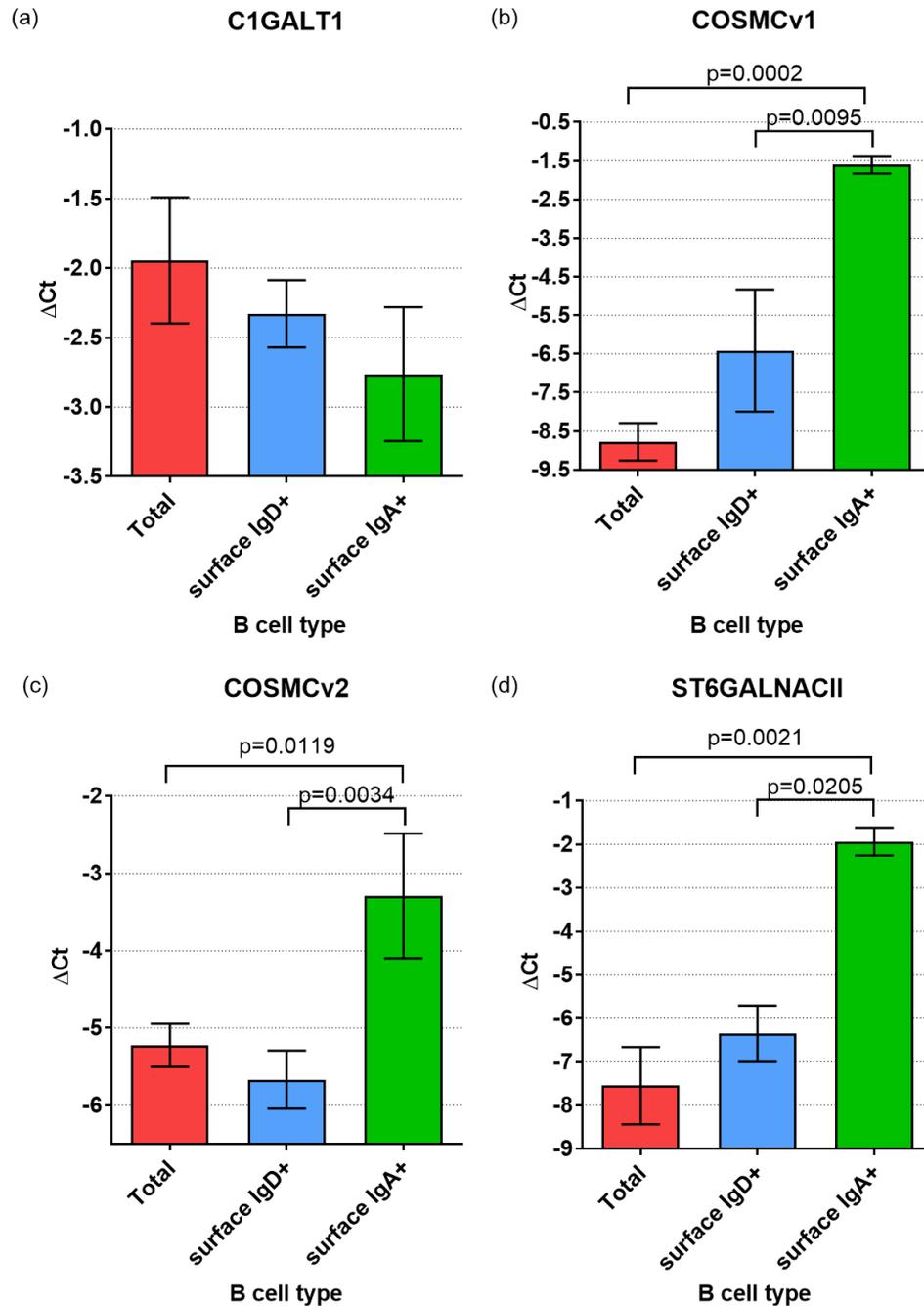


Figure 4.5: C1GALT1, COSMCv1, COSMCv2 and ST6GALNACII mRNA expression in total, surface IgA+ and surface IgD+ B cells: mRNA expression of (a) C1GALT1; (b) COSMC transcript variant 1; (c) COSMC transcript variant 2 and (d) ST6GALNACII in 9 healthy volunteers. A trend for lower expression of C1GALT1 in IgA+ cells is seen. Significant differences between cell types are seen in the expression of both transcript variants of COSMC and ST6GALNACII. n=9 for each bar on the graphs. Graphs show mean \pm SD. Analysis was performed on the normalised Δ Ct values with PSMB6 as the ERG and using one-way ANOVA with post-hoc Tukey tests.

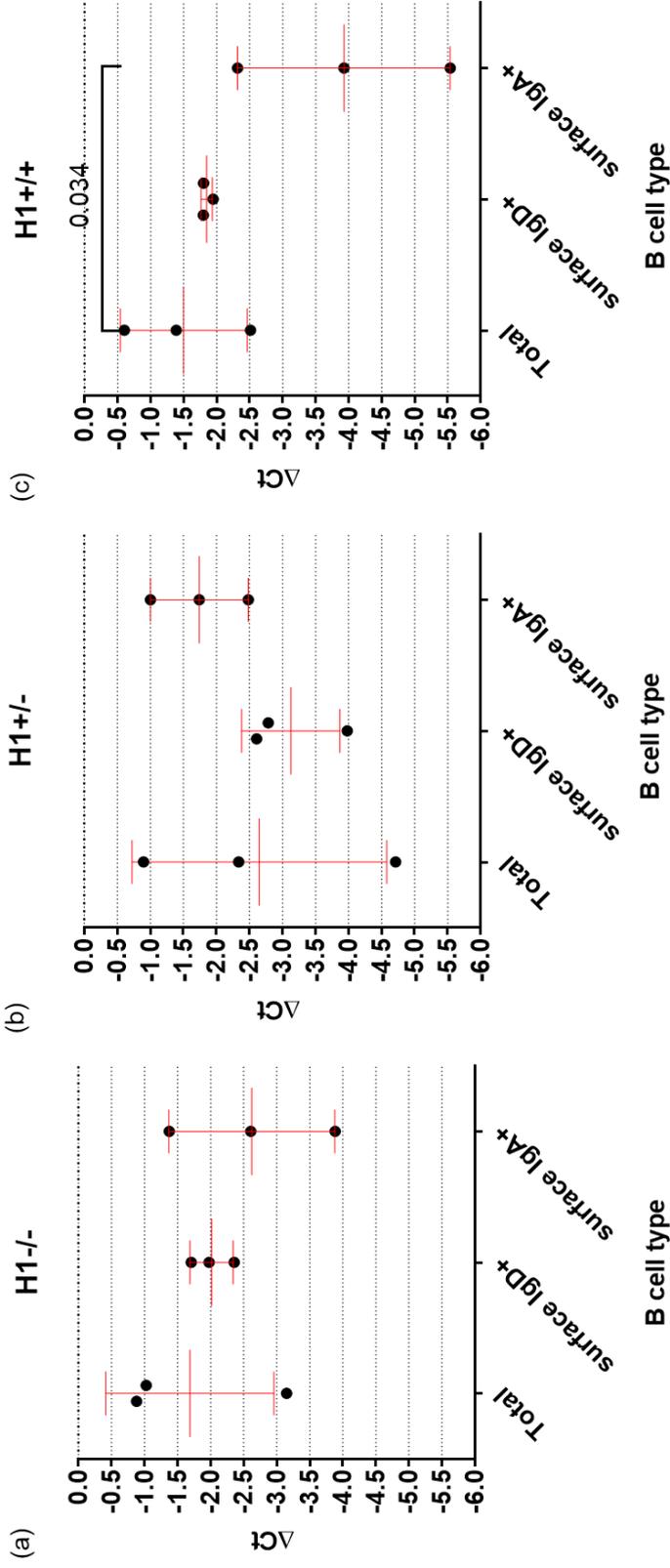


Figure 4.6: C1GALT1 mRNA expression in total, surface IgA+ and surface IgD+ B cells by number of copies of the H1 haplotype: Expression of C1GALT1 in cells from 9 healthy volunteers; (a) 3 homozygous for H1; (b) 3 heterozygous and (c) 3 homozygous negative for H1. Lower expression of C1GALT1 seen in the combined 9 healthy subjects (Fig. 4.5 (a)) is significantly accentuated with homozygosity for the H1. n=3 for each bar on the graphs. Graphs show mean \pm SD. Analysis was performed on the normalised ΔC_t values with PSMB6 as the ERG and using one-way ANOVA with post-hoc Tukey tests.

4.3.6 – Allele-specific qPCR for H1-linked C1GALT1 coding region SNP

The H1 haplotype itself is in the non-coding region of C1GALT1. A SNP in the coding region of C1GALT1 (rs10251492) was found to be associated with both the H1 haplotype and Gd-IgA1 levels (Supplementary table S4.1). The 50 volunteers used here were genotyped for this SNP. In these subjects, the linkage of this SNP did not match the H1 haplotype; one of the H1 homozygous negatives and two of the H1 homozygous positives were heterozygous for the linked SNP. However, when reanalysing serum Gd-IgA1 levels based on copies of the H1 linked SNP, the same pattern seen with copies of the H1 haplotype of increasing serum Gd-IgA1 levels with copies of the linked SNP was seen (Supplementary figure S4.1). When also reanalysing the C1GALT1 mRNA expression in the sorted cells based on copies of the H1-linked SNP, homozygosity for the linked SNP produced the lowest C1GALT1 mRNA specifically in surface IgA+ B cells; as was seen with the H1 haplotype analysis (Supplementary figure S4.2). Statistical analysis could not be made on this, as only one of the nine volunteers that cells were sorted from was found to be homozygous for the H1-linked SNP.

The allele-specific qPCR methodology used here was intended to measure allele-specific expression differences in C1GALT1 in heterozygotes for the H1 haplotype. This method was confirmed to work as expected in cDNA, as the results matched the genotype in samples from total B cells. However, the small amount of cDNA available in surface IgD+ B cells and especially surface IgA+ B cells meant that a complete data set was not able to be produced (Supplementary table S4.2).

4.3.7 – Protein expression of glycosylation enzymes

Several methods were attempted to measure the levels of translated C1GalT1 in lysates from the sorted cells, in order to see if the maturation-dependent regulation extended from transcription of mRNA to translation of the protein. First, Western blotting of serially diluted recombinant C1GalT1 (ProSpec) showed that the lower limit of detection was around 5ng, equating to around 20,000 cells (Supplementary figure S4.3). As this was not sensitive enough to measure C1GalT1 in the sorted cell populations, an ELISA method was

developed and optimised with a detection range of 10ng/ml to 0.1ng/ml, using a standard curve constructed from serially diluted C1GalT1 (section 2.5.4). However, this still proved not sensitive enough to measure C1GalT1 in the lysates from the low cell number populations sorted.

The next attempt to measure C1GalT1 protein involved first sending test samples (around 20,000 surface IgD+ B cells each) to Proteomics RTP at the University of Warwick to attempt quantification of C1GalT1 using mass spectrometry. While a small signal was observed for general cellular proteins in these samples, this was too weak for any quantification of C1GalT1 itself (see Appendix III for mass spectrometry report).

In a final attempt to measure C1GalT1 in these samples, Merck were contracted to develop an assay using their Single Molecule Counting (SMC) technology. In brief, this technology is similar to traditional ELISAs, but utilises a unique elution step and digital counting to achieve much improved signal to noise ratios; providing possible quantification at a much lower level. A prototype assay was developed and optimised by Merck, achieving a low limit of quantification of 25pg/ml. Unfortunately, most of the samples sent to Merck for testing were below this limit (see Appendix IV for SMC assay development and sample testing report); meaning that quantification of C1GalT1 protein in these samples was unsuccessful, regardless of the method attempted.

4.4 – Discussion

Several studies in IgAN have looked at the expression and activity of C1GalT1 and Cosmc in unsorted B cells, finding decreased expression of C1GALT1 and COSMC mRNA, and decreased activity translated C1GalT1 and Cosmc (Allen *et al*, 1997; Qin *et al*, 2005; Buck *et al*, 2008; Inoue *et al*, 2010). However, the studies themselves are inconsistent with the findings. They also focussed only on total B cells, not taking into account any effect of maturation-dependent transcriptional regulation; as is the case in IgAN with no aberrancy in IgD hinge region O-glycosylation observed (Smith, De Wolff *et al*, 2006). The work in this project, while limited by numbers of subjects for analysis, is the first such looking at transcription differences in glycosyltransferases between naïve B cells and IgA producing B cells.

The significant differences seen in this project in the expression of COSMC and ST6GALNACII between surface IgD+ and surface IgA+ B cells reinforce the idea that in the process of naïve B cells (IgD+) differentiating and undergoing class switch recombination to IgA producing cells, alterations occur in transcriptional regulation of the mechanism of O-glycosylation. While no significant difference in C1GALT1 mRNA expression was seen between surface IgA+ and surface IgD+ B cells, there was an observable lower apparent expression in the surface IgA+ B cells; consistent with maturation-dependent transcriptional regulation.

Previously published data linking genetic variation with gene expression in lymphoblastoid cell lines has shown that SNPs on the H1 haplotype of C1GALT1 are strongly associated with reduced transcription of C1GALT1 (Yu, Pal and Moul, 2016). Other in silico analysis has shown that some SNPs of the H1 haplotype are predicted to lie within transcription factor binding elements; one at a predicted SOX2-OCT4 site and another within the promotor region of C1GALT1 and predicted to affect binding of RUNX3 (J. Wang *et al*, 2012; Watanabe *et al*, 2010). The H1 haplotype of C1GALT1 is strongly associated with serum Gd-IgA1 levels, and this project has shown a significant reduction of C1GALT1 mRNA with homozygosity for the H1 haplotype specific to IgA producing cells, which begins to confirm the prediction from the in silico studies; with the caveat that higher numbers of samples are required for full confirmation of this.

With regards to the SNP in the coding region of C1GALT1 linked to the H1 haplotype; the subjects in this study that did not seem to show this association are of South Asian ancestry. The linkage of this SNP has only been determined so far in Caucasians, which could account for the seeming lack of linkage in these subjects. Regardless of this, the pattern of reduced C1GALT1 mRNA expression was the same whether organised by H1 or H1-linked SNP.

In summary, the work in this chapter has confirmed the action of maturation-dependent transcriptional regulation of glycosyltransferase enzymes in the development of B cells. It has also been shown that transcription of C1GALT1 is reduced by the H1 haplotype of C1GALT1, which in turn is also

maturation-dependent. During this work, new assays for quantification of translated C1GalT1 protein have been developed, namely an ELISA method and an SMC assay, which could aid in future studies of this nature.

Chapter 5 – Modulation of IgA1 O-glycosylation

5.1 – Introduction and aims

The mechanism behind the aberrant production of Gd-IgA1 in IgAN patients is still largely not well understood. Several studies looking at cytokine control of this process have been carried out, demonstrating that galactosylation of the IgA1 hinge region can be reduced by the action of a variety of cytokines; in an immortalised IgA1 producing cell line (DAKIKI cells) by stimulation with a major virulence factor of *Helicobacter pylori*, CagA, and with the cytokines IL-4, TGF- β and IL-17 (Yang *et al*, 2014; Yamada *et al*, 2010; Xiao *et al*, 2017; Lin *et al*, 2018), and also in immortalised IgA1 producing cells isolated from healthy subjects and IgA nephropathy patients that were stimulated with IL-4 and IL-6 (H. Suzuki *et al*, 2014). All studies so far have identified the reduction of galactosylation at the IgA1 hinge region as a direct result of reductions in C1GalT1 leading to less galactose addition. Some have also found this to be compounded indirectly by reductions in Cosmc resulting in less activated C1GalT1, and others found increased levels of ST6GalNAcII to be present leading to blocking of galactosylation by premature sialylation of GalNAc residues.

The aim of the work in this chapter was to study the effect of novel cytokines and chemokines on the mechanism of O-glycosylation of the IgA1 hinge region in IgA1 producing cells.

5.2 – Methods

The DAKIKI cell line (ATCC) is a surface IgA1+ B lymphoblast cell line immortalised by transformation with EBV (Steinitz and Klein, 1980), making them a suitable cell line for experiments involving IgA1 O-glycosylation. To characterise the DAKIKI cells, 1×10^5 cells were cultured in 5ml media for 48 hours in a 25cm² culture flask. The culture supernatants were analysed for total IgA and Gd-IgA1 levels by ELISA (sections 2.5.1 and 2.5.2). The cells were phenotyped flow cytometrically (section 2.7, without cell collection); with surface staining for CD19 as the marker of B cell lineage, CD20 as a positive marker of memory B cells, CD38 and CD138 as markers of plasmablasts and plasma cells (Robillard

et al, 2014), as well as IgA and IgD. Details of the antibodies and the working volumes used here are shown in Supplementary table S2.1. An aliquot of cells was also lysed in TRIzol and mRNA isolated for gene expression analysis by qPCR (section 2.9) of C1GALT1, COSMC, ST6GALNACII, IGHA1 and IGHD. A comparison of mRNA expression was made with IgA+ B cells from Chapter 4. Genomic DNA from these cells was also genotyped (section 2.3) for the H1 haplotype identified in Chapter 3.

For the first experiment in modulating IgA1 O-glycosylation, a dose-response method was used. Cytokines were selected based on their functional role in B cell activation, regulation of IgA synthesis or homing of B cells:

- TGF- β (Sigma T7039) for its function in inducing C α 1/2 germline transcripts, in effect inducing the IgA response (Sonoda *et al*, 1992), and as the positive control based on previously published data on DAKIKI stimulation (Xiao *et al*, 2017).
- CD40L (Sigma GF101) for its function as a T cell-dependent activator of naïve B cells (Elgueta *et al*, 2009; Lafarge *et al*, 2011).
- TNF α (Sigma H8916) for its role in regulation of IgA synthesis (Tezuka *et al*, 2007).
- APRIL (Sigma SRP3008) and BAFF (Sigma GF136) for their similar role in T cell independent activation of naïve B cells (Litinskiy *et al*, 2002; Darce *et al*, 2007). APRIL for its role in the survival of plasmablasts in the bone marrow (Belnoue *et al*, 2008) and BAFF also for its role in the survival of transitional B cells (Rowland *et al*, 2010).
- IL-10 (Sigma SRP3071) for its role in inducing differentiation of IgA plasma cells (Marconi *et al*, 1998; Hirano *et al*, 2003; Lafarge *et al*, 2011).
- MAdCAM-1 (R&D 6065-MC-050) and BCA-1 (Sigma B2929) as ligands for mucosal homing receptors on B cells (Briskin *et al*, 1997; Legler *et al*, 1998; Sakai and Kobayashi, 2015).

Cytokine concentration ranges were selected based on published serum concentrations (Table 5.1). Stimulation took place for 48 hours before harvesting (section 2.11). Dose-response experiments were repeated three times.

Cytokine:	Concentration range:
TGF- β 1	5-80ng/ml
CD40L	5-80ng/ml
TNF α	5-80pg/ml
APRIL	0.4-6.4ng/ml
BAFF	0.2-3.2ng/ml
IL-10	1-16pg/ml
MAdCAM-1	0.2-3.2ng/ml
BCA-1	20-320pg/ml

Table 5.1: Cytokines and concentration ranges for dose-response stimulation of DAKIKI cells: Concentration ranges of cytokines for DAKIKI stimulation were determined based on previously reported serum concentrations.

For the second experiment, cytokines with significant results from the dose-response experiment were selected for a time-course study. Time points for harvesting samples of cells and culture supernatant were at 1 hour, 12 hours, 24 hours and 48 hours. Time-course experiments were repeated six times.

Samples were analysed for total IgA and Gd-IgA1 levels by ELISA in culture supernatants, results presented as IgA concentration ($\mu\text{g/ml}$) and fold difference to unstimulated cells in Gd-IgA1/ μg IgA. Due to a shortage of HA lectin for use in the ELISA method of Gd-IgA1 measurement, there has been a shift to another lectin from *Vicia villosa* (VV). This lectin directly replaces HA in the ELISA and has been used as such in studies of IgAN, as it binds the same terminal O-linked GalNAc epitope (Tollefsen and Kornfeld, 1983). It was also used recently to measure Gd-IgA1 levels in the study from which the positive control for this chapter, TGF- β , was selected (Xiao *et al*, 2017). The Gd-IgA1 measurement made using VV lectin was found to correlate extremely strongly with the same measurement made with HA lectin in both serum and DAKIKI culture supernatant (data not shown). For the dose-response experiments, comparison ELISAs were used with HA and VV lectin binding; after which only VV lectin was used in the time-course.

After isolation of mRNA and protein from the lysed cells in the time-course experiments, mRNA expression of GALNT2, C1GALT1, COSMC, ST6GALNACII and IGHA1 was measured by qPCR (section 2.9.1.3), with IGHA1 used as the ERG. Levels of translated C1GalT1 protein from the time-course experiment were analysed in cell lysates by Western blot and normalised to β -actin on the same blot (section 2.10, see Supplementary table S2.2 for antibody dilutions). Selected mRNA samples from the time-course experiments were subjected to multiplex gene expression analysis using the Nanostring nCounter Immunology Panel (section 2.9.3).

5.3 – Results 1 – Characteristics of DAKIKI cells

5.3.1 – mRNA expression compared to IgA+ B cells

To firstly assess the expression levels of glycosylation enzymes in DAKIKI cells, the mRNA expression of C1GALT1, COSMC, ST6GALNACII, IGHA1 and IGHD in DAKIKI cells was measured and compared to the expression data from surface IgA+ B cells isolated from healthy subjects (see Chapter 4) (Figure 5.1). Significant differences were seen in C1GALT1 and ST6GALNACII; C1GALT1 was higher in DAKIKI cells and ST6GALNACII was lower in DAKIKI cells. No IGHD expression was seen in DAKIKI cells.

5.3.2 – Flow cytometric phenotyping of DAKIKI cell surface markers

In order to phenotypically characterise the DAKIKI cells, they were stained for a range of B cell surface markers for flow cytometric profiling. The surface markers of the DAKIKI cells led to characterisation as follows: cells are of B cell lineage (CD19+), share characteristics of memory B cells and plasma cells (CD20+, CD38+ and CD138+) and are completely class switched to IgA (IgA+ and IgD-) (Figure 5.2).

5.3.3 – FPLC chromatogram of purified DAKIKI IgA

It was next necessary to identify whether the IgA produced by DAKIKI cells was monomeric or polymeric, and how it compared to normal serum IgA. To this end, IgA was purified from DAKIKI culture supernatant after one week of incubation. This IgA was then separated by size using an FPLC and the resulting chromatogram compared with a chromatogram of normal serum IgA. DAKIKI IgA was found to be mostly monomeric, with a small amount of polymeric most likely formed by self-aggregation in the culture supernatant after secretion (Figure 5.3). This analysis was performed by Jasraj Bhachu.

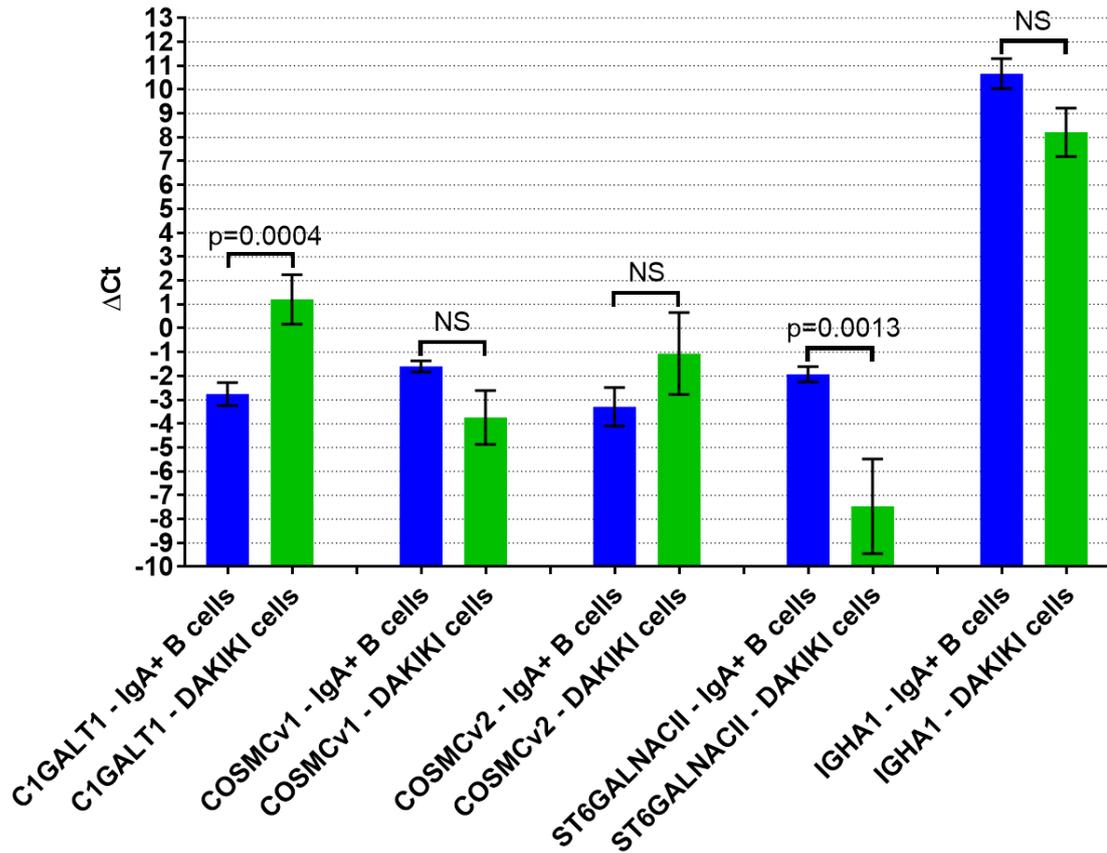


Figure 5.1: mRNA expression in DAKIKI cells compared to surface IgA+ B cells: Significant differences in mRNA expression are seen between surface IgA+ B cells from healthy subjects and DAKIKI cells in C1GALT1 and ST6GALNACII. mRNA for both transcript variants of COSMC and IGH A1 show no significant differences. Graph shows mean \pm SD. Analysis was performed on the normalised ΔCt values with PSMB6 as the ERG and using unpaired t-tests.

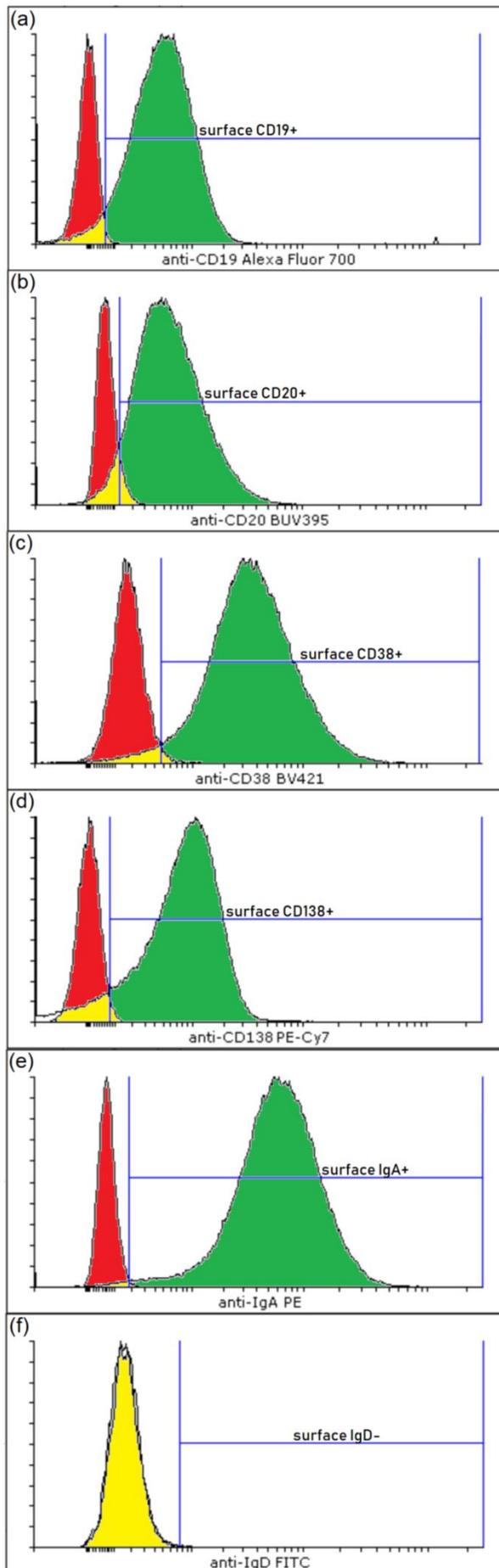


Figure 5.2: Flow cytometric phenotyping of DAKIKI cell surface markers: Green histograms are cells stained with fluorescently conjugated antibodies for surface markers, red histograms show unstained cells (yellow parts delineate overlap of positive and negative staining):

- (a) anti-CD19 AlexaFluor700
- (b) anti-CD20 BUV395
- (c) anti-CD38 BV421
- (d) anti-CD138 PE-Cy7
- (e) anti-IgA PE
- (f) anti-IgD FITC

Characteristics of the cells are as follows: B cell lineage (surface CD19+), share characteristics of memory B cells and plasma cells (surface CD20+, CD38+ and CD138+), completely class switched to IgA (surface IgA+ and IgD-).

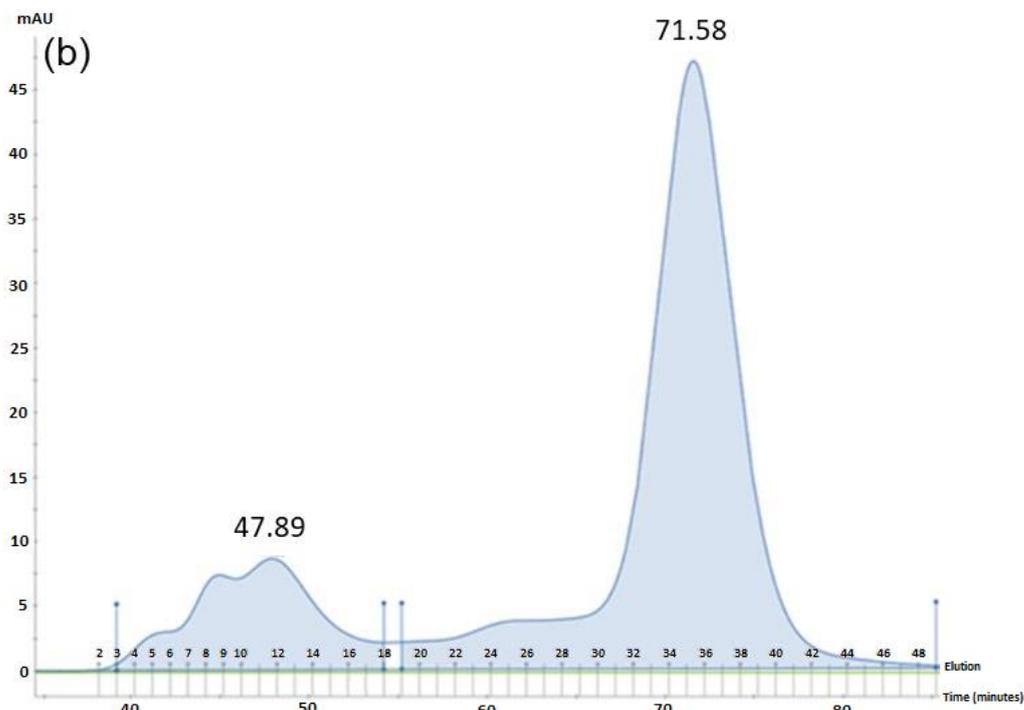
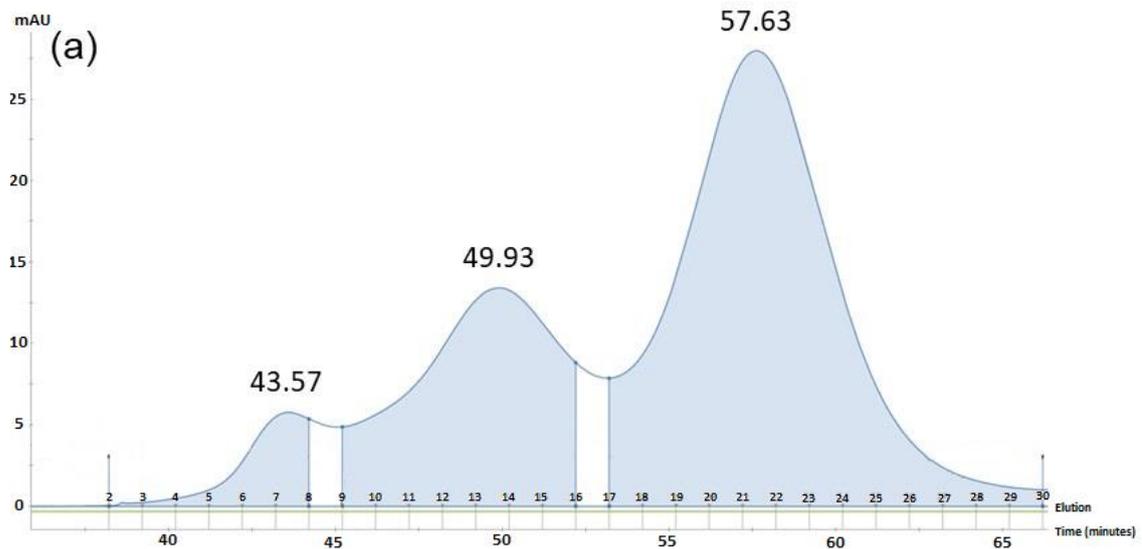


Figure 5.3: FPLC chromatograms of IgA purified from human serum and DAKIKI cell culture supernatant: Comparison of IgA purified from serum from a healthy subject (a) and IgA purified from DAKIKI cell culture supernatant after one week of culture (b). Serum IgA (a) shows normal pattern, with polymeric IgA (peak 43.57), dimeric IgA (peak 49.93) and monomeric IgA (peak 57.63). IgA produced by DAKIKI cells (b) shown to be mostly monomeric (peak 71.58); with the multiple peak at 47.89 potentially due to self-aggregation of IgA in the supernatant.

5.3.4 – IgA synthesis rate in DAKIKI cells and hinge region glycosylation of DAKIKI IgA

An estimate of the IgA1 synthesis rate was next made by measuring the amount of total IgA produced into culture supernatant by 1×10^5 DAKIKI cells cultured for 48 hours by ELISA. This was found to be around $0.3 \mu\text{g}$ (data not shown). HA lectin binding ELISA of the same culture supernatant showed this IgA1 to be highly galactosylated, giving low OD readings (data not shown).

5.4 – Results 2 – Dose-response stimulation of DAKIKI cells with cytokines

5.4.1 – Secreted IgA levels after 48hr stimulation of DAKIKI cells with cytokines

After 48hrs of stimulation with the cytokines in a dose-response manner, as described in Table 5.1, no significant differences in the amount of IgA produced was seen compared to unstimulated cells with any of the conditions (Figure 5.4). This means that none of the cytokines studied here had an impact on the production of IgA, unlike the previous report of TGF- β causing a decrease in IgA1 synthesis. This could be due to methodological differences such as differing numbers of cells used for the experiment, the size of the culture plate used, the measurement of IgA1 itself or the supplier of the TGF- β used for stimulation.

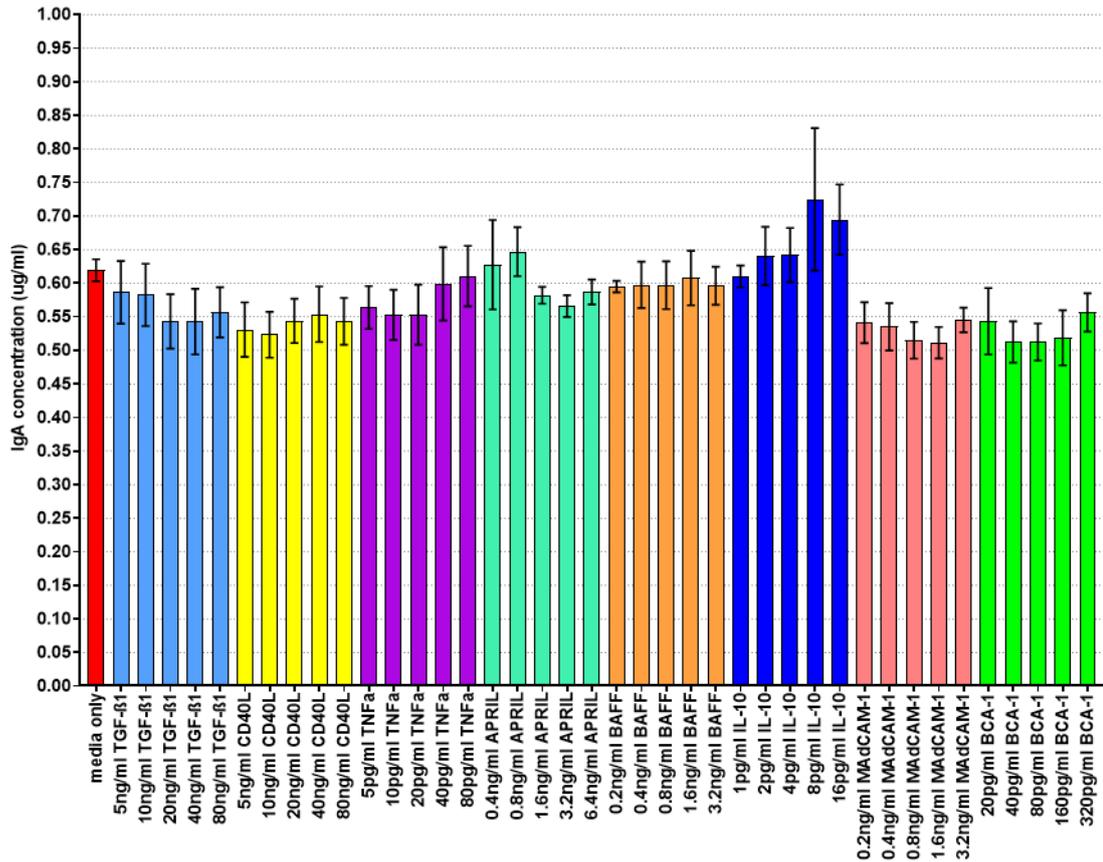


Figure 5.4: Secreted IgA levels after 48hr dose-response stimulation of DAKIKI cells with cytokines: No significant differences seen in IgA in culture supernatants after 48 hour stimulation of DAKIKI cells with cytokines in a dose-response manner as measured by IgA ELISA. Graph shows mean \pm SD. n=3, data analysed by individual unpaired t-tests comparing each condition to unstimulated.

5.4.2 – Secreted Gd-IgA1 levels after 48hr stimulation of DAKIKI cells with cytokines

Upon measuring the levels of Gd-IgA1 in the culture supernatants, five of the nine cytokines used to stimulate DAKIKI cells showed significant increases in Gd-IgA1 levels (fold change Gd-IgA1/ μ g IgA compared to unstimulated) using both HA and VV lectin binding ELISAs. These were: TGF- β (80, 40 and 20ng/ml), 3.2ng/ml APRIL, 0.4ng/ml BAFF, 1.6ng/ml MAdCAM-1 and BCA-1 (160, 80, 40 and 20pg/ml) (Figures 5.5 & 5.6). One concentration of each of these was utilised in the time-course experiment.

Differences between the binding of lectin from HA and from VV were evident here, this is potentially due to the different specificities of the two lectins. HA lectin has been found to have specific binding to IgA1 O-linked GalNAc, while VV lectin can also bind to other proteins such as IgA2 and IgG (Moore *et al*, 2007); and while neither of these are produced by DAKIKI cells, there may be other proteins produced by these cells or in the culture supernatant itself that can complex with the secreted IgA1 and have binding affinity for VV lectin.

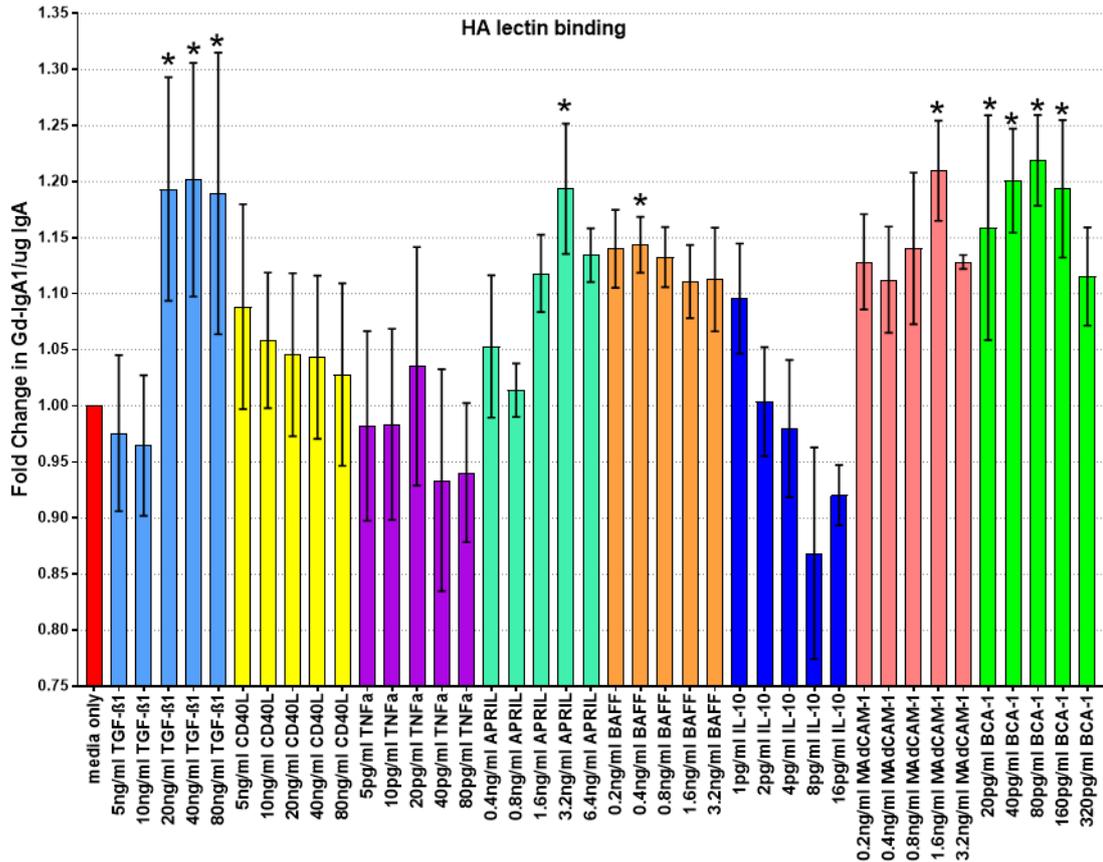


Figure 5.5: Secreted Gd-IgA1 levels after 48hr dose-response stimulation of DAKIKI cells with cytokines: Graph showing changes in Gd-IgA1 levels in culture supernatants from DAKIKI cells stimulated for 48 hours with cytokines in a dose-response manner (* indicates significant change compared to unstimulated cells). Gd-IgA1 levels measured by HA lectin binding ELISA, normalised to total IgA and presented as fold change in Gd-IgA1/μg IgA compared to unstimulated cells (media only). Graph shows mean ± SD. n=3, data analysed by individual unpaired t-tests comparing each condition to unstimulated.

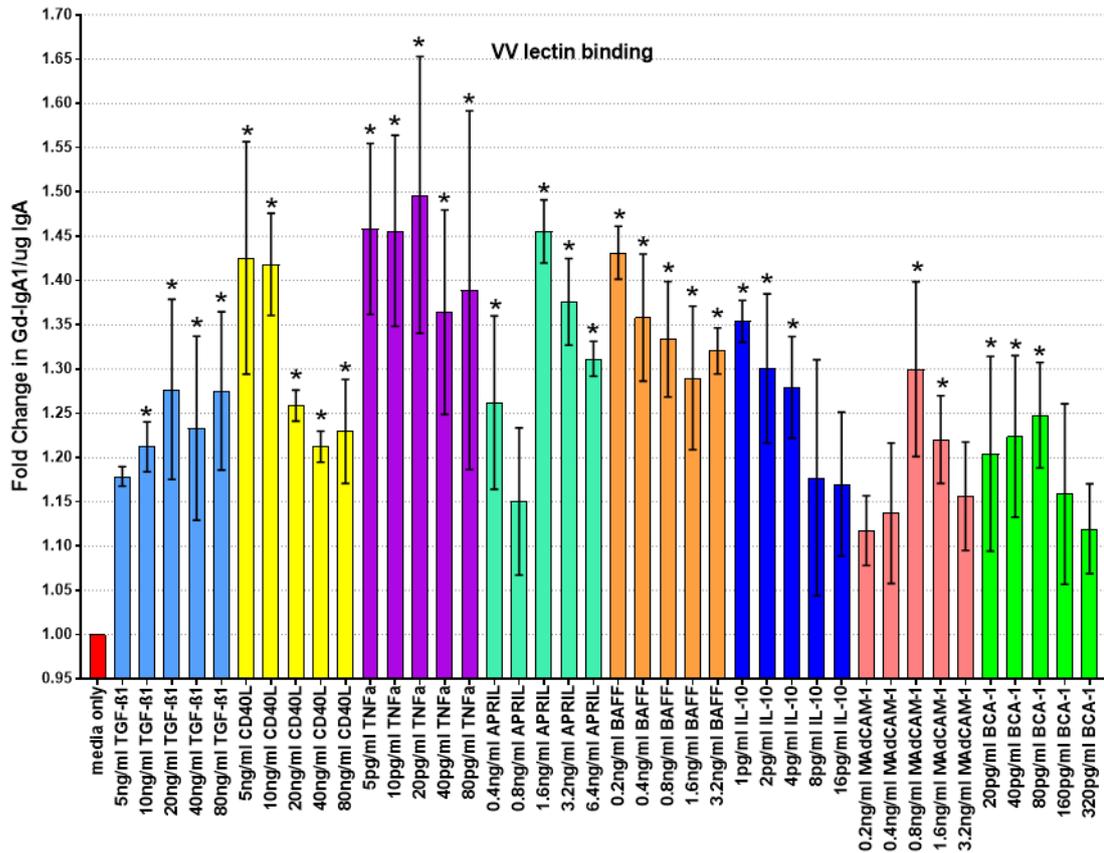


Figure 5.6: Secreted Gd-IgA1 levels after 48hr dose-response stimulation of DAKIKI cells with cytokines: Graph showing changes in Gd-IgA1 levels in culture supernatants from DAKIKI cells stimulated for 48 hours with cytokines in a dose-response manner (* indicates significant change compared to unstimulated cells). Gd-IgA1 levels measured by VV lectin binding ELISA, normalised to total IgA and presented as fold change in Gd-IgA1/μg IgA compared to unstimulated cells (media only). Graph shows mean ± SD. n=3, data analysed by individual unpaired t-tests comparing each condition to unstimulated.

5.5 – Results 3 – Time-course stimulation of DAKIKI cells with cytokines

5.5.1 – Secreted IgA and Gd-IgA1 levels after 48hr stimulation of DAKIKI cells with cytokines

Consistent with the dose-response experiment, the five cytokines used to stimulate DAKIKI cells in the time-course experiment (40ng/ml TGF β , 3.2ng/ml APRIL, 0.4ng/ml BAFF, 1.6ng/ml MAdCAM-1 and 80pg/ml BCA-1) did not cause any changes in IgA production compared to unstimulated cells after 48hrs (Figure 5.7), while still causing a significant increase in Gd-IgA1 levels (Figure 5.8). This serves to validate these cytokines as factors that can reduce the galactosylation of IgA1 in DAKIKI cells.

5.5.2 – Effect of time-course cytokine stimulus on DAKIKI glycosylation enzyme mRNA expression

To further examine the effect of the cytokine stimulus on the reduced galactosylation of DAKIKI IgA1 mRNA expression of the enzymes involved in IgA1 O-glycosylation was measured. Firstly, after 24hr stimulation all of the cytokines caused an increase in GALNT2 mRNA expression compared to unstimulated cells; however, only MAdCAM-1 showed a significant increase (Figure 5.9). GALNT2 encodes the GalNAc transferase enzyme GalNT2, increases of which would potentially lead to more GalNAc addition at the IgA1 hinge region and more available sites for galactosylation.

Expression of mRNA for the galactosyltransferase enzyme C1GalT1 was analysed next. Significant decreases in C1GALT1 mRNA compared to unstimulated cells were seen after 12hrs stimulation of DAKIKI cells with 0.4ng/ml BAFF and after 24hrs with 1.6ng/ml MAdCAM-1 and 80pg/ml BCA-1 (Figure 5.10). Reductions in C1GalT1 potentially would directly lead to less galactosylation of the IgA1 hinge region.

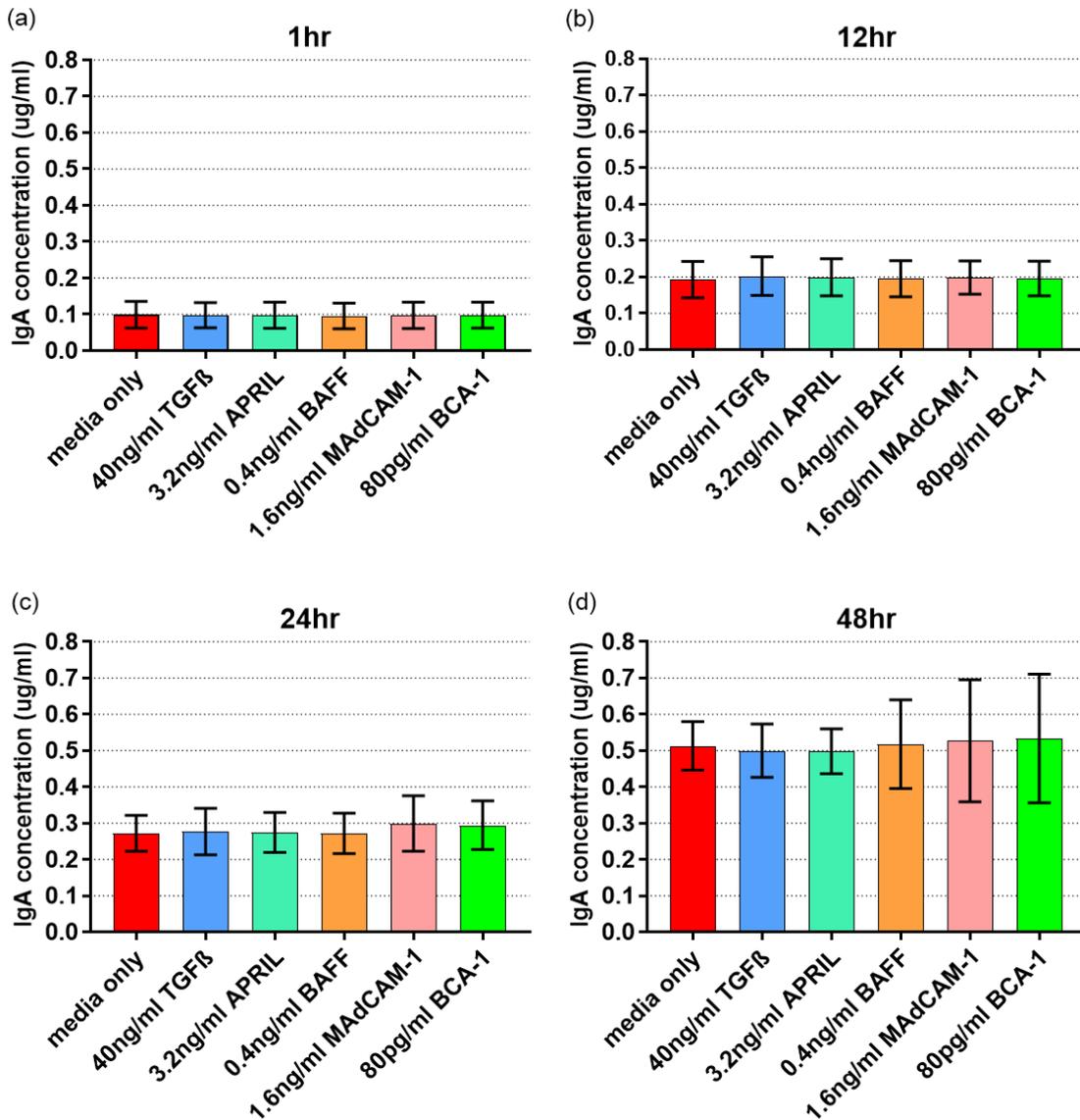


Figure 5.7: Secreted IgA levels after time course stimulation of DAKIKI cells with cytokines: Levels of secreted IgA in DAKIKI culture supernatant increase over time after cells are stimulated with cytokines, but no differences are seen between cells stimulated with any condition and unstimulated cells at all time points. (a) 1hr; (b) 12hr; (c) 24hr; (d) 48hr. Graphs show mean \pm SD. n=6, data analysed by individual unpaired t-tests comparing each condition to unstimulated.

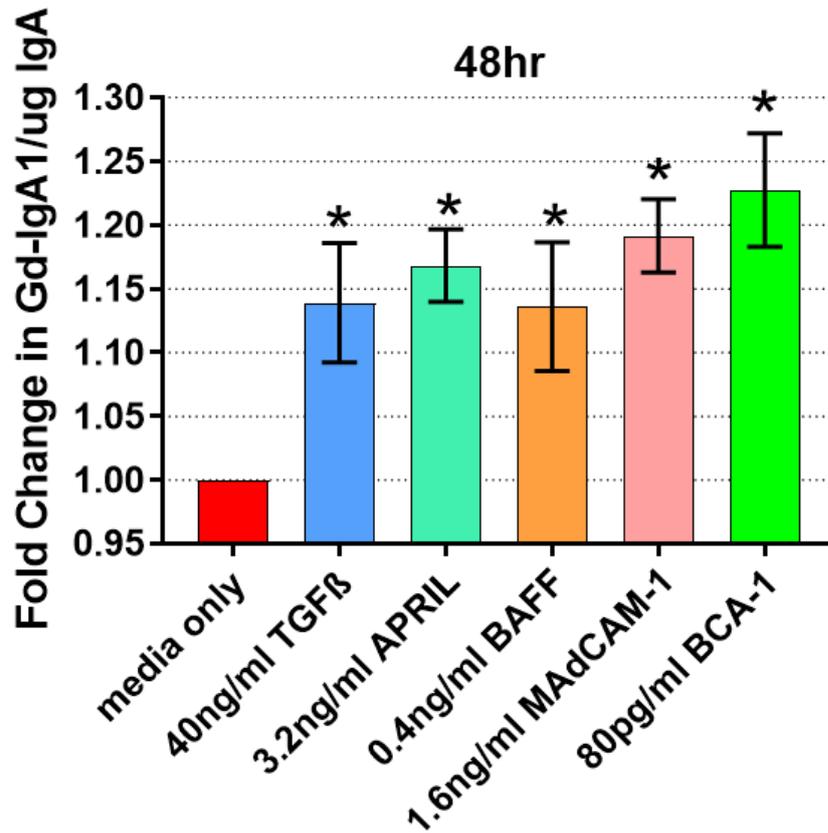


Figure 5.8: Secreted Gd-IgA1 levels after 48 hour stimulation of DAKIKI cells with cytokines: Consistent with the dose-response, stimulation of DAKIKI cells with 80ng/ml TGF- β 1, 3.2ng/ml APRIL, 0.4ng/ml BAFF, 1.6ng/ml MAdCAM-1 or 160pg/ml BCA-1 produces significantly elevated levels of Gd-IgA1 after 48 hours compared to unstimulated as measured by VV lectin binding ELISA. Graph shows mean \pm SD. n=6, Gd-IgA1 levels normalised to total IgA and presented as fold change in Gd-IgA1/ μ g IgA. Graph shows mean \pm SD. n=6, data analysed by individual unpaired t-tests comparing each condition to unstimulated.

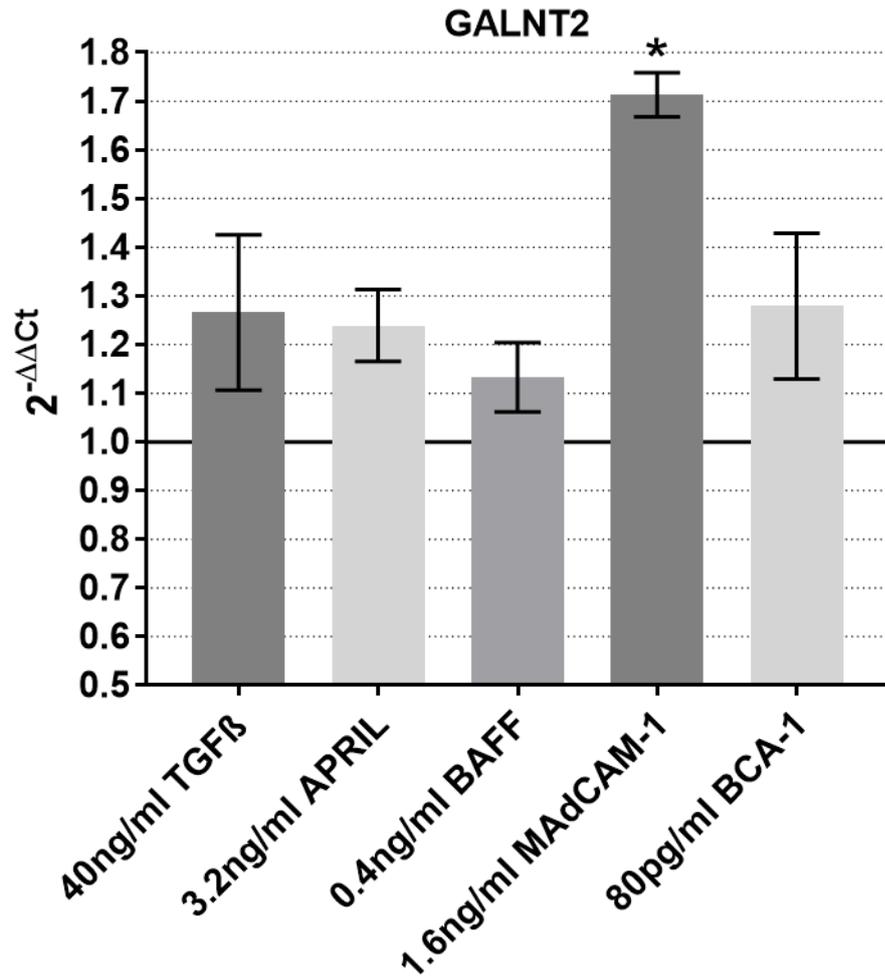


Figure 5.9: GALNT2 mRNA expression in DAKIKI cells after 24hr stimulation with cytokines: Significant increase seen in GALNT2 mRNA expression after 24hr stimulation of DAKIKI cells with 1.6ng/ml MAdCAM-1 compared to unstimulated cells. Graph shows mean \pm SD. n=6, data analysed by the $2^{-\Delta\Delta Ct}$ method and individual unpaired t-tests comparing each condition to unstimulated (1.0).

C1GALT1 mRNA expression

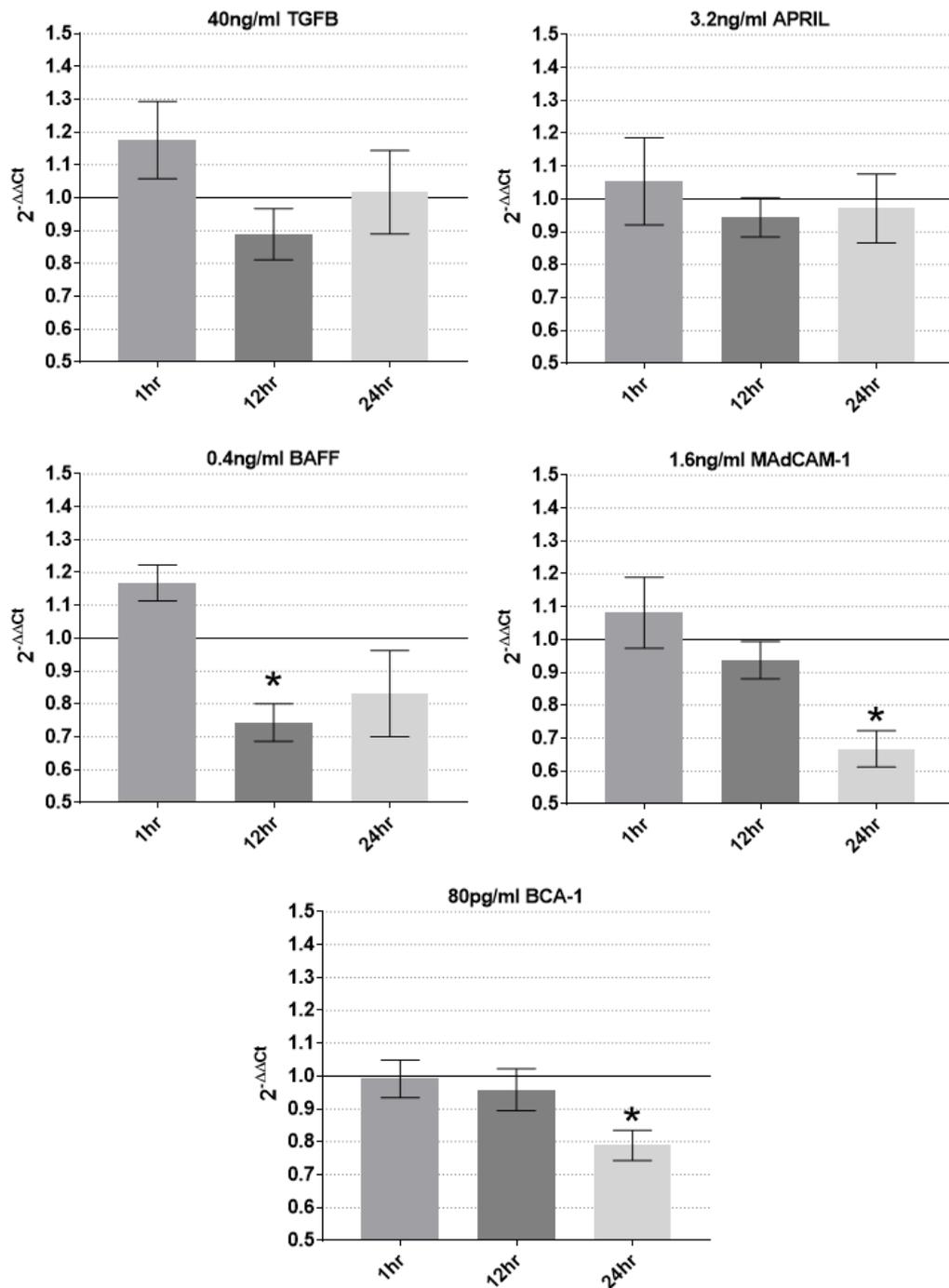


Figure 5.10: C1GALT1 mRNA expression in DAKIKI cells after 1, 12 and 24hr stimulation with cytokines: Significant decreases compared to unstimulated cells in C1GALT1 mRNA seen after 12hrs stimulation of DAKIKI cells with 0.4ng/ml BAFF and after 24hrs with 1.6ng/ml MAdCAM-1 and 80pg/ml BCA-1. Graph shows mean \pm SD. n=6, data analysed by the $2^{-\Delta\Delta Ct}$ method and individual unpaired t-tests comparing each condition and time point to unstimulated at the matching time point (1.0).

Next, mRNA expression of both transcript variants for the C1GalT1 chaperone protein, *Cosmc*, was analysed. Non-significant reductions in COSMC transcript variant 1 mRNA were seen compared to unstimulated cells after 12hrs stimulation with all cytokines (Figure 5.11). Reductions in COSMC transcript variant 2 mRNA were seen at 12 and 24hrs with all conditions, only reaching statistical significance with 24hr stimulation with MAdCAM-1 (Figure 5.12). Indirect reductions in galactosylation could potentially occur with COSMC decreases; less translated *Cosmc* would mean less activated C1GalT1.

Finally, mRNA expression of the sialyltransferase enzyme ST6GalNAcII was analysed. Significant increases compared to unstimulated cells in ST6GALNACII mRNA expression were seen after 12 and 24hrs with 80ng/ml TGF- β and after 24hrs with 3.2ng/ml APRIL, 1.6ng/ml MAdCAM-1 and 160pg/ml BCA-1 (Figure 5.13). ST6GalNAcII adds sialic acid to O-linked GalNAc residues; increases in ST6GalNAcII would therefore potentially cause premature sialylation of GalNAc and block the addition of galactose.

5.5.3 – Reduction in C1GalT1 protein after 24hr stimulation of DAKIKI cells with cytokines

All of the cytokines used in the time-course stimulation of DAKIKI cells caused a significant decrease in the amount of translated C1GalT1 protein compared to unstimulated cells after 24hrs (Figure 5.14). This is a greater decrease than was seen in C1GALT1 mRNA in all conditions and could be the result of the combined decreases in C1GALT1 and COSMC mRNA, but could also involve other factors such as microRNAs targeting C1GALT1 preventing translation, or an effect on increasing the degradation of translated C1GalT1 or *Cosmc*.

COSMCv1 mRNA expression

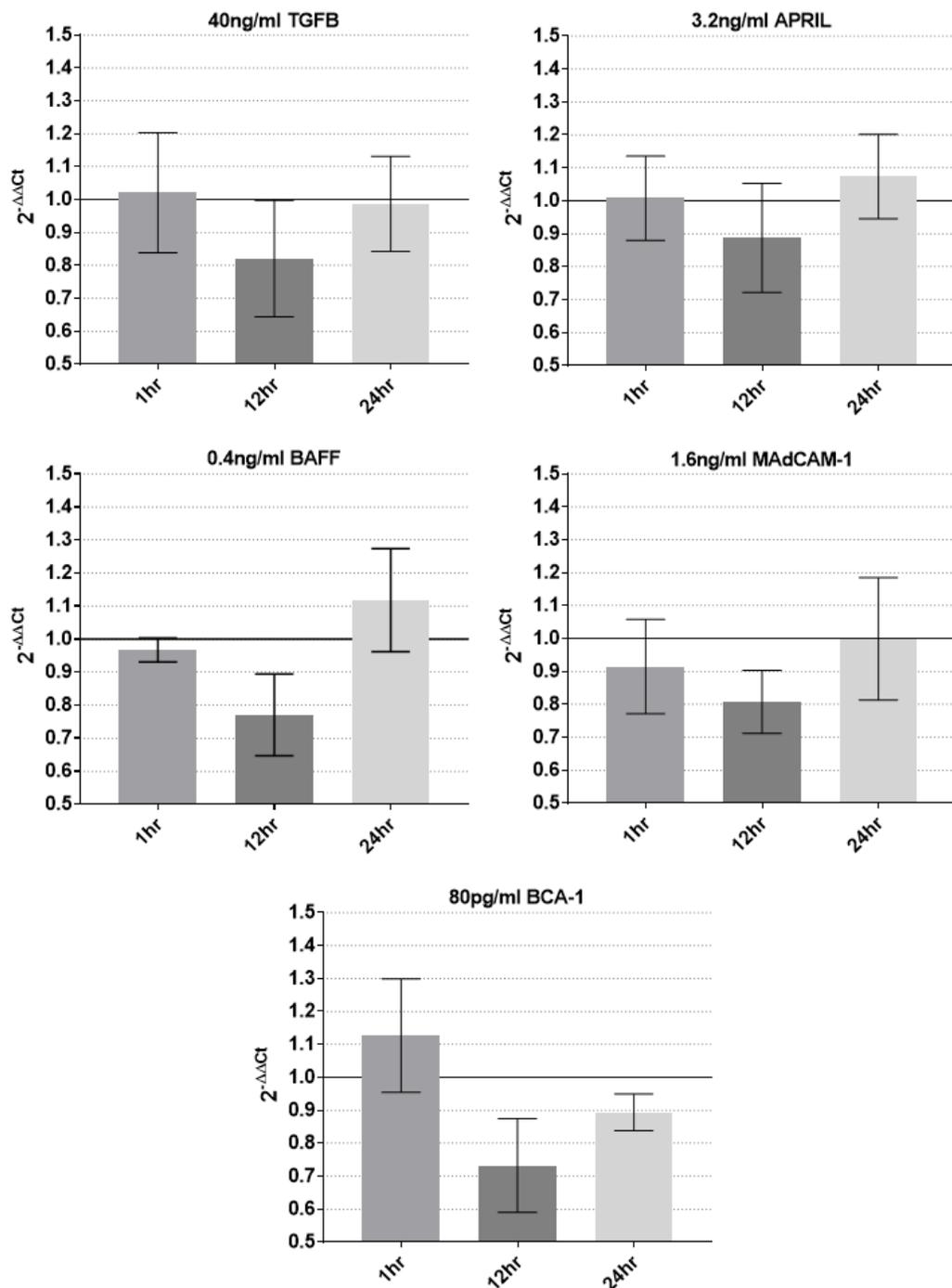


Figure 5.11: COSMC transcript variant 1 mRNA expression in DAKIKI cells after 1, 12 and 24hr stimulation with cytokines: No significant differences seen in Cosmc transcript variant 1 mRNA expression after stimulation of DAKIKI cells with cytokines compared to unstimulated cells. Graph shows mean \pm SD. n=6, data analysed by the $2^{-\Delta\Delta Ct}$ method with IGHA1 as the ERG and individual unpaired t-tests comparing each condition and time point to unstimulated at the matching time point (1.0).

COSMCv2 mRNA expression

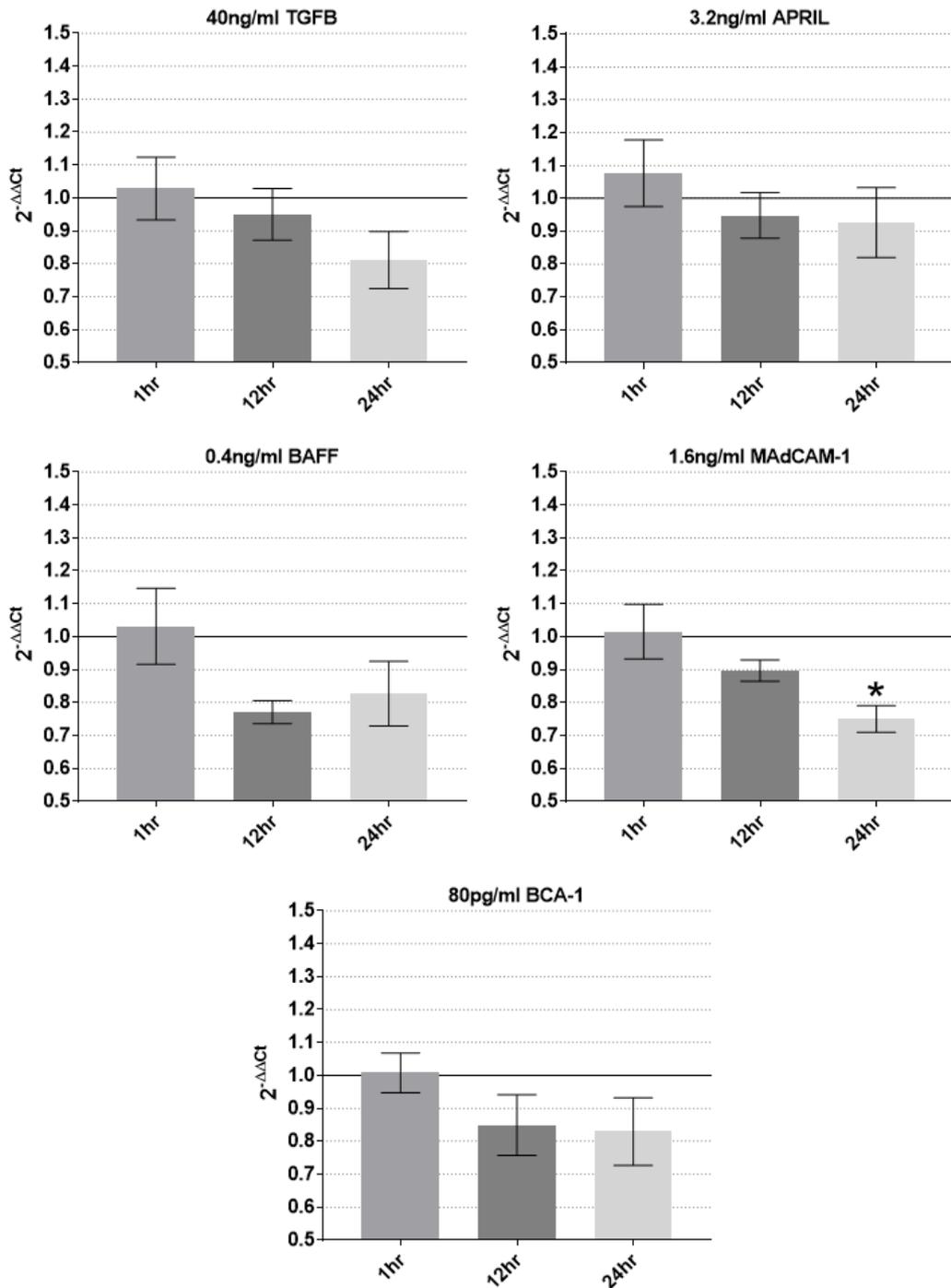


Figure 5.12: COSMC transcript variant 2 mRNA expression in DAKIKI cells after 1, 12 and 24hr stimulation with cytokines: Significant decrease compared to unstimulated cells in COSMCv1 mRNA expression seen after 24hrs stimulation of DAKIKI cells with 1.6ng/ml MAdCAM-1. Graph shows mean \pm SD. n=6, data analysed by the $2^{-\Delta\Delta Ct}$ method with IGHA1 as the ERG and individual unpaired t-tests comparing each condition and time point to unstimulated at the matching time point (1.0).

ST6GALNACII mRNA expression

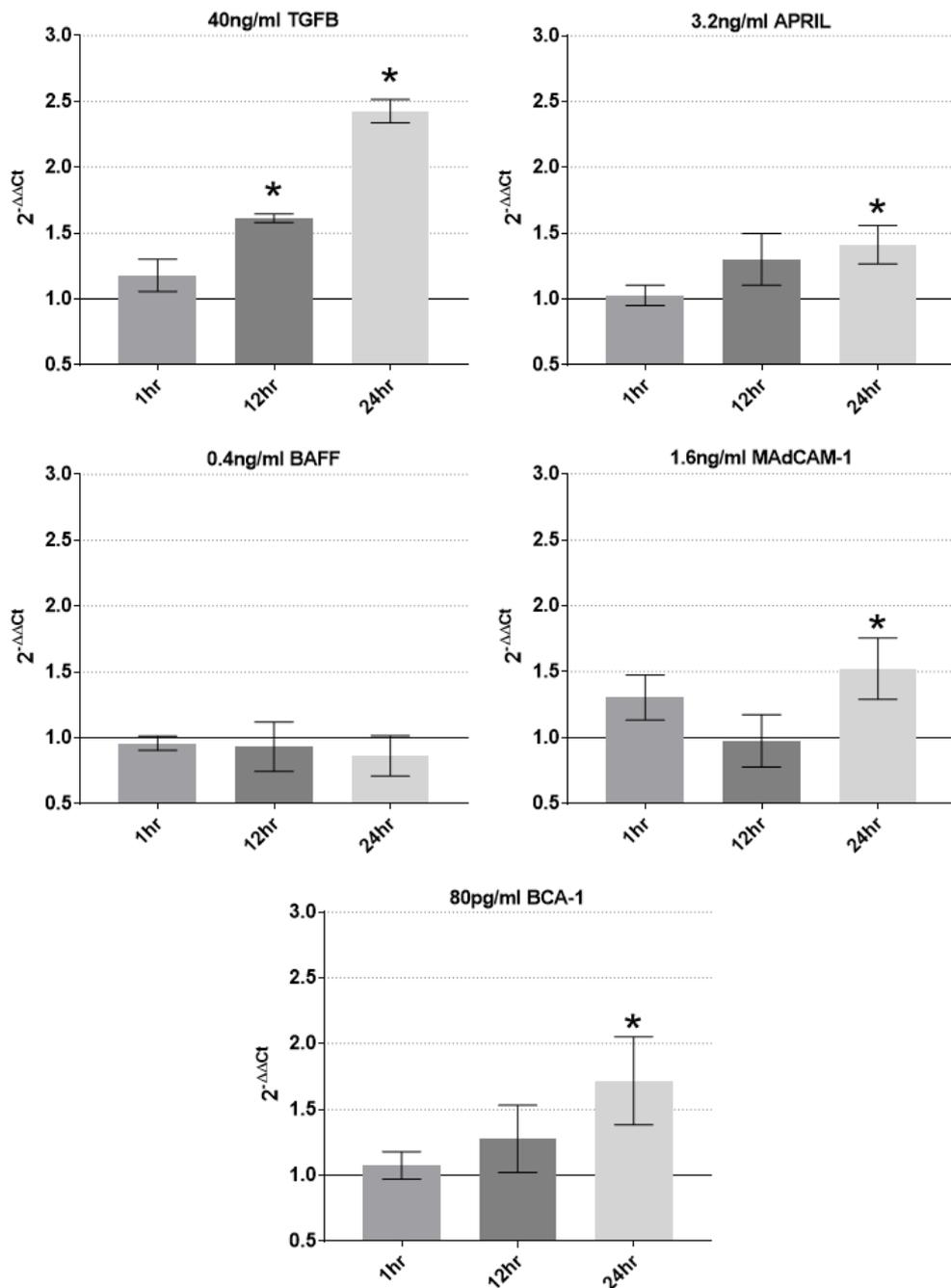


Figure 5.13: ST6GALNACII mRNA expression in DAKIKI cells after 1, 12 and 24hr stimulation with cytokines: Significant increases compared to unstimulated cells in ST6GALNACII mRNA expression seen after 12 and 24hrs with 80ng/ml TGF- β and after 24hrs with 3.2ng/ml APRIL, 1.6ng/ml MAdCAM-1 and 160pg/ml BCA-1. Graph shows mean \pm SD. n=6, data analysed by the $2^{-\Delta\Delta Ct}$ method with IGHA1 as the ERG and individual unpaired t-tests comparing each condition and time point to unstimulated at the matching time point (1.0).

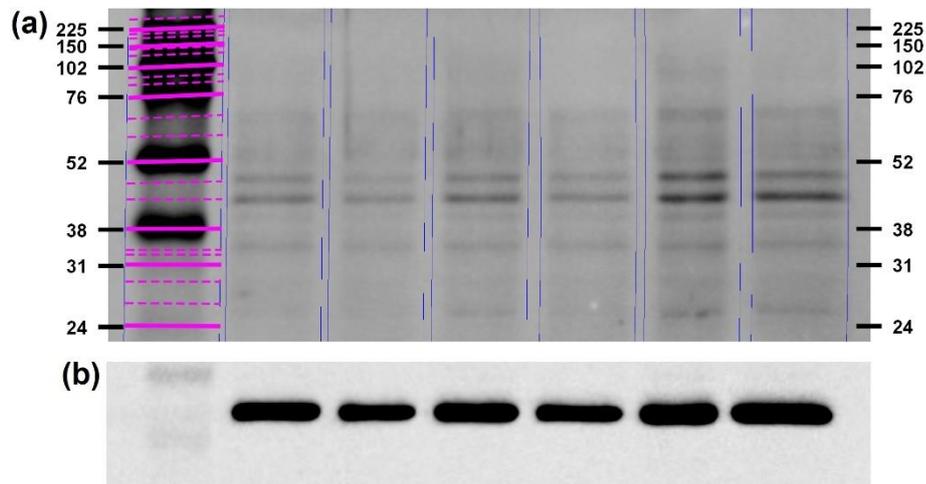
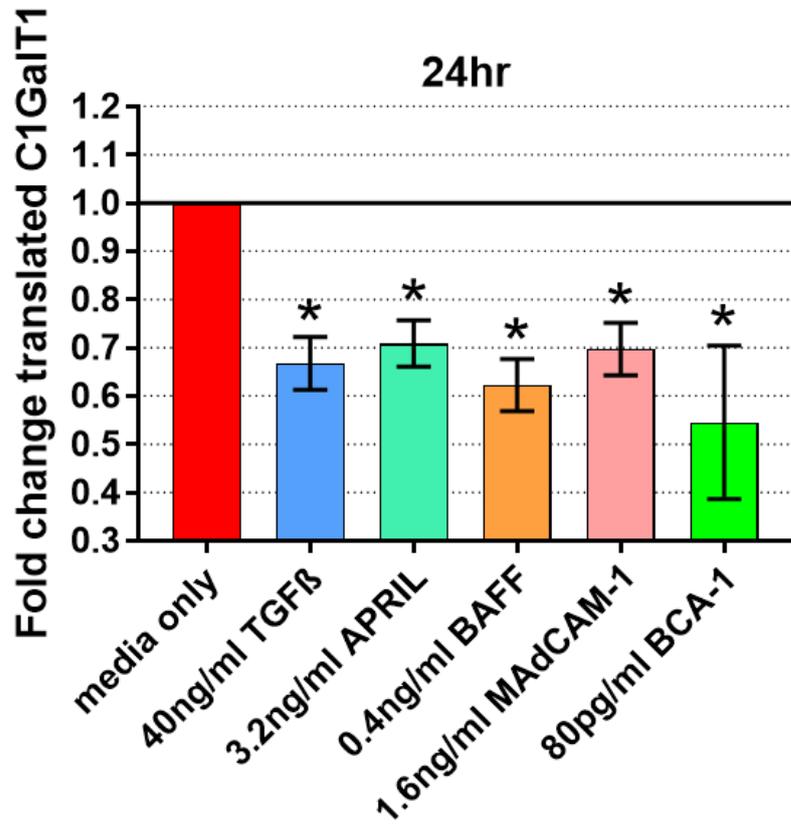


Figure 5.14: C1GalT1 protein expression in DAKIKI cells after 24hr stimulation with cytokines: All cytokines stimulate a significant reduction in translated C1GalT1 in DAKIKI cells after 24 hours compared to unstimulated (media only). Results corrected to β -actin and fold changes to unstimulated calculated. Graph shows mean \pm SD. $n=6$, data analysed using individual unpaired t-tests comparing each condition to unstimulated (1.0). Representative blots are shown below the graph; (a) C1GalT1 with molecular weight markers and (b) β -actin.

5.5.4 – mRNA analysis using NanoString Immunology Panel after 24hr stimulation of DAKIKI cells with cytokines

NanoString technology allows for direct analysis of up to 600 mRNA targets using molecular “barcoding” and microscopic imaging in a single sample. The Immunology Panel includes genes for the major classes of cytokines and their receptors, enzymes and specific gene families such as the major chemokine ligands and receptors, interferons and their receptors, the TNF-receptor superfamily, and the KIR family genes.

Upon analysing the mRNA samples from the 24hr time point after stimulus of DAKIKI cells with cytokines, a total of 50 mRNA targets showed significant fold changes between stimulated and unstimulated (Table 5.2). These mainly clustered with functions involved in cytokine signalling, host-pathogen interaction, lymphocyte activation and the innate immune system (Tables 5.3a & 5.3b). The nSolver analysis software automatically discounts results with high standard deviations between replicates, but validation of these targets is still required before conclusions can be made. Some of the 50 mRNA targets found to have been altered here after stimulation (IL-6, IL-17A and TGF- β) have been previously reported to alter IgA1 O-glycosylation on their own.

Gene	40ng/ml TGFβ1	3.2ng/ml APRIL	0.4ng/ml BAFF	1.6ng/ml MAdCAM-1	80pg/ml BCA-1
ABL1		p=0.0209			
APP	p=0.0138				
ARG1	p=0.0482				
ATG7			p=0.0492		
B3GAT1	p=0.0294				
BCL3				p=0.0193	
C14orf166	p=0.0309				p=0.0413
C1R	p=0.0243				
CCL22			p=0.0428		
CD1D					p=0.0102
CD5				p=0.0337	
CD55					p=0.0466
CD59			p=0.0435		
CD9	p=0.0250				p=0.0369
CFP			p=0.0296		
CMKLR1				p=0.0326	
CSF1				p=0.0306	
CTNNB1	p=0.0448		p=0.0436		
CXCL13			p=0.0238		
DEFB103B		p=0.0372			
EGR1				p=0.0088	
HLA-DPA1				p=0.0317	
IKZF1				p=0.0302	
IL17A			p=0.0475		
IL22			p=0.0403		
IL29			p=0.0404		
IL2RB					p=0.0201
IL4R	p=0.0326				
IL6	p=0.0252	p=0.0160			
IL9		p=0.0377			
ITGA5					p=0.0019
JAK1	p=0.0488				
KIT			p=0.0070		
LILRB2	p=0.0355				
LILRB5					p=0.0491
NCAM1			p=0.0261		
NFATC3		p=0.0297			
NOTCH2	p=0.0147			p=0.0176	
PLAUR					p=0.0056
PML				p=0.0442	
PRF1	p=0.0473				
PSMB5	p=0.0086			p=0.0119	p=0.0123
PSMD7	p=0.0206	p=0.0176			p=0.0223
SPP1		p=0.0248			
STAT5B		p=0.0115			
TGFB1			p=0.0202		
TNFRSF17	p=0.0437			p=0.0470	
TP53				p=0.0301	p=0.0174
XBP1	p=0.0222			p=0.0257	
ZBTB16				p=0.0457	

Table 5.2: NanoString nCounter immunology panel results after 24hr stimulation of DAKIKI cells with cytokines: Table showing 50 mRNA targets with significant fold changes between stimulated and unstimulated DAKIKI cells after 24hrs; green for upregulation and red for downregulation, p values generated by the analysis software included. n=3, mRNA levels measured using NanoString and analysed using nSolver software.

Gene	Cytokine Signaling	Host-pathogen Interaction	Lymphocyte Activation	Immature Immune System	Hemostasis	Adaptive Immune System	Oxidative Stress	Transcriptional Regulation	Complement System	Immunometabolism	Th2 Differentiation	Apoptosis	MHC Class I Antigen Presentation	T Cell Receptor Signaling	Type II Interferon Signaling
ABLI	-	+	+	+	+	-	+	-	-	-	-	-	-	-	-
APP	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-
ARG1	+	+	+	+	+	-	+	-	-	+	-	-	+	-	-
ATG7	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
B3GAT1	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
BCL3	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-
CL6orf166	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-
CLR	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
CCL22	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-
CD1D	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
CD5	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
CD55	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-
GD59	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-
CD9	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
CFP	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-
CNKLR1	-	+	+	+	-	-	-	-	-	+	-	-	-	-	-
CSF1	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
CTNNA1	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
CXCL13	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
DEFB103B	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
EGF1	+	+	+	+	-	+	-	+	-	-	-	-	-	+	-
HLA-DPA1	+	+	+	+	-	+	-	+	-	-	-	-	-	+	-
IRZF1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL17A	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-
IL22	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-
IL29	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL2RB	+	+	+	+	+	-	-	-	-	-	+	+	-	-	-
IL4R	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-
IL6	+	+	+	+	-	-	+	-	-	+	+	-	-	-	-
IL9	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
ITGA5	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
JAK1	+	+	+	+	+	-	-	-	-	+	+	-	-	-	+
KIT	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
LILRB2	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
LILRB5	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-
NCAM1	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
NPATC3	-	+	+	+	-	+	-	+	-	-	-	-	-	+	-
NOTCH2	-	+	+	+	-	-	-	-	-	-	+	+	-	-	-
PLAUR	-	+	+	+	+	-	-	-	+	-	-	-	-	-	-
PML	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-
PRF1	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
PSMB5	+	+	+	+	-	+	+	-	-	+	+	+	+	+	-
PSMD7	-	+	+	+	-	+	+	-	-	+	+	+	+	+	-
SPP1	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
STAT5B	+	+	+	+	-	+	-	+	-	-	+	+	+	+	-
TGFB1	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-
TNFRSF17	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-
TP53	+	+	+	+	+	-	+	+	-	-	-	+	+	+	-
XBP1	-	+	+	+	+	-	+	+	-	-	-	-	-	-	-
ZBTB16	-	-	+	+	-	+	+	+	-	-	-	-	+	+	-

Table 5.3a: Functional clustering of genes identified by NanoString to be differentially regulated upon stimulation with cytokines: The functions of the mRNA identified by NanoString to be up- or down-regulated in DAKIKI cells after stimulation by cytokines compared to unstimulated. Functions are shown in the top row, genes that play a role in the functions are indicated with a + in the relevant column.

Gene	B cell Receptor Signaling	Chemokine Signaling	NLR signaling	Phagocytosis and Degradation	TNF Family Signaling	TLR Signaling	Cell Adhesion	NF- κ B Signaling	Treg Differentiation	Type I Interferon Signaling	Autophagy	Inflammasomes	Lymphocyte Trafficking	MHC Class II Antigen Presentation	TGF β Signaling	Th1 Differentiation
ABL1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
APP	-	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-
ARG1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ATG7	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
B3GAT1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BCL3	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Clonorf66	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
CLR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CCZ2	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD1D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD59	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CFP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CMKLR1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CSF1	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
CTNMB1	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
CYLL13	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DEFB103B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EGRC1	-	-	-	+	-	-	+	-	-	+	-	-	-	+	-	-
HLA-DPA1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IKZF1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL17A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL2RB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL2RB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL4R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL6	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
IL9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ITGA5	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
JAK1	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
KIF	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LILRB2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LILRB2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LILRB5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NCAM1	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
NFATC3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NOTCH2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PLAUR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PLAUR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PMIL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PRF1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PSMB5	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
PSMB5	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
PSMD7	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SPP1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
STAT5B	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
TGFB1	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-
TNFRSF17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TP53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
XBP1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ZBTB16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 5.3b: Functional clustering of genes identified by NanoString to be differentially regulated upon stimulation with cytokines: The functions of the mRNA identified by NanoString to be up- or down-regulated in DAKIKI cells after stimulation by cytokines compared to unstimulated. Functions are shown in the top row, genes that play a role in the functions are indicated with a + in the relevant column.

5.6 - Discussion

The mechanism behind O-glycosylation at the IgA1 hinge region is complex, with the involvement of several enzymes and proteins; and the aberrancy in this leading to reduced galactosylation of the IgA1 hinge region in IgAN is not well understood. Reductions or increases in mRNA of any of the enzymes involved, and any subsequent change in the translated protein, by the action of abnormal levels of cytokines or chemokines could alter the O-glycan structure. The first part of the O-glycosylation mechanism is the addition of GalNAc by the enzyme GalNT2 (Iwasaki *et al*, 2003a). An increase in GalNT2 in IgAN would potentially lead to more GalNAc addition at the IgA1 hinge region and provide more available sites for galactosylation. C1GalT1 is the enzyme that catalyses the addition of galactose to GalNAc (T. Ju *et al*, 2002). Reductions in C1GalT1 in IgAN would directly reduce the amount of galactose added; and if there were increases in GalNT2 but no corresponding increase in C1GalT1, there would be more non-galactosylated GalNAc residues. C1GalT1 itself requires a molecular chaperone protein, Cosmc, for correct folding and activation of its enzymatic function (T. Z. Ju and Cummings, 2002). Any decreases in Cosmc in IgAN would decrease the amount of functional C1GalT1, indirectly leading to a reduction in galactosylation. Finally, ST6GalNAcII is the enzyme that catalyses the addition of sialic acid to GalNAc (Samyn-Petit *et al*, 2000). An increase in ST6GalNAcII in IgAN could potentially cause premature sialylation of GalNAc residues, blocking the subsequent addition of galactose. Any differences in sialylation of IgA1 could be detected by the lectin binding ELISA either by running the ELISA twice (with and without removal of sialic acid residues by neuraminidase treatment) or with a different lectin specific for sialic acid residues.

APRIL plays a role in T cell independent activation of naïve B cells and in the survival of plasmablasts in the bone marrow (Litinskiy *et al*, 2002; Belnoue *et al*, 2008). APRIL has been found to be present at elevated levels in serum from IgAN patients, and concurrently in the same study was shown to correlate with serum levels of Gd-IgA1 (Zhai *et al*, 2016). The work in this project validates the correlation of APRIL with Gd-IgA1 levels by showing that APRIL stimulation of IgA1 producing cells causes a reduction in galactosylation of the IgA1 hinge region.

BAFF also plays a role in T cell independent activation of naïve B cells, and also in the survival of transitional B cells (Litinskiy *et al*, 2002; Rowland *et al*, 2010). A study has shown that BAFF overexpressing transgenic mice developed a commensal flora-dependent IgA-associated nephropathy, with elevated serum levels of polymeric IgA that was aberrantly glycosylated (McCarthy *et al*, 2011). Two stimulation studies in cultured tonsillar mononuclear cells have also shown overexpression of BAFF and increases in Gd-IgA1 production; one by stimulation with capsaicin and the other using vibration (Shao *et al*, 2014; Ye *et al*, 2016). Serum levels of BAFF have also been found to be elevated in IgAN and correlate with renal function (Xin *et al*, 2013). The work in this project has shown that BAFF itself can cause an increase in Gd-IgA1 levels.

It has long been known that hinge regions of IgA1 produced at mucosal surfaces are less galactosylated than that of circulating IgA1, and it has been theorised that the higher levels of circulating Gd-IgA1 seen in IgAN is produced by cells that have been miss-trafficked from the mucosa (Royle *et al*, 2003; Smith, Molyneux *et al*, 2006). In this chapter it has been shown that MAdCAM-1 and BCA-1, ligands for mucosal homing receptors (Sakai and Kobayashi, 2015), can reduce the galactosylation of IgA1 in DAKIKI cells; to a greater extent with MAdCAM-1. If extrapolated to in vivo processes, this could in effect be seen as priming the cells for roles at mucosal surfaces. This could further imply that defects in MAdCAM-1, BCA-1 or their receptors on B cells in IgAN could lend credence to the theory of circulating Gd-IgA1 in IgAN being mucosal IgA1. Both MAdCAM-1 and BCA-1 are expressed on cell surfaces of high endothelial venules in lymph nodes and mucosal lymphoid tissues (Nakache *et al*, 1989; Briskin *et al*, 1997; Mark Ansel *et al*, 2000), therefore excess shearing of them from the cell surface into circulation in IgAN could potentially prime circulating B cells for mucosal function, thus decreasing the galactosylation of the IgA1 produced. However; both MAdCAM-1 and BCA-1 have not as yet been studied in IgAN, and further work would be needed to assess any potential role it, or its receptor, may play in affecting the pathophysiology of the disease.

The decrease seen in C1GalT1 protein expression under stimulus by all of the cytokines studied here could wholly account for the higher levels of Gd-IgA1 produced in all cases. However, there is a discrepancy between the slight

differences seen in C1GALT1 mRNA and the larger differences in translated protein. This indicates that other factors are involved that could be causing degradation of C1GalT1 after translation. Further investigation into where this occurs is necessary to understand the mechanism behind alterations in galactosylation of the IgA1 hinge region. The decreases that were seen in C1GALT1 mRNA and protein were consistent with those changes seen in previous studies of modulation of IgA1 O-glycosylation in DAKIKI cells (Yang *et al*, 2014; Yamada *et al*, 2010; Xiao *et al*, 2017; Lin *et al*, 2018), one of which also showed analysed activity of C1GalT1 and found a decrease in this (Yamada *et al*, 2010).

The data from the NanoString Immunology Panel analysis of mRNA provides a lot of data to begin elucidating the mechanism behind the observed increases in Gd-IgA1. Both TGF- β and APRIL caused an upregulation in IL6, which a previous study has shown by itself upregulates the production of Gd-IgA1 by dysregulation of C1GalT1 and ST6GalNAcII (H. Suzuki *et al*, 2014). TNFRSF17 encodes BCMA, a B cell receptor for both APRIL and BAFF (Coquery and Erickson, 2012), and was found to be downregulated by both TGF- β and MAdCAM-1. It is also interesting to note that while BAFF and APRIL share two B cell receptors, TACI and BCMA (Marsters *et al*, 2000), the NanoString data shows no similar regulation between stimulation of DAKIKI cells with BAFF and APRIL. The mRNA targets identified as being altered by the stimulation conditions in this work require validation before conclusions about the potential regulatory pathways involved can be made.

To summarise, the work in this chapter has identified novel cytokines that can directly reduce galactosylation of the IgA1 hinge region. Two of these (APRIL and BAFF) have previously been found to be associated with IgAN, and a further two have yet to be investigated in IgAN (MAdCAM-1 and BCA-1).

Chapter 6 – Final discussion

6.1 – Summary of findings

This work in this project aimed to investigate the genetic control of IgA1 hinge region O-glycosylation, how the O-glycosylation mechanism itself can be altered during B cell development and also how it can be modulated by the action of cytokines on IgA1 producing cells.

Consistent with previous studies, this project has shown that the aberrant IgA1 O-glycosylation seen in IgAN is evident in both Caucasian and Chinese patients. As has also been shown in previous studies, serum Gd-IgA1 levels show an association with progressive forms of IgAN; this association being strengthened in Caucasians as Gd-IgA1 levels in serum were found to remain stable over time. Alongside this, this work has also shown for the first time lower overall levels in a Chinese population compared to Caucasians. Gender differences in serum Gd-IgA1 levels have also been shown for the first time in both the Caucasian and Chinese IgAN cohorts; however, the reasons behind these gender differences are still unclear.

It has been demonstrated here that a common variation of C1GALT1, named the H1 haplotype, strongly affects Gd-IgA1 level in the population; which potentially could be a factor that can influence the risk of progression in IgAN. Association of the H1 haplotype with IgAN has yet to be confirmed, requiring a much larger cohort for an effective GWAS. A possible association of COSMC with Gd-IgA1 levels was also seen in this project, but this association did not reach genome-wide statistical significance. However, a separate GWAS did find an association between Gd-IgA1 levels and a COSMC SNP, along with one of the C1GALT1 SNPs described here (Kirylyuk *et al*, 2017).

The only immunoglobulin that can be O-glycosylated in a similar fashion to IgA is IgD. However, in IgAN no aberrancy in IgD O-glycosylation has been observed (Smith, De Wolff *et al*, 2006). In the sorted cell populations in this project, the observation of significantly elevated COSMC and ST6GALNACII mRNA expression only seen in surface IgA+ B cells, as well as the trend for reduced C1GALT1 mRNA expression in the same, clearly shows maturation-

dependent transcriptional regulation during the class switching of naïve B cells to IgA producing B cells; a process highly regulated by a variety of cytokines and transcription factors (Calame and Shapiro-Shelef, 2005). The lower mRNA expression of C1GALT1 being significantly accentuated by homozygosity for the H1 haplotype also fits the theory of maturation-dependent transcriptional regulation (Gale *et al*, 2017). The H1 haplotype has been predicted to affect binding of the transcription factor RUNX3, which is necessary for class switching of naïve B cells to IgA producing B cells (Watanabe *et al*, 2010).

In characterising the DAKIKI cells, it was observed that surface markers showed shared characteristics of memory B cells and plasma cells, which is most likely due to the fact that the cells were initially isolated from a carcinoma before being immortalised with EBV transformation. They were found to produce mostly monomeric IgA, with the small amount of polymeric IgA seen potentially due to self-aggregation of the IgA after time in the culture supernatant.

Previous studies (Yamada *et al*, 2010; Yang *et al*, 2014; Xiao *et al*, 2017; Lin *et al*, 2018) have shown that supplementation of DAKIKI cells with cytokines can modulate the levels of Gd-IgA1 produced by altering C1GALT1, COSMC and ST6GALNACII expression. This work has identified novel cytokines that can increase Gd-IgA1 production (APRIL, BAFF, MAdCAM-1 and BCA-1), and furthermore has shown this can be by altering the regulation of GALNT2 transcription, as well as C1GALT1, COSMC and ST6GALNACII. An increase in GALNT2 would lead to more GalNAc addition, providing more O-glycan sites for galactosylation. Reductions in C1GALT1 would directly reduce the amount of galactose added to the IgA1 hinge region, and reductions in COSMC indirectly by lowering the amount of correctly folded translated C1GalT1. ST6GALNACII increases would have the effect of blocking the addition of galactose by sialic acid residues being added to GalNAc before galactosylation. It has been theorised that the higher levels of circulating Gd-IgA1 seen in IgAN is produced by cells that have been miss-trafficked from the mucosa, as mucosal IgA1 is known to be less galactosylated than systemic (Smith, Molyneux *et al*, 2006). MAdCAM-1 and BCA-1 are ligands for mucosal homing receptors and have not yet been studied in relation to IgAN, but could lend support to the miss-trafficking theory if they are present at higher levels in circulation.

To summarise the hypothesis and findings of this thesis; firstly, when naïve B cells class switch to IgD or IgA producing B cells, the action of maturation dependant transcriptional regulation alters the levels of glycosylation enzymes. Here there is a potential greater impact specifically in IgA producing B cells on C1GALT1 mRNA with homozygosity for the H1 haplotype shown in this project. Next, IgA producing cells can then develop further in the mucosal or systemic compartments. In the systemic compartment, the action of cytokines can alter the glycosylation of IgA1, and higher levels of ligands for mucosal homing receptors in circulation could miss-prime circulating IgA producing B cells for mucosal function; which produces more undergalactosylated IgA naturally. Finally, this IgA1 with reduced galactose can form immune complexes which can then deposit in the renal mesangium leading to renal injury and IgA nephropathy. This process is summarised in Figure 6.1.

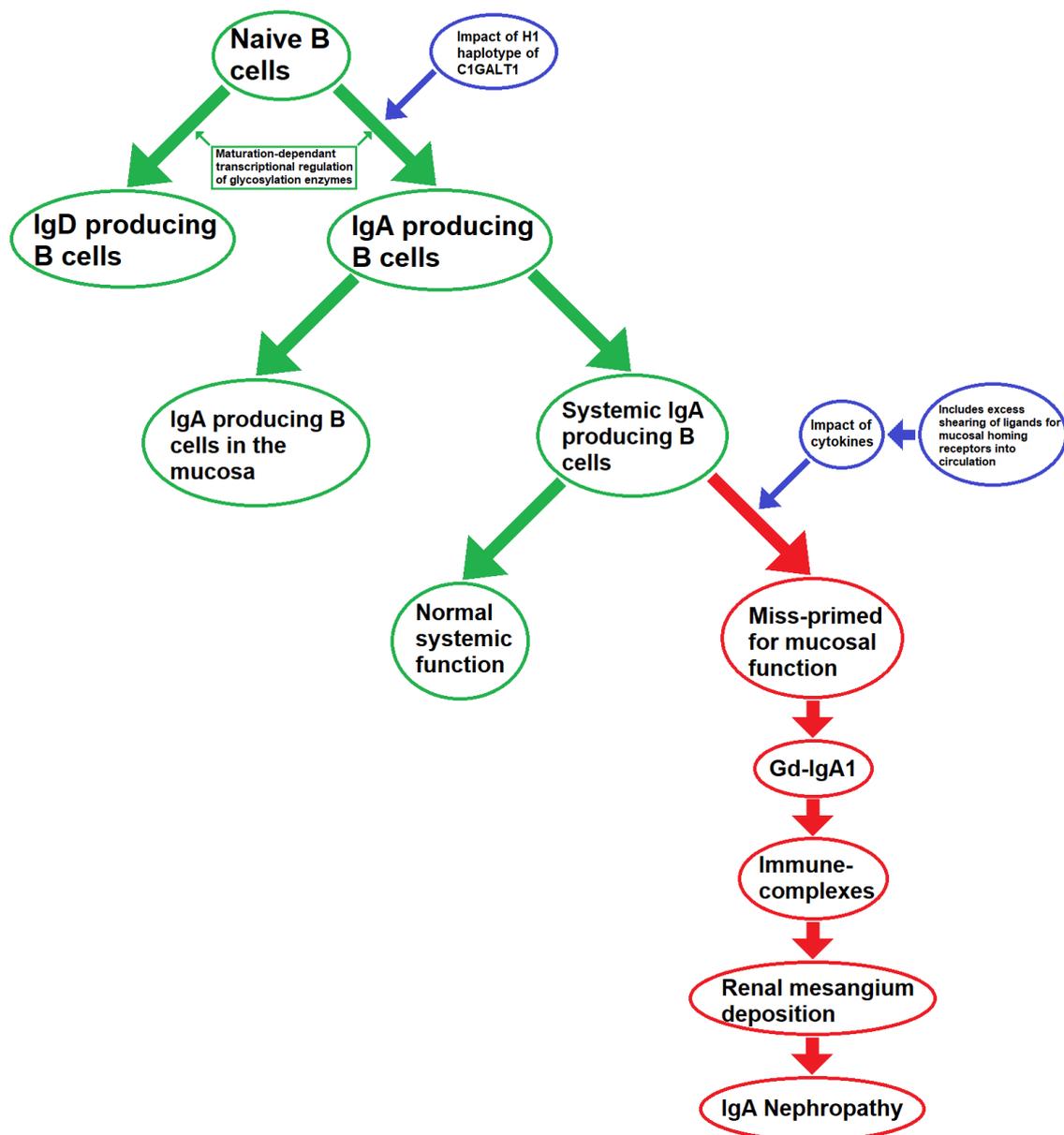


Figure 6.1: Summary of thesis hypothesis and findings: A flow diagram summary of the hypothesis and findings from this thesis. Naïve B cells class switch to IgD or IgA producing B cells, with maturation dependant transcriptional regulation altering the levels of glycosylation enzymes. Here there is an impact specifically in IgA producing B cells with homozygosity for the H1 haplotype of C1GALT1. IgA producing cells can then develop in the mucosal or systemic compartments. In the systemic compartment, cytokines can alter the glycosylation of IgA1, and higher levels of ligands for mucosal homing receptors in circulation could miss-prime circulating IgA producing B cells for mucosal function; producing more Gd-IgA1. Gd-IgA1 can form immune complexes which can then deposit in the renal mesangium leading to renal injury and IgA nephropathy.

6.2 – Limitations

6.2.1 – Limitations of the serum Gd-IgA1 study

The first limitation in this chapter involved the lack of matching samples and data in the Chinese cohort compared to the Caucasian cohort. No ten-year follow up data was available for the Chinese IgAN patients, so the definition of progressive and non-progressive IgAN used in the Caucasian cohort could not be made in the Chinese patients. There were also no Chinese MN patient samples available for a disease control group.

A limitation with the HA lectin binding method involves the observed overlap in the observed serum Gd-IgA1 levels between IgAN and healthy subjects, as the IgA1 hinge region contains up to six sites which can be O-glycosylated, with many potential combinations of glycoforms. Many of these will normally lack galactose at some sites, and in health and IgAN serum contains IgA1 with a mixture of glycoforms which bind the HA (or VV) lectin to differing extents, which makes the use of lectin binding methods as diagnostic tools more difficult.

A further limitation was observed in the identification of the H1 haplotype of C1GALT1 associated with serum Gd-IgA1 levels. This limitation is the lack of clear evidence of an association of the H1 haplotype with IgAN. The data produced here shows that for each SD increase in Gd-IgA1, the odds of IgAN increased by a ratio of around 1.52. In the Caucasian population studied here, the R^2 value for the association of the H1 haplotype with serum Gd-IgA1 levels was 0.033, which implies that 3.3% of the variation in serum Gd-IgA1 levels is due to the number of copies of the H1 haplotype. In order to detect an association with IgAN, power calculations indicate that a candidate gene study in Caucasians would require more than 5300 participants for greater than 80% power to detect any effect of the H1 haplotype on the risk of IgAN; and a GWAS would require around five times greater numbers. The H1 haplotype was found to be less common in the Chinese population studied here, with an R^2 value of 0.019, indicating that even greater numbers would be required to detect an association between the H1 haplotype and IgAN in Chinese patients.

6.2.2 – Limitations of the B cell O-glycosyltransferase study

The first limitation in this chapter was the number of people found to be homozygous for the H1 haplotype of C1GALT1. Out of the 50 people genotyped for H1, only three were found to be homozygous for it; this means that the analysis was not highly powered and more numbers are needed to be recruited to validate the observed differences.

The major limitation in this chapter revolved around the low numbers of cells sorted from 40ml of whole blood. This meant that the low amounts of mRNA that could be isolated from these cells required preamplification of the cDNA produced for viable gene expression analysis. This also meant that there was too little mRNA for allele-specific qPCR and too little protein for quantification of translated C1GalT1. Furthermore, the low cell numbers led to being unable to attain more phenotypically distinct subtypes of B cells. Naïve B cells, memory B cells, plasmablasts and plasma cells differ in that naïve and memory B cells have higher cell surface expression of immunoglobulins and do not produce any immunoglobulins for secretion. Once activated and undergoing differentiation to plasmablasts, this begins to reverse, with less cell surface immunoglobulins and more secretion. After fully maturing into plasma cells, most cell surface immunoglobulin expression is lost, and the cell is fully devoted to producing secreted forms of immunoglobulins. These distinct stages of development could have further differences in the transcriptional regulation of glycosyltransferase genes.

6.2.3 – Limitations of the IgA1 O-glycosylation modulation study

The main limitation in this chapter was in the measurement of Gd-IgA1 produced by DAKIKI cells. Due to a worldwide shortage of HA lectin for use in the ELISA method, a switch to using VV lectin had to be made. The Gd-IgA1 measurement made using VV lectin was found to correlate extremely strongly with the same measurement made with HA lectin in both serum and DAKIKI culture supernatant. However, the signal produced by the VV lectin was much weaker; and with the DAKIKI IgA being highly galactosylated, displaying low lectin binding, the development of readable results from the reaction in this method took time. A monoclonal antibody, KM55, raised against an IgA1 hinge

region completely lacking in galactose has recently been produced (Yasutake *et al*, 2015); however, in testing KM55 for use in this study, there was a lack of reproducibility of results so it was discounted as a viable method of Gd-IgA1 measurement for this project.

The largest mRNA change after stimulation of DAKIKI cells to modulate IgA1 O-glycosylation was seen in ST6GALNACII. No genetic association was found in the first chapter of this thesis between ST6GALNACII and Gd-IgA1 levels; however, the measurement of Gd-IgA1 levels was made by the GalNAc-specific HA lectin and therefore would not detect variation in the sialylation of IgA1.

6.3 – Future work

6.3.1 – Serum Gd-IgA1 levels and the H1 haplotype of C1GALT1

Due to the worldwide shortage of HA lectin to measure serum Gd-IgA1 levels, future studies of this nature would require use of a different lectin to assess serum Gd-IgA1 levels. This project utilised VV lectin as a substitute for HA lectin in the cell culture experiments; however, some differences were observed between the two. When testing the suitability of VV lectin for this project, serum Gd-IgA1 levels measured by VV lectin were found to have a high correlation with HA lectin binding of the same samples. While this could make VV lectin suitable for serum Gd-IgA1 level studies, there are other lectins available that may be a closer match to HA lectin; these would require testing before further work on serum Gd-IgA1 levels could be performed.

The next work for this section will include assessing the levels of serum Gd-IgA1 in patients with IgAN and healthy subjects from a variety of other ethnicities and comparing them to the Caucasian and Chinese cohorts already studied. Wherever possible, serum samples from MN patients in the other ethnicities will be included as the disease controls in order to match the analysis of the specificity of elevated serum Gd-IgA1 levels in IgAN already performed here in Caucasians.

These extra cohorts will also be genotyped to assess the presence and frequency of the H1 haplotype of C1GALT1; along with identifying any other

potential alleles linked to higher serum Gd-IgA1 levels not present in Caucasian or Chinese populations. It would be interesting to attempt recruiting parents of patients from these other ethnic cohorts, including a Chinese cohort as parent samples were not available for this project, to see if or to what degree serum Gd-IgA1 levels are heritable in different ethnicities. The stability of serum Gd-IgA1 levels over time in ethnicities other than Caucasians will also need to be assessed.

To identify any association of the H1 haplotype of C1GALT1 with susceptibility to IgAN, a large cohort of 10,000 Chinese IgAN patients and 15,000 Chinese healthy subjects are currently being genotyped. Such large numbers are a necessity for sufficient power in a study of this nature, given that the H1 haplotype is less common in Chinese populations. However, recruitment of these numbers of subjects is easier in China due to the larger population size and greater prevalence of IgAN.

Some SNPs of the H1 haplotype are predicted to lie within transcription factor binding sites, one of which is within the promoter region of C1GALT1. Our collaborators at UCL who initially identified the H1 haplotype are currently investigating this. They have so far shown that two variants on the H1 haplotype reduce expression of reporter gene, and that there is less protein binding to this allotpe. They are currently investigating which protein(s) bind differently according to genotype that can potentially affect transcription of C1GALT1.

6.3.2 – O-glycosyltransferase expression during B cell development

The most pressing future work for this section will be to increase the numbers of subjects studied; this would allow a clearer picture of the difference between those homozygous for the H1 haplotype of C1GALT1, those heterozygous and those homozygous negative. Ideally, future subjects will have more than 40ml whole blood taken from which to sort surface IgA+ and surface IgD+ B cells; giving an increase in the final number of collected cells and aiding in ease of the downstream analyses and potentially collecting more phenotypically distinct cell populations. It would also be beneficial to recruit IgAN patients to include in this study, to assess whether the presence or absence of the H1 haplotype has any differential effect on these cells in IgAN.

Further on, experiments will be planned to work out where the differences in the O-glycosylation mechanism between surface IgA⁺ and surface IgD⁺ B cells occur. This will involve development of an in vitro model of differentiation of naïve B cells (surface IgD⁺) to IgA secreting plasma cells to attempt to track the changes in the expression or activity of the enzymes and proteins involved in O-glycosylation as the cells undergo activation, class-switch recombination and maturation. These experiments will be performed on cells with and without the H1 haplotype of C1GALT1.

6.3.3 – Modulation of IgA1 O-glycosylation

As the largest mRNA change after stimulation with cytokines was in ST6GALNACII, it would be beneficial to measure the sialylation of IgA1 in the supernatants of these experiments. Lectins are available that bind the α 2-6 sialic acid residues at the IgA1 hinge region. To this end, a selection of these will be tested in the lectin binding ELISA and the best candidate used to measure sialylation of the IgA1 in the culture supernatants from this project.

To further elucidate the mechanism behind changes in IgA1 O-glycosylation the mRNA from samples in the time course experiments will be sent for Next Generation Sequencing of total mRNA and micro RNAs. Aliquots of dried cell pellets from the same samples will also be used for full proteomic analysis by mass spectrometry. Alongside qPCR validation of any targets identified by sequencing, validation of some or all of the fifty targets found to be up- or downregulated by the NanoString Immunology Panel will be performed. Validation of protein targets identified by mass spectrometry will also be carried out by Western blotting.

As the DAKIKI cells were found to be homozygous negative for the H1 haplotype of C1GALT1, experiments are being planned to transfect these cells with heterozygous and homozygous positive H1 C1GALT1 genes, to see any different effect on the action of these cytokines on C1GALT1 with variations in the H1 haplotype.

In cell culture experiments, IgA isolated from IgAN patients and healthy subjects is known to stimulate renal cells to different degrees (Ebefors *et al*, 2016). Large scale experiments on DAKIKI cells with and without cytokine

stimulus will be performed in order to isolate and purify IgA with different glycosylation states from each stimulus. This IgA will then be used to stimulate various types of renal cells in culture and assess any differential effect purely based on the same IgA expressing a different glycosylation pattern.

For greater clinical relevance, all or part of this work on DAKIKI cells will be repeated on cultured PBMCs from IgAN patients and healthy subjects; with potential further on to repeat again with immortalised IgA+ B cells from the same. It is the hope that through the sum of this work, potential drug targets can be identified with the aim of reducing the amount of pathogenic Gd-IgA1 found in IgAN.

The identification of MAdCAM-1 and BCA-1 as factors that can affect IgA1 O-glycosylation is an exciting discovery, as neither cytokine has been studied in IgAN previously. The serum samples analysed in the work in Chapter 3 of this thesis would be the most readily available samples to measure levels of circulating MAdCAM-1 and BCA-1 and compare those in IgAN patients with healthy subjects. This would allow identification of whether they are raised in circulation in IgAN, thus potentially causing B cells to be miss-trafficked (or miss-primed) for mucosal function and therefore lead to the increase in circulating Gd-IgA1 levels seen in IgAN.

6.4 – Concluding remarks

Aberrant O-glycosylation of IgA1, namely galactose deficiency in the hinge region, is a well documented finding in IgAN and thought to be a main factor in the pathogenesis of the disease. However, the biological basis for this aberrancy is still not well understood despite much research. The work from this project serves to elucidate further control of the mechanism behind O-glycosylation at the IgA1 hinge region. This work raises questions for future IgAN studies about how differences in the O-glycosylation mechanism between genders in IgAN and between ethnicities cause the observed differences in serum levels of Gd-IgA1, and about how this mechanism can be regulated genetically and modulated with cytokines.

Appendix I – Ethical approval



University Ethics Sub-Committee for Medicine and
Biological Sciences

03/12/2015

Ethics Reference: 3803-dhbw2-infectionimmunityinflamm

TO:

Name of Researcher Applicant: David Wimbury

Department: 3I

Research Project Title: Transcriptional control of IgA1 O-galactosylation in IgA nephropathy

Dear David Wimbury,

RE: Ethics review of Research Study application

The University Ethics Sub-Committee for Medicine and Biological Sciences has reviewed and discussed the above application.

1. Ethical opinion

The Sub-Committee grants ethical approval to the above research project on the basis described in the application form and supporting documentation, subject to the conditions specified below.

2. Summary of ethics review discussion

The Committee noted the following issues:

All approved

3. General conditions of the ethical approval

The ethics approval is subject to the following general conditions being met prior to the start of the project:

As the Principal Investigator, you are expected to deliver the research project in accordance with the University's policies and procedures, which includes the University's Research Code of Conduct and the University's Research Ethics Policy.

If relevant, management permission or approval (gate keeper role) must be obtained from host organisation prior to the start of the study at the site concerned.

4. Reporting requirements after ethical approval

You are expected to notify the Sub-Committee about:

- Significant amendments to the project
 - Serious breaches of the protocol
 - Annual progress reports
 - Notifying the end of the study
5. Use of application information

Details from your ethics application will be stored on the University Ethics Online System. With your permission, the Sub-Committee may wish to use parts of the application in an anonymised format for training or sharing best practice. Please let me know if you do not want the application details to be used in this manner.

Best wishes for the success of this research project.

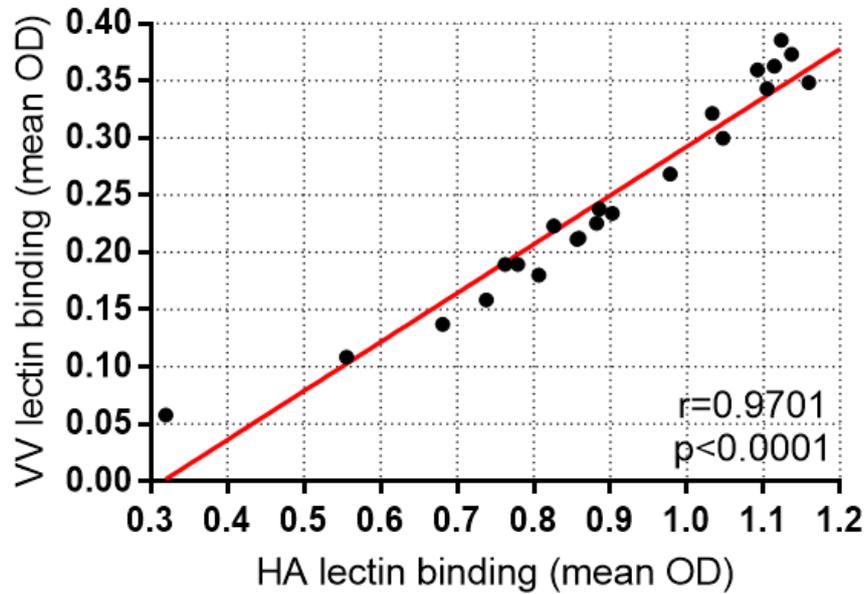
Yours sincerely,

Dr. Susan Wallace

Chair

Appendix II – Supplementary data

Chapter 2 – Methods and materials



Supplementary figure S2.1: Correlation of Gd-IgA1 levels in serum between HA and VV lectin binding: Significant correlation seen between HA and VV lectin binding ELISA measurements of Gd-IgA1 levels in serum from 22 healthy subjects. Data was correlated using the Pearson method; $r=0.9701$, $p<0.0001$.

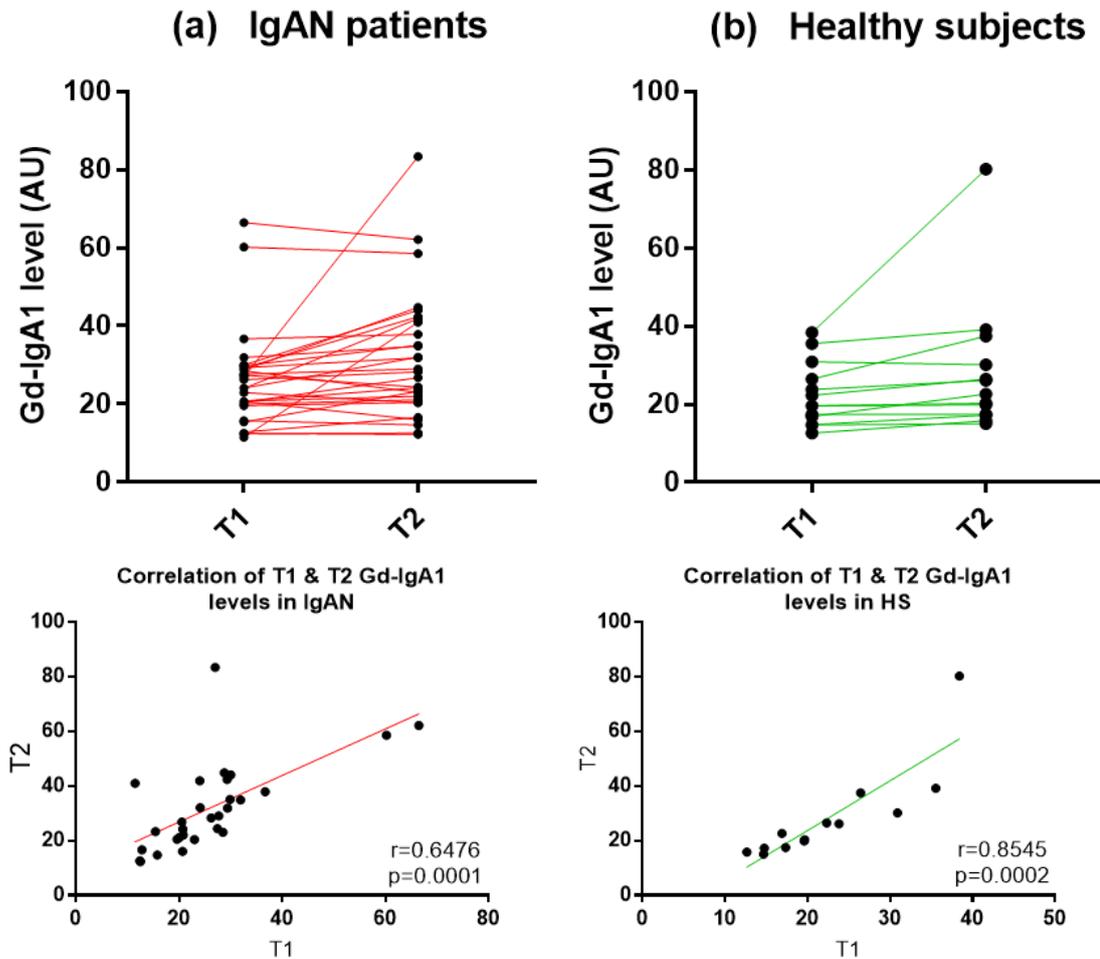
Antibody	Fluorescent conjugate	Supplier	Catalogue no.	Volume per 200µl cell suspension
anti-CD19	AlexaFluor 700	BD	557921	10µl
anti-CD20	BUV395	BD	563782	10µl
anti-CD38	BV421	BD	562444	10µl
anti-CD138	PE-Cy7	eBioscience	25-1389	10µl
anti-IgA	PE	Miltenyi Biotec	130-093-128	20µl
anti-IgD	FITC	BD	555778	10µl

Supplementary table S2.1: Fluorescently conjugated antibodies used for FACS: Details of the fluorescently conjugated antibodies used for FACS in Chapters 4 and 5 along with the working volumes used are shown.

Antibody	Conjugate	Supplier	Catalogue no.	Dilution factor
rabbit anti-human C1GalT1	-	Santa Cruz	sc-98433	1:200
anti-rabbit Igs	HRP	Dako	P0448	1:1000
mouse anti-human β -actin	-	abcam	ab6276	1:2000
anti-mouse Igs	HRP	Dako	P0260	1:1000

Supplementary table S2.2: Antibodies used for Western blotting: Details of the antibodies and dilutions for Western blotting in Chapters 4 and 5.

Chapter 3 – Systematic analysis of aberrant IgA1 O-glycosylation in IgAN

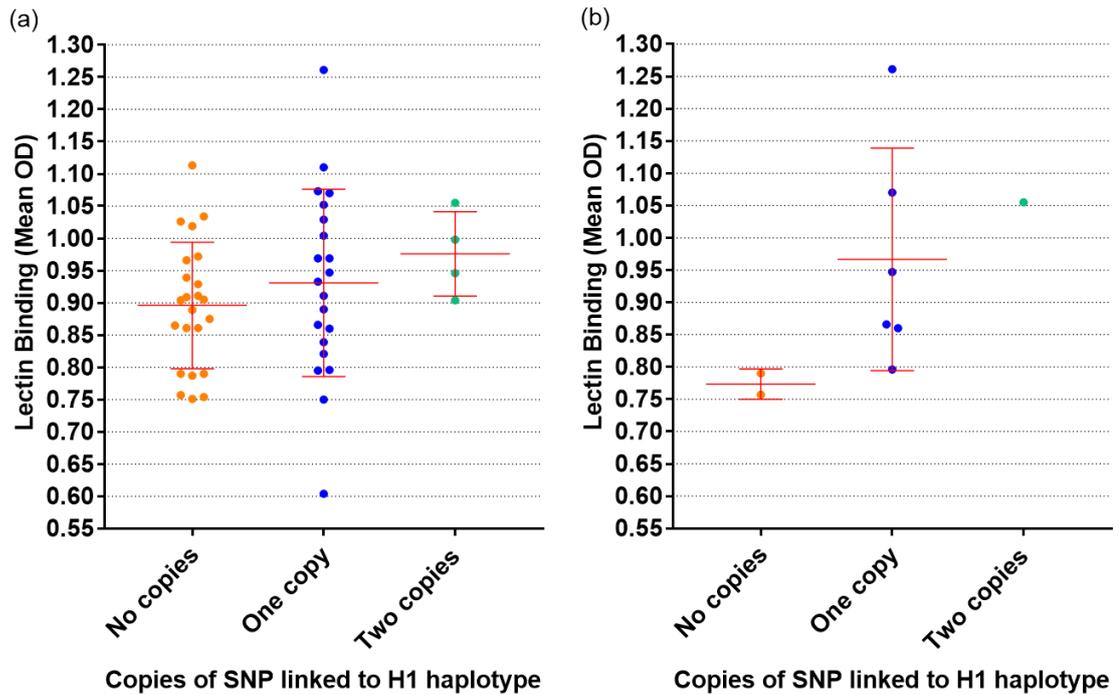


Supplementary figure S3.1: Stability of Gd-IgA1 levels over time: Gd-IgA1 levels in serum remain stable over time in samples from the Leicester IgAN Research Archive as measured by HA lectin binding ELISA (a) Healthy subjects; (b) IgAN patients. Data analysed by calculating the coefficient of variation (CV) between Gd-IgA1 levels at the earliest and latest time points (T1 and T2), and also by correlation of the same data (Cheshire, 2011).

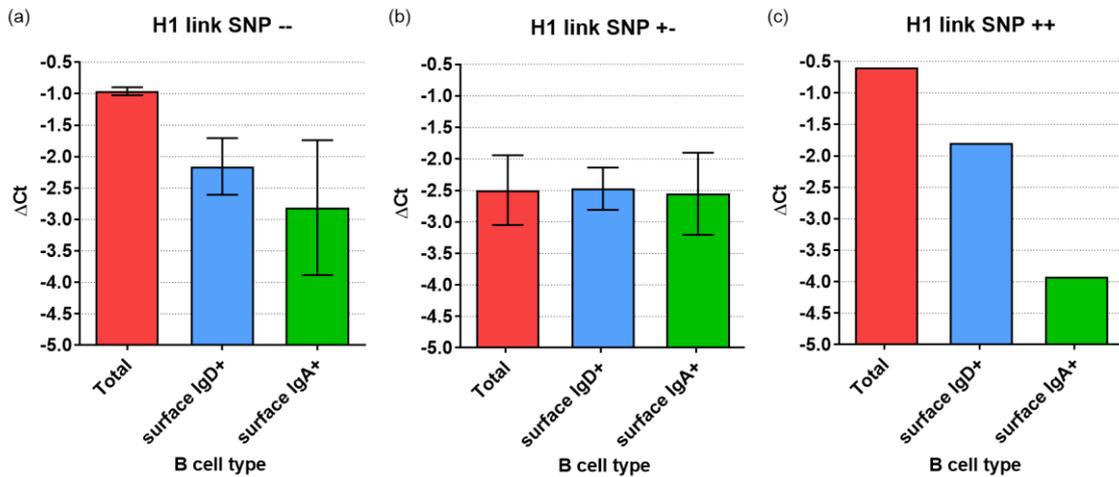
Chapter 4 - Expression of glycosylation enzymes in IgA and IgD producing cells

rs	CHR_POS_REF_ALT	Chisq	p.value	H1_ALT_AF	non_H1_ALT_AF	Chi2	Imputed P	GRAPHIC_p	Log10(Chi2stat)	-Log10(P_Gd_IgA1)
rs1047763	7_7283569_G_A	315.58415	1.32675E-70	0.01031	0.27433	315.58415	0.00001034	0.00002018	2.49912	5.98548
rs35362940	7_7284537_T_C	321.94864	5.45065E-72	0.00825	0.27500	321.94864	0.912	0.25357	2.50779	0.04001
rs10251492	7_7284546_G_A	4874.34640	0	0.01649	0.97890	4874.34640	1.421E-10	3.701E-11	3.68792	9.84741
rs11764290	7_7284591_T_C	541.98174	6.9873E-120	0.01134	0.39901	541.98174	0.00003194	6.472E-10	2.73398	4.49567
rs73049979	7_7284890_C_T	337.63583	2.08809E-75	0.00103	0.27294	337.63583	0.002599	0.507901	2.52845	2.58519
rs73049985	7_7285708_A_G	691.77887	1.8342E-152	0.15052	0.00065	691.77887	0.0003603	0.0326678	2.83997	3.44334
rs572541196	7_7285856_A_AAT	518.72919	7.9988E-115	0.00208	0.37661	518.72919	#N/A	#N/A	2.71494	#N/A
rs5010183	7_7286198_C_T	5015.51104	0	0.01343	0.98342	5015.51104	1.122E-10	8.938E-10	3.70032	9.95001
rs10246303	7_7286445_A_T	1593.76137	0	0.01237	0.70693	1593.76137	6.488E-10	3.906E-08	3.20242	9.18789
rs13225339	7_7288776_T_C	326.60449	5.27648E-73	0.00619	0.27455	326.60449	0.8114	0.630197	2.51402	0.09076

Supplementary table S4.1: Strength of linkage of SNPs in coding region of C1GALT1 to H1 and Gd-IgA1 levels: GWAS data from a Caucasian cohort showing SNPs in the 3' UTR region of C1GALT1 with significant linkage to the H1 haplotype of C1GALT1 and also to Gd-IgA1 levels. Data courtesy of D. Gale. SNP rs10251492 selected for allele-specific qPCR.



Supplementary figure S4.1: Gd-IgA1 levels by SNP linked to H1 haplotype of C1GALT1 (rs10251492): Gd-IgA1 levels increase with copies of the H1 linked SNP in the coding region of C1GALT1 in 50 healthy volunteers genotyped for this SNP. (a) Whole cohort of 50; (b) Volunteers selected genotyped as 3 homozygous for H1, 3 heterozygous for H1 and 3 homozygous negative for H1.

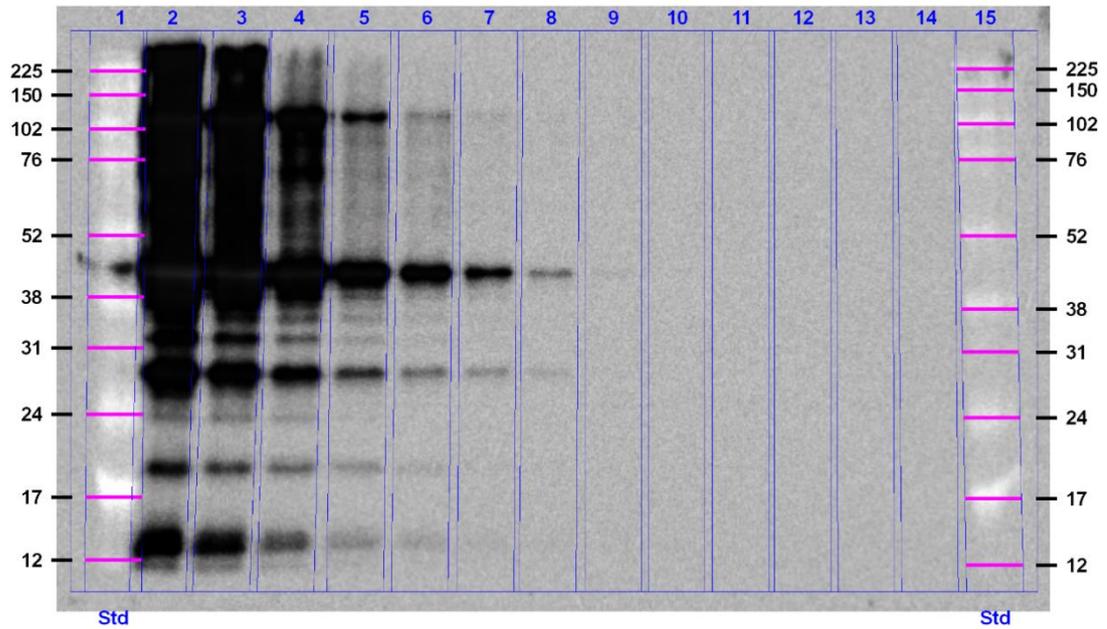


Supplementary figure S4.2: C1GALT1 mRNA expression in total, surface IgA+ and surface IgD+ B cells by SNP linked to H1 (rs10251492):

Expression of C1GALT1 in cells from the 9 healthy volunteers; 3 homozygous for H1 haplotype of C1GALT1, 3 heterozygous and 3 homozygous negative for H1. Linkage mis-match between H1 haplotype and linked SNP gives 1 homozygous for H1 linked SNP, 6 heterozygous and 2 homozygous negative; and is possibly due to ethnic differences. Data shown is the normalised ΔC_t values with PSMB6 as the ERG. Analysis of the effect of homozygosity for the H1 linked SNP on C1GALT1 mRNA expression was not possible due to only one sample being homozygous for this SNP.

			Ct values in mRNA							
Sample	Copies of H1	link SNP	Whole B cells		IgD+ B cells		IgA+ B cells			
			A	G	A	G	A	G		
16	0	A/A	39.031		35.725		42.326			
2	0	A/G	35.828	34.561	40.603	39.733				
17	0	A/G	32.411	31.561			40.239	39.324		
3	1	A/A	33.488		46.944					
7	1	A/G	34.500	33.609	39.117	38.409	42.349	42.046		
9	1	A/G	36.474	35.532	42.163	44.285				
107	2	A/G	42.964	40.068	40.982	41.499				
108	2	A/G	31.805	30.895	41.944	41.258	42.826	48.415		
104	2	G/G		28.778		38.374				

Supplementary table S4.2: Allele-specific qPCR of H1 linked SNP in mRNA from sorted cells surface IgA+ and surface IgD+ B cells: Ct values in whole B cell mRNA confirms method for allele-specific qPCR of this SNP is viable, as data matches the genotype of the SNP. Lack of some results in surface IgD+ B cells and lack of most results in surface IgA+ B cells reflects fewer numbers of starting cells and therefore less mRNA.



Supplementary figure S4.3: Western blot of purified recombinant C1GalT1: Acquired using BioRad ChemiDoc Touch and analysed using Image Lab software. Lanes 1 & 15: Molecular weight marker. C1GalT1 concentrations loaded in serial dilution: 2.5µg – 0.61ng. Lowest possible detection at lanes 9 and 10 (19.53 and 9.77ng).

Appendix III – C1GalT1 quantification by mass spectrometry

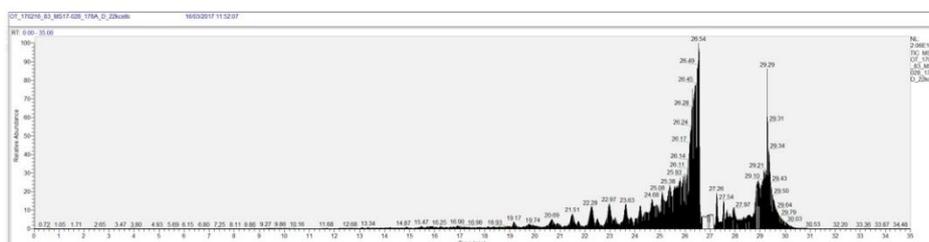


report

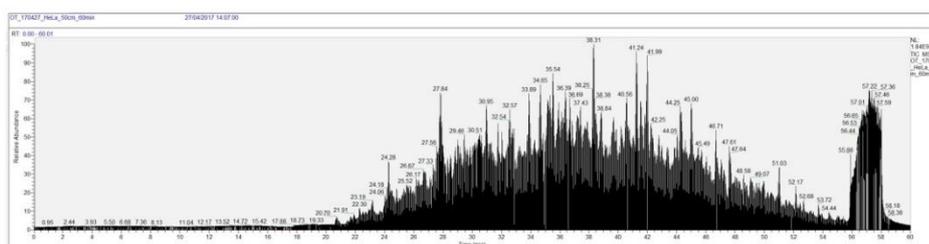


“The authors would like to acknowledge the contribution of the WPH Proteomics RTP, Gibbet Hill Road, University of Warwick, UK.

Samples	Submitted	Completed	Cost (£)	Number
2	22/2/2017		-	MS17-028
Analysis required:	<i>Identify and/or quantify C1GALT1 and other target proteins</i>			
Main conclusion:	<p><i>The sample has been methanol chloroform precipitated because we ignored the composition of the buffer (from a kit, the company say that the buffer is not MS compatible). After the sample was digested with trypsin and 1/3 of the sample analysed in a DDA mode in the Mass spectrometer Fusion. The signal was very poor and only 130 proteins were identified and C1GALT1 was not present. As a reference 50 ng of peptides from HeLa digest (approx. 150 ng of total protein) delivers an average intensity of 10e9. Below you can compare both chromatograms.</i></p> <p><i>Additionally we used targeted (SRM, single reaction monitoring) proteomics in a triple quadrupole (Quantiva mass spectrometer) where the known peptides are fragmented and some fragments are quantify. We used previous mass spectrometry databases to find out the transitions. With them we can build the method and we run again the samples using 1/3 of the total sample injections. We observed a small signal for some transitions but they are so week that quantification is not possible.</i></p>			
Next steps:	<i>Better sample is needed, a different lysis buffer to avoid methanol chloroform precipitation, more cells and improved trypsin digestion (doi:10.1038/nmeth.2834)</i>			
Analysed by	<i>Juan Ramon Hernandez Fernaud</i>			



Chromatogram from 22,000 cells



Chromatogram from 50 ng of peptides from HeLa

Appendix IV – C1GalT1 SMC assay development report



ASSAY DEVELOPMENT REPORT

Prototype Assay Development and Sample Testing
of an SMCTM Immunoassay for C1GALT1 in Human
B-Cell Lysate

Submitted To

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Prepared By

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Custom Assays & Sample Testing, Molecular & Cell Biology Systems

MilliporeSigma

A business of Merck KGaA, Darmstadt, Germany

25801 Industrial Blvd, Hayward CA 94545, USA

Principal Investigator(s): Sarah Hamren

Study Personnel: Victoria Torres

Notebooks Referenced: 00742

Report Date: September 28, 2018

Number of Pages: 15

CONFIDENTIAL

Appendix V – Journal publication arising from this work

Galactosylation of IgA1 Is Associated with Common Variation in C1GALT1

Gale, D.P., Molyneux, K., **Wimbury, D.**, Higgins, P., Levine, A.P., Caplin, B.,
Ferlin, A., Yin, P., Nelson, C.P., Stanescu, H., Samani, N.J., Kleta, R., Yu, X.
and Barratt, J.

Journal of the American Society of Nephrology, 2017, 28(7), pp. 2158-2166

<https://jasn.asnjournals.org/content/28/7/2158.long>

Appendix VI – Conference presentation abstracts arising from this work

The H1 risk haplotype significantly accentuates maturation-dependent transcriptional regulation of the C1GALT1 gene in IgA committed B cells

Wimbury, D.; Molyneux, K. and Barratt, J.

Oral presentation, 15th International Symposium on IgA Nephropathy 27-29/09/2018

Kidney Diseases 2018; 4(3): 109

Introduction: We have previously reported a linear regression genome wide association study (GWAS) which identified alleles at a single locus spanning the C1GALT1 gene that were strongly associated with levels of poorly O-galactosylated IgA1 (H1 haplotype). C1GALT1 encodes the enzyme core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 (C1GALT1), which catalyses the transfer of Galactose (Gal) from UDP-Gal to N-acetylgalactosamine (GalNAc) O-linked esters of Threonine and Serine residues of IgA1. This requires the chaperone Cosmc, encoded by the X-linked gene COSMC, which prevents rapid degradation of C1GALT1. In a separate quantitative GWAS for serum levels of poorly O-galactosylated IgA1 two genome-wide significant loci were identified, in C1GALT1 (as in our GWAS) and COSMC. Imputation of all the alleles common to the H1 haplotype using 1000 Genomes data indicated that there were no common coding variants that were over- or under-represented on the H1 haplotype compared with other common haplotypes, implying that the biological effect on enzyme activity is mediated by differences in gene expression in the presence of the different allotypes. Consistent with this we have reported that the changes in immunoglobulin O-glycosylation seen in IgAN only occur after class switching to IgA synthesis and we hypothesise that this is secondary to maturation-dependent transcriptional regulation of the C1GALT1 gene.

Objectives: The objectives here were to determine the effect of class switching on expression of C1GALT1 and the impact of the H1 haplotype on maturation-dependent transcriptional regulation of the C1GALT1 gene.

Materials and Methods: Peripheral CD19+, CD19+ IgD+ and CD19+ IgA+ B cells were sorted from healthy volunteers (n=9) genotyped for the H1 haplotype

(using rs4720724, rs758263, rs4263662, rs10259085, rs2190935 and rs1008897: 3 homozygous for H1, 3 heterozygous and 3 homozygous negative for H1). Total mRNA was isolated from sorted B cell populations and expression of C1GALT1, both transcript variants of COSMC and ST6GALNACII, IgA1 heavy chain and IgD heavy chain mRNA measured by qPCR.

Results: Compared to naïve B cells (CD19+ IgD+), CD19+ IgA+ B cells expressed lower levels of C1GALT1 mRNA, which was significantly accentuated with homozygosity for the H1 haplotype. Furthermore, the H1 haplotype exerted no effect on levels of C1GALT1 mRNA in naïve B cells. CD19+ IgA+ B cells also displayed significantly higher levels of both transcript variants of COSMC and ST6GALNACII when compared to naïve B cells.

Discussion: These results are consistent with our previous work reporting differential O-glycosylation of IgD and IgA1 and are consistent with maturation-dependent transcriptional regulation of the C1GALT1 gene, and a significant influence of the H1 haplotype on this process, in IgA-committed B cells.

Conclusion: Elucidation of the pathways involved in transcriptional regulation of the C1GALT1 gene and the impact of the H1 haplotype on these pathways are essential as we try to improve our understanding of the role of IgA1 O-glycosylation in IgAN.

O-glycosylation of IgA1 is associated with genetic variation of C1GALT1
Gale, D.; Wimbury, D.; Yin, P.; Higgins, P.; Kleta, R.; Yu, X.; Molyneux, K. and Barratt, J.

Poster presentation, American Society of Nephrology Kidney Week 31/10-05/11/2016

IgA nephropathy (IgAN) is characterised by IgA deposition in the glomeruli and is an important cause of kidney failure. Its cause is not known but recent work has revealed differences in glycosylation of the IgA molecule in patients compared with controls. We used a high throughput lectin-based assay of IgA galactosylation to measure levels of Galactose-deficient IgA1 (Gd-IgA1) in a discovery cohort of 503 UK patients with biopsy-proven IgAN with >5 year follow-up data and 250 of their healthy parents in 137 complete trios. Findings were

replicated in 309 UK patients with membranous glomerulopathy and 1000 Chinese patients with biopsy proven IgAN.

Gd-IgA1 levels were higher in UK IgAN patients with progressive kidney damage than in any of the other groups. Notably Gd-IgA1 was especially low in membranous patients. Heritability (h^2), estimated by comparing mid-parental and off-spring Gd-IgA1 levels, was 0.28.

Linear regression genome wide association study in 613 founder members of the discovery cohort identified alleles at a single locus, spanning the C1GALT1 gene, that was strongly associated with Gd-IgA level (Beta = 0.04; $p < 10^{-8}$), with no other hits across the genome. C1GALT1 encodes a galactosyltransferase enzyme known to be important in galactosylation of O-linked glycoproteins. This association was replicated in separate cohorts of UK patients with membranous glomerulonephritis ($p < 10^{-6}$; combined cohorts $p = 10^{-14}$) and in a candidate gene study Chinese patients with IgAN ($p < 10^{-6}$). The same extended haplotype was associated with elevated Gd-IgA1 levels in all cohorts studied.

In addition to providing robust validation of the assay, these findings demonstrate that common variation at C1GALT1 strongly affects Gd-IgA1 level in the population, which may influence risk of progression in IgA nephropathy.

Systematic Analysis of IgA1 Glycosylation in IgA Nephropathy, Membranous Nephropathy and Healthy Subjects and the Effects of Ethnicity

Molyneux, K.; Wimbury, D.; Gale, D.; Higgins, P.; Yin, P.; Yu, X. and Barratt, J.

Oral presentation, American Society of Nephrology Kidney Week 31/10-05/11/2016

Background: IgA nephropathy (IgAN) is characterised by the deposition of galactose deficient IgA1 (Gd-IgA)-containing immune complexes in the mesangium. IgAN is especially common in East Asia, and while the diagnostic criteria are the same worldwide, there are marked regional differences in gender distribution and clinical outcome, suggesting that the biology of the condition is not uniform. The aim of this study was to compare levels of Gd-IgA in serum from Caucasian and Chinese patient and control cohorts.

Methods: An elisa based method was used to measure binding of the lectin Helix Aspersa agglutinin to IgA captured from serum from: 1091 UK IgAN patients, 998

Chinese IgAN patients, 360 UK membranous nephropathy (MN) patients, 193 UK controls and 80 Chinese controls.

Results: Gd-IgA was lower in UK MN patients compared to both IgAN patients ($p < 0.0001$) and the healthy controls ($p < 0.0001$) from the UK. Among Chinese individuals, Gd-IgA levels were higher in IgAN patients compared to healthy controls ($p < 0.05$), but levels were lower in the Chinese compared to the UK cohort, in both IgAN patients ($p < 0.0001$) and controls ($p < 0.0001$).

Discussion: Gd-IgA1 levels are associated with IgAN and in Caucasian and Chinese patients but the difference in prevalence of IgAN cannot be attributed to differences in Gd-IgA levels between these populations. Results also presented at this meeting show that a C1GALT1 haplotype, common in Caucasians but rare in Chinese people, is strongly associated with elevated Gd-IgA1 levels and the reduced Gd-IgA in the Chinese population is consistent with reduced frequency of this haplotype. These data support the hypothesis that the causes of IgAN vary across the world.

Gender differences in serum IgA1 O-galactosylation levels and risk of developing progressive IgA nephropathy

Wimbury, D.; Molyneux, K.; Higgins, P.; Gale, D. and Barratt, J.

Oral presentation, 5th East Midlands Renal Showcase 18/11/2015

IgA nephropathy is a primary glomerulonephritis with variable prognosis characterised by the deposition of aberrantly galactosylated IgA in the renal mesangium. A number of cases of familial IgAN have been reported suggesting a genetic component in the pathophysiology of the condition, though most cases are sporadic. A 2:1 gender bias in the diagnosis of IgAN, favouring males, is seen in European but not Chinese populations; the cause of this imbalance is not understood. Changes in the pattern of serum IgA1 O-galactosylation in IgAN patients have been widely reported, however differences between male and female patients have not been investigated.

The aims of this study were to investigate;

1. A link between serum IgA1 O-galactosylation and IgAN prognosis
2. The heritability of IgA1 serum O-galactosylation
3. Gender differences in serum IgA1 O-galactosylation

Relative levels of serum IgA1 O-galactosylation for 1090 IgAN serum samples from the UKGDB, including 296 male and 140 female patients; 136 complete parent-child trios, 39 parent-child pairs and 193 unrelated spouses were measured using an established ELISA-based Helix aspersa (HA) lectin binding assay. The unrelated spouses were taken as healthy subjects. The data was normally distributed and gender differences analysed using ANOVA and unpaired t-tests. For heritability analysis, the data was standardised between patients and parents, and analysed by correlation and linear regression; with a narrow sense heritability (h^2) value closer to 1 giving stronger evidence for the involvement of genetic factors.

We found patients with progressive IgAN have significantly higher IgA1-HA lectin binding than both non-progressors and controls ($p < 0.005$ vs non-progressors; $p < 0.02$ vs healthy subjects) strongly indicating that changes in the pattern of serum IgA1 O-galactosylation influence the risk of developing significant renal impairment in IgAN. Interestingly, when patients are split by gender, only female progressors had significantly higher IgA1-HA lectin binding than all the other groups. Heritability analysis of the whole patient data set shows $h^2 = 0.2954$, implying that genetic factors have a role in determining the pattern of IgA1 O-galactosylation. However, when patients were split by gender, the difference in heritability was marked, in males, $h^2 = 0.03696$ while in females, $h^2 = 0.8314$, giving a very strong indicator the genetic factors involved in the pattern of IgA1 O-galactosylation play a larger role in IgAN in females compared to males. These results suggest that differences in genetic factors involved in IgA1 O-galactosylation between males and females could result in the higher prevalence of IgAN seen in males.

The levels of aberrantly galactosylated IgA1 are associated with the risk of developing progressive renal disease in IgA nephropathy

Molyneux, K.; Wimbury, D.; Higgins, T.; Gale, D. and Barratt, J.

Poster presentation, 52nd ERA-EDTA Renal Congress

Nephrology Dialysis Transplantation 2015; 30 (suppl 3): iii33

The UK Glomerulonephritis DNA Bank (UKGDB) IgAN cohort was established in 2000. Participants of European ancestry were recruited through four UK centres

(Glasgow, Leicester, London, and Oxford) through probands with renal biopsy-proven IgAN <50 years of age at the time of diagnosis and >18 years of age at the time of recruitment. Individuals with evidence of liver disease or Henoch-Schonlein purpura were excluded. Diagnosis was confirmed in all cases by direct review of renal biopsy histopathology reports and clinical case records. Where available, samples were also collected from the parents of affected individuals and, in some cases, other nuclear family members, to enable a study of family-based association. The UKGDB IgAN cohort comprises 1090 serum samples which include 136 complete parent-child trios, 39 parent-child pairs and 193 unrelated spouses.

Ten year follow-up data is now available on this cohort and we have used this to identify patients with non-progressive IgAN (<10% change in serum creatinine over a minimum of 10 years follow-up) and progressive IgAN (>100% increase in serum creatinine or ESRD during follow-up). Of the cohort 160 patients (33.3%) developed progressive IgAN, 194 patients (40.4%) were non-progressors, 54 (11.25%) were indeterminate at 10 years follow-up, 72 patients (15%) had no follow-up data available.

Changes in the pattern of IgA1 O-galactosylation in IgAN have been widely reported but there is little information in Caucasian populations on the relationship of IgA1 O-galactosylation and severity of IgAN. In this study we measured relative levels of serum IgA1 O-galactosylation using an established ELISA-based *Helix aspersa* (HA) lectin binding assay. In this assay increasing HA lectin binding to IgA1 correlates with lower O-galactosylation of the IgA1 hinge region. The samples were analysed in duplicate by 2 independent scientists achieving a coefficient of variance of <5% between samples. The unrelated spouses were taken as healthy subjects. The data was normally distributed and was analysed using unpaired t-tests.

Patients with non-progressive IgAN exhibited the same pattern of IgA1 hinge region O-galactosylation as healthy subjects (serum IgA1-HA binding: non-progressors 0.885 ± 0.01 , healthy subjects 0.896 ± 0.01 , $p=>0.05$). By contrast, patients who went onto develop progressive renal impairment had significantly higher IgA1-HA lectin binding compared to both the non-progressors and healthy subjects at recruitment (progressors 0.928 ± 0.01 ; $p=<0.005$ vs non-progressors; $p=<0.05$ vs healthy subjects).

These observations strongly suggest that changes in the pattern of serum IgA1 O-galactosylation influence the risk of developing significant renal impairment in IgAN. This is consistent with observations correlating IgA1-HA lectin binding with severity of renal biopsy features and risk of progression in Chinese cohorts. The lack of an association between serum IgA1 O-galactosylation and IgAN in patients with non-progressive IgAN suggests that factors in addition to IgA1 hinge region O-galactosylation are important in initiating IgA immune complex deposition in IgAN.

Serum IgA1 hinge region O-galactosylation is a heritable trait in Caucasians

Molyneux, K.; Wimbury, D.; Higgins, T.; Gale, D. and Barratt, J.

Poster presentation, 52nd ERA-EDTA Renal Congress

Nephrology Dialysis Transplantation 2015; 30 (suppl 3): iii384

The UK Glomerulonephritis DNA Bank (UKGDB) IgAN cohort was established in 2000. Participants of European ancestry were recruited through four UK centres (Glasgow, Leicester, London, and Oxford) through probands with renal biopsy-proven IgAN <50 years of age at the time of diagnosis and >18 years of age at the time of recruitment. Individuals with evidence of liver disease or Henoch-Schonlein purpura were excluded. Diagnosis was confirmed in all cases by direct review of renal biopsy histopathology reports and clinical case records. Where available, samples were also collected from the parents of affected individuals and, in some cases, other nuclear family members, to enable a study of family-based association. The UKGDB IgAN cohort comprises 1090 serum samples, including 136 complete parent-child trios and 39 parent-child pairs.

Changes in the pattern of IgA1 O-galactosylation in IgAN have been widely reported. We have presented data previously to show that the pattern of serum IgA1 O-galactosylation remains unchanged over time in both healthy subjects and patients with IgAN. This observation, along with reports of large pedigrees of familial IgAN, increased occurrence of the disease in relatives of affected individuals and geographical variations in disease prevalence suggest a genetic contribution to the pathogenesis of IgAN and possibly IgA1 O-galactosylation.

In this study we assessed the heritability of serum IgA1 O-galactosylation by measuring relative levels of serum IgA1 O-galactosylation in 136 parent-child

trios identified from the UKGDB IgAN cohort using an established ELISA-based *Helix aspersa* (HA) lectin binding assay. In this assay, increasing HA lectin binding to IgA1 correlates with lower O-galactosylation of the IgA1 hinge region. The samples were analysed in duplicate by 2 independent scientists achieving a coefficient of variance of <5% between samples. The data was standardised between patients and parents, and analysed by correlation and linear regression. Narrow-sense heritability (h^2) was 0.2954, strongly implying that genetic factors play an important role in determining the pattern of IgA1 O-galactosylation in Caucasians. These results are consistent with findings in familial IgAN and sporadic IgAN in other ethnic groups.

Separately, we have also collected data showing that the pattern of serum IgA1 O-galactosylation in patients with biopsy-proven IgAN and non-progressive disease is not significantly different from that seen in healthy subjects. The extent of IgA1 O-galactosylation was however significantly associated with the severity of renal injury and risk of developing progressive renal failure in IgAN. IgA1 O-galactosylation therefore appears to be more strongly associated with disease severity (or risk of progression) than it is with susceptibility. Together, these findings imply that as yet undefined genetic determinants of IgA1 O-galactosylation may influence the risk of renal failure in IgAN.

Appendix VII – Buffers and solutions

General

1x PBS

- 8g NaCl
- 0.2g KCl
- 1.15g Na₂HPO₄
- 0.2g KH₂PO₄
- Dissolved in 1l distilled water
- Adjusted to pH7.4

1x TBS

- 6.06g Tris base
- 8.77g NaCl
- Dissolved in 1l distilled water
- Adjusted to pH7.6

ELISA

ELISA coating buffer (0.05M carbonate/bicarbonate, pH9.6)

- 50ml H₂O
- 0.027g Na₂CO₃
- 0.189g NaHCO₃

ELISA wash buffer (PBS/0.3M NaCl/0.1% TWEEN20)

- 20.75g NaCl
- 1ml TWEEN20
- Dissolved in 1l 1x PBS

OPD substrate

- 1 OPD tablet (Thermo 34006)
- Dissolved in 9ml distilled water
- Addition of 1ml stable peroxide solution (Thermo 34062) immediately before use

OPD stop solution (1N H₂SO₄)

- 972ml distilled water
- Add 28ml concentrated H₂SO₄ gradually in a fume hood

SDS-PAGE

10% (w/v) SDS

- 10g SDS
- Dissolved in 100ml distilled water

10% APS

- 0.1g APS
- Dissolved in 1ml distilled water

0.5M Tris-HCl, pH6.8

- 30.285g Tris-HCl
- Dissolved in 500ml distilled water
- Adjusted to pH6.8

1.5M Tris-HCl, pH8.8

- 90.855g Tris-HCl
- Dissolved in 500ml distilled water
- Adjusted to pH8.8

Stacking and resolving gels

Component	Stacking gel	10% resolving gel	12.5% resolving gel
Distilled water	3.03ml	3.1ml	3.19ml
30% (w/v) acrylamide	0.65ml	2.5ml	4.17ml
0.5M Tris-HCl, pH6.8	1.25ml	-	-
1.5M Tris-HCl, pH8.8	-	1.875ml	2.5ml
10% (w/v) SDS	0.05ml	0.075ml	0.1ml
10% APS	0.025ml	0.0375ml	0.05ml
TEMED	0.005ml	0.001ml	0.005ml

Non-reducing sample buffer

- 4.6ml distilled water
- 1ml 0.5M Tris-HCl pH6.8
- 0.8ml glycerol
- 1.6ml 10% (w/v) SDS
- Bromophenol blue

Reducing sample buffer

- 4.2ml distilled water
- 1ml 0.5M Tris-HCl pH6.8
- 0.8ml glycerol
- 1.6ml 10% (w/v) SDS
- 0.4ml β -mercaptoethanol (added in fume hood)
- Bromophenol blue

10x running buffer

- 30.3g Tris base
- 144g glycine
- 10g SDS
- Dissolved in 1l distilled water

10x transfer buffer

- 30.3g Tris base
- 144g glycine
- Dissolved in 1l distilled water

TTBS (1x TBS/0.1% TWEEN20)

- 1ml TWEEN20
- Dissolved in 1l 1x TBS

Cell work

B cell storage buffer (HBSS/2% FBS)

- 490ml HBSS
- 10ml FBS

DAKIKI culture media (RPMI 1640/10% FBS)

- 450ml RPMI 1640 (Gibco)
- 50ml FBS

Appendix VIII – List of suppliers

Applied Biosystems

ATCC

BD Biosciences

Bio-Rad

Dako

eBioscience

Gibco

Merck

Miltenyi Biotec

NanoString

New England Biolabs

NIBSC

Nunc

Promega

ProSpec

R&D Systems

Santa Cruz Biotechnology

Sarstedt

Sigma-Aldrich

Stemcell Technologies

The Binding Site

Thermo Fisher Scientific

Zymo Research

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