STUDIES ON ANTIBODIES THAT FORM IMMUNE COMPLEXES WITH LIPOPROTEIN(a) [Lp(a)] IN PLASMA

A THESIS PRESENTED BY

SABARINATH P.S

TO

SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
Thiruvananthapuram

IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

2014

DECLARATION

I, **Sabarinath P.S**, hereby declare that I had personally carried out the work depicted in the thesis entitled **"Studies on antibodies that form immune complexes with lipoprotein(a) [Lp(a)] in plasma".** No part of the thesis has been submitted for award of any other degree or diploma prior to this date.

Signature:

Date:

Dr. P. S. Appukuttan,

Professor and Head, Department of Biochemistry,

Sree Chitra Tirunal Institute for Medical Sciences & Technology,

Medical College P.O, Trivandrum,

Kerala, India- 695 011.

CERTIFICATE

This is to certify that Mr. Sabarinath P.S, in the Department of Biochemistry of this institute, has fulfilled the requirements prescribed for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum.

The thesis entitled **"Studies on antibodies that form immune complexes with lipoprotein(a) [Lp(a)] in plasma"** was carried out under my direct supervision. No part of thesis was submitted for the award of any degree or diploma prior to this date.

Clearance was obtained from the Institutional Ethics Committee (IEC-511) for carrying out the study.

Signature:

Date:

The thesis entitled

STUDIES ON ANTIBODIES THAT FORM IMMUNE COMPLEXES WITH LIPOPROTEIN(a) [Lp(a)] IN PLASMA

Submitted by

SABARINATH P.S

for the degree of

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE

FOR

MEDICAL SCIENCES AND TECHNOLOGY,
TRIVANDRUM

Thiruvananthapuram

Is evaluated and approved by

____________________ ____________________

Name of guide Name of thesis examiner

// ACKNOWLEDGEMENTS

It’s my pleasure to thank all those people who made this thesis possible.

I am greatly indebted to my Ph.D. supervisor Dr. P.S.Appukuttan, Professor and Head, Dept. of Biochemistry, SCTIMST, for his constant encouragement and enthusiastic guidance for the entire duration of my study. I am fortunate to have had him as my mentor for it was he who taught me scientific experimentation.

I express my sincere gratitude to the members of my Doctoral Advisory Committee, Dr. K. Shivakumar, Scientist G, Division of Cellular & Molecular Cardiology, SCTIMST and Dr. P. Remani, Additional Professor, Division of Cancer Research, Regional Cancer Centre, for their valuable suggestions and constant encouragement.

I am thankful to SCTIMST for providing me laboratory facility and financial assistance in the form of SCTIMST- Institute Fellowship from 2009-2013.

I would like to thank Dr. N. Jayakumari, Professor, Dept. of Biochemistry, SCTIMST, for her valuable suggestions and for permitting me the use of fluorescence microplate reader.

I am thankful to all staff of the Dept. of Blood Transfusion Services, Dept. of Microbiology and Central Clinical Laboratory of this Institute for providing me outdated plasma, whole blood and sheep blood samples at any time all throughout the duration of my study.

My sincere thanks to Annamma K.I, Scientific Officer, Geetha.M, Scientific Officer, Sumitha, Technical staff and Dr. Anu Paul, my senior, for their guidance with regard to experimental procedures.

My sincere gratitude to T.K. Shyamala, Sasidharan, C. Raman Kutty Nair and Sheela. B for assisting me during the course of my work.

Special thanks to Dr.Srinivas. G, Scientist E, Department of Biochemistry and my colleagues Kalaivani V, Genu George, Karthi K, Dr. Suboj Babykutty, Dr. Priya. P.S, Dr. Vinod.V, Padmakrishnan C.J, Nandini R J, Raji, Mary T, Sowmya Rani, Sini S, Reema G and Dr.Deepa. I would also like extend my thanks to Scientists, Staff and friends in other departments for their timely help and good company.

I am thankful to all staff of the Medical Illustration Department for helping me with the documentation.

I am indebted to my parents and in-laws for their prayers and for the support they have provided me with.

Lastly but most importantly, I acknowledge my wife Swapna Nandakumar for patiently proofreading my thesis and for her timely suggestions.

Sabarinath.P.S

TABLE OF CONTENTS

Contents	Page no.
Title page	i
Declaration by the student	ii
Certificate of guide	iii
Approval of Thesis	iv
Acknowledgement	v-vi
List of Figures	1 - 2
List of Tables	3
Abbreviations	4 - 5
Synopsis	6 - 12
Introduction	13 - 21
Literature Review	22 - 60
Materials and Methods	61 - 84
Results and Discussion	85 - 127
Summary and Conclusion	128 - 134
Future directions	135
Bibliography	136 – 151
List of publications	152

LIST OF FIGURES

Figure No.	Caption	Page No.
1.	Structure of major aspargine linked oligosaccharides	25
2.	Structure of sialyl-Lewis antigen	29
3.	N-glycan pattern in Fetuin	34
4.	Schematic model of Lp(a)	55
5.	N-glycan distribution in apoB	56
6.	Immunoglobulin distribution in LIg	88
7.	Haemagglutination of human O-group RBC by LIg	89
8.	Inhibition ELISA to demonstrate the sugar specificity of LIg	91
9.	LIg binding to α- and β-anomers of galactose	92
10.	Effect of removal of TAG on LIg recognition of glycoconjugates	93
11.	Agglutination of unmodified human and sheep RBC by LIg	94
12.	Effect of deletion of N- and O-glycans of AF on LIg recognition	95
13.	LIg binding to desialylated Lp(a) and LDL	97
14.	Incorporation of FITC-conjugated LIg into IC	98
15.	Immunoglobulin distribution in Lp(a) IC	104

Figure No.	Caption	Page No.
16.	Immunoglobulin distribution in apoB IC	105
17.	Immunoglobulin distribution in apoA1 IC	107
18.	Dominance of Lp(a) in IC	108
19.	To assay the desialylation status of Lp(a) in IC	110
20.	Haemagglutination assay of de novo IC	116
21.	Agglutination by de novo IC from different plasma samples	117
22.	Effect of polyagglutinin titre in formation of agglutinating IC	119
23.	Extent of Lp(a) removal from N-L1	122
24.	Contribution of Lp(a) in agglutinating IC	122
25.	Jacalin-based enzyme immunoassay assay to determine Lp(a) levels in plasma	124
26.	Effect of Lp(a) size on agglutination by lipoprotein IC	124

LIST OF TABLES

Table No.	Caption	Page No
1.	Mucin type O-glycans	27
2.	Antigen and antibody characteristics that affect the pathogenicity of IC	50
3.	Inhibition of LIg-mediated agglutination of desialylated RBC by sugars	90
4.	PNA response per unit anti-apo(a) response for IC with or without 8 M urea treatment and pure Lp(a)	110
5.	Agglutination inhibition of de novo IC to demonstrate the involvement of anti-carbohydrate antibodies	120

ABBREVIATIONS

AF – Asialofetuin

AGE – Advanced Glycation End Product

Anti-Gal – Anti alpha-galactoside binding antibody

β2-GPI – Beta-2 Glycoprotein I

CAD – Coronary Artery Disease

CHD – Coronary Heart Disease

ConA – Concanavalin A

CRP – C-reactive proteins

CVD – Cardiovascular Diseases

FITC – Fluorescin isothiocyanate

GS I-B4 – *Griffonia simplicifolia* isolectin B4

HDL – High Density Lipoprotein

HRP – Horse radish peroxidise

IC – Immune Complex

L1 – Lipoprotein layer

LacNAc – N-acetyl lactosamine

LDL – Low Density Lipoproteins

LIg – Lactose binding immunoglobulin

Lp(a) – Lipoprotein(a)

MDA - Malondialdehyde

MUC1 – Mucin peptides

N-L1 – Neuraminidase-treated lipoprotein layer

NH-L1 – Heat-inactivated neuraminidase-treated lipoprotein layer

OPD – Orthophenylenediamine

OxLDL – Oxidised LDL

OxLp(a) – Oxidised Lp(a)

PEG – Polyethylene glycol 6000

PNA – Peanut agglutinin

STPS – Serine- and Threonine-rich peptide sequences

Tg – Thyroglobulin

TAG – Terminal alpha-galactoside

TIM – Soybean trypsin inhibitor-Melibiose

TIL – Soybean trypsin inhibitor-Lactose

VNTR – Variable Number Tandem Repeats

SYNOPSIS

Background

Atherosclerosis is a complex, multi-factorial disease of the large and medium sized arteries. It is the major underlying cause of mortality and morbidity in cerebrovascular and cardiovascular diseases. Though several theories have been proposed to explain its pathogenesis recent studies implicate a role for lipoprotein immune complexes in foam cell formation and inflammatory events leading to atherosclerosis. Studies in this direction have identified oxidation of lipoproteins and subsequent recognition by their cognate antibodies as the most common event leading to immune complex (IC) formation. However, most studies have focused on low density lipoprotein (LDL) immune complex and very little is known about immune complexes formed by lipoprotein(a) [Lp(a)].

Lp(a) is a structural variant of LDL formed by covalent linkage of an apo(a) subunit by disulfide bond to the apoB subunit of LDL. Apo(a) is composed of kringle IV (type 1-10), V and an inactive protease domain, homologous to plasminogen. *In vitro* experiments have demonstrated that Lp(a) can interfere with normal fibrinolytic function of plasminogen and prevent thrombolysis. Apo(a) exhibits size polymorphism due to variation in kringle IV type 2 repeats which can range from 3-40. N-glycans of complex biantennary structure present on the kringle region and O-glycans of core-1 type present at the inter-kringle regions vary with kringle IV type 2 repeats so that carbohydrate content of apo(a) may reach up to 28% of its mass. N-glycans of high mannose, complex and hybrid type constitute 10% by

weight of apoB. High serum Lp(a) has been correlated with disease conditions like stroke, aneurism, migrane, pre-eclampsia, cerebro- and cardiovascular diseases. Although Lp(a) has been implicated in vascular pathology its mechanism of action still remains unknown with lipid lowering drugs and diet having no effect on Lp(a) levels in circulation.

Hypothesis

Serum sialic acid and sialidase levels are reported to be elevated in cardiovascular disease. *In vitro* studies have demonstrated that desialylated Lp(a) and LDL induce lipid accumulation in human aortic intimal cells. Lp(a) from coronary artery disease (CAD) patients are 2.5 fold more desialylated than from normal subjects. Humoral immune response to desialylated Lp(a) and its mechanism of tissue deposition remains unexplored.

Desialylation of Lp(a) by microbial or endogenous sialidases will expose epitopes which are otherwise cryptic, namely Thomsen-Friedenreich (TF or T) antigen (Galβ1-3GalNAc) in O-glycans and LacNAc groups (Galβ1-4GlcNAc) in N-glycans. Lactose binding antibody (LIg) that recognizes LacNAc is a candidate anti-carbohydrate antibody to form IC with plasma Lp(a).

When an IC is formed between multivalent antigens and antibodies, the complex extends three dimensionally to form a lattice leaving some antigen binding sites on the antibody unoccupied due to steric hindrance or due to binding stochiometry driven by concentrations of participating components. The unoccupied binding site may be available to accommodate a different antigen containing same cognate

epitope in different spatial arrangements. Desialylated Lp(a) which contains dozens of exposed LacNAc and T-antigen moieties is a potential candidate antigen for formation of such IC with circulating anti-carbohydrate antibodies such as LIg and anti-T. Multiple valency of Lp(a) offers scope for unoccupied binding sites on these ICs capable of anchoring on to desialylated cell surface. This may be a plausible mechanism of Lp(a) deposition on to vessel wall.

Objectives

1. Determine the immunoglobulin composition of Lp(a) IC in circulation.
2. Determine the desialylation status of Lp(a) in circulating IC.
3. Elucidate specificity of plasma lactose binding antibody.
4. Examine desialylation-dependent recognition of Lp(a) by lactose binding antibody to form IC.
5. Elucidate possible mechanism of tissue deposition of desialylated Lp(a) IC.

Methods

Lectins (PNA, ConA, jacalin) and lactose binding immunoglobulin (LIg) were isolated by affinity chromatography. Lp(a) was isolated from human plasma by affinity precipitation with jacalin, followed by Tris Borate EDTA electrophoresis and electroelution. Lp(a) was assayed by jacalin-based enzyme immunoassay. Circulating lipoprotein IC from human plasma was isolated by precipitation with polyethylene glycol (PEG) at designated concentrations. Alternatively, after free

plasma lipoproteins were removed in top 20% layer following ultracentrifugation at 535000 g with density adjusted to 1.24 g/cc with KBr, bottom 80% devoid of free lipoproteins was used as source of lipoprotein IC. The immunoglobulin distributions in lipoprotein ICs were assayed by sandwich ELISA using anti-apoprotein antibody coated wells for capturing IC and anti-human Ig-HRP for quantitation. IC was dissociated with 8 M urea and liberated lipoproteins were collected in top 20% layer by ultracentrifugation at 535000 g and density 1.24 g/cc. Desialylation status of Lp(a) liberated into top 20% layer by ultracentrifugation after urea treatment was assayed by direct coating and probing with PNA-HRP. Ligand binding properties of LIg were determined by haemagglutination assay and direct ELISA using appropriate glycoconjugates, sugar inhibitors, and exo/endo-glycosidase. Desialylation-dependent recognition of Lp(a) by LIg was assayed by probing ELISA well-coated Lp(a) with biotinylated-LIg followed by avidin-HRP or by measuring IC between FITC-LIg and desialylated plasma samples with varying concentrations of Lp(a). Denovo IC formed between desialylated lipoprotein and plasma immunoglobulins was collected by PEG precipitation and its agglutination of desialylated human RBC was taken as a measure of its free valency and this system served as a prototype of IC binding to host cells.

Major findings

Among the two different concentrations of PEG (2 and 3.5%), 3.5% PEG precipitated more Lp(a) IC than 2% while HDL ICs were precipitated only at 3.5%. Antibodies of IgA, IgG, IgM type are involved in formation of IC with Lp(a), with IgM being the most dominant immunoglobulin type followed by IgG and IgA.

Unlike in serum, dominance of IgM in Lp(a) IC indicates that primary natural antibodies are involved in IC formation.

Although serum concentration of LDL is several times greater than that of Lp(a), results from two different experiments show that 1) apo(a)-ICs out number apoB-ICs suggesting that Lp(a) has greater tendency to associate with antibodies than LDL; 2) Ratio of response towards anti-apo(a) to that towards anti-apoB in IC prepared from plasma devoid of free lipoproteins was close to the ratio of same parameters in purified Lp(a) indicating Lp(a) is the predominant lipoprotein in plasma IC.

Urea treatment dissociated IC and liberated free Lp(a) to top 20% volume in ultracentrifugation. Comparison of binding of PNA (lectin specific to desialylated core-1 O-glycans) to Lp(a) liberated from IC and to purified Lp(a) revealed that IC Lp(a) is far more desialylated.

IgG is the most dominant immunoglobulin type in LIg with IgA, IgM contributing only one-fourth of IgG. In addition to lactose and LacNAc moieties, LIg exhibits extended specificity towards terminal alpha-galactoside groups as suggested by results from 3 independent experiments: 1) LIg agglutinated unmodified sheep RBC while human O-group RBC was agglutinated only after desialylation. Haemagglutination was inhibited by lactose at 12 mM, while 1-O-methyl α-galactoside and 1-O-methyl β-galactoside were equally inhibitory at 50 mM concentration. 2) Lactose and melibiose were equally inhibitory in LIg binding to bovine thyroglobulin and asialofetuin. Specificity of LIg towards α-galactoside groups is greater than its β-anomer when ligands are presented in glycoconjugate form than in solution. 3) Removal of terminal alpha-galactoside group from bovine

thyroglobulin and rabbit RBC membrane glycoprotein by α-galactosidase reduced LIg binding; the residual LacNAc groups were much less efficient ligands than α-galactoside moieties.

LIg recognized Lp(a) only after desialylation of the latter and binding was specific to LacNAc groups on Lp(a) since truncation of LacNAc moieties by α-mannosidase decreased LIg binding. In support to this conclusion selective removal of O-glycan on asialofetuin by O-glycanase did not affect LIg binding while removal of LacNAc groups using α-mannosidase reduced LIg binding. Further, incorporation of fluorescence tagged LIg into IC fraction was dependent on desialylation of plasma and increased with plasma Lp(a) concentration, while FITC-labeled non specific antibody was not incorporated into IC fraction upon desialylation.

De novo IC formed between desialylated lipoproteins and plasma antibodies were capable of agglutinating desialylated human O-group RBC and not unmodified RBC which suggests that antibodies involved in the formation of IC with Lp(a) retain part of their sugar binding sites unoccupied. IC formed from non-desialylated lipoprotein sample as control did not agglutinate desialylated RBC. Agglutination was inhibitable with sugar and β-anomers of galactose viz. lactose and 1-O-methyl β-galactoside were the most efficient inhibitors, confirming the involvement of β-galactose-specific antibody. Agglutination titre of de novo IC was proportional to the titre of serum anti-carbohydrate antibodies capable of agglutinating desialylated human RBC.

Among serum lipoproteins, Lp(a) formed majority of agglutinating IC since removal of Lp(a) reduced agglutination by 4 to 8 fold, which again confirms that Lp(a) has greater tendency to form IC upon desialylation. Variation in Lp(a) molecular size also reflected in agglutinability. Given the same amount of Lp(a), de novo IC formed using low molecular weight Lp(a) agglutinated 2 fold higher than high molecular weight Lp(a).

Significance

Dominance of apo(a) in atherosclerotic plaques and its accumulation with disease progression has necessitated research to understand mechanism of tissue deposition of Lp(a). The present study shows that Lp(a) dominates in IC and desialylation is one of the factors leading to IC formation. Immune complex formed between multivalent antigen and anti-carbohydrate antibodies retain some of the biding sites on antibodies unoccupied which can in turn bind to appropriate ligands on cell surfaces. Since areas more prone to lesions on arteries are desialylated and alpha-galactoside-specific antibodies are detected in atheromatous plaques, tissue deposition of IC formed between desialylated Lp(a) and anti-carbohydrate antibodies is a novel mechanism by which Lp(a) can get deposited on vessel wall. Incidentally diabetes that considerably augments the atherosclerosis process is marked by extensive desialylation of plasma components. So far physiological role of LIg has not been elucidated. This study suggests that LIg recognises desialylated Lp(a) leading to IC formation which may be a mechanism by which Lp(a) is sequestered from the system and transferred to extravascular tissues.

INTRODUCTION

Atherosclerosis

Atherosclerosis is a progressive disease of the large and medium sized arteries. It is the major underlying cause of mortality and morbidity in cerebrovascular and cardiovascular diseases. Hallmarks of atherosclerotic lesions are presence of lipid laden macrophages (foam cells), calcium deposits, extracellular lipid core, fibrin, antibodies, complement components and peripheral mononuclear cells in the intimal region of the lesion-prone areas of arterial wall. As the lesion progresses, plaque becomes more vulnerable to rupture and thrombus formation leading to ischemia of the organ [Galkina and Ley, 2009; Hansson et al., 2006]. Atherosclerosis is a complex, multi-factorial disease since no single factor has been identified as cause of the disease, by itself. However, clinical and epidemiological studies have identified several risk factors that predispose individuals to atherogenesis [Tegos et al., 2001]. Although several theories have been proposed to explain its pathogenesis, recent studies indicating that atherosclerosis may be a chronic inflammatory disease have gained wide acceptance [Ross, 1999; Libby et al., 2002; Hansson et al., 2006].

Immune complex-mediated vascular pathology

Immune complex (IC) formed between microbial or self antigens and their cognate antibodies have been implicated in pathogenesis of disease conditions like serum sickness, connective tissue autoimmune diseases and systemic infections. The deposition or formation of IC on the vascular wall initiates a type III hypersensitive reaction, where the Fc-region of the antibody binds to effector molecules like complement components and Fc-receptors, which in turn triggers an inflammatory cascade involving mast cell degranulation, neutrophil chemotaxis and

release of lytic enzymes leading to vascular injury [Jancar and Crespo, 2005]. The discovery of microbial antigens (*Chlamydiae pneumoniae, Helicobacter pylori, Herpesvirus, Cytomegalovirus*), modified lipoproteins, heat shock proteins (HSPs), beta-2 glycoprotein I (β2-GPI) and antibodies against them, both in circulation and in atherosclerotic plaques have led to the hypothesis that IC may promote inflammatory events in atherosclerosis [Hansson et al., 2006; Burut et al., 2010].

Lipoprotein immune complex

The proteins, lipids and carbohydrate moieties in lipoproteins are susceptible to spontaneous modifications. The most common modifications are lipid peroxidation, glycation and desialylation. Polyunsaturated fatty acids in cholesteryl esters and phospholipids undergo oxidation by Fenton's reaction, to give rise to reactive aldehydes like malondialdehyde (MDA) and 4-hydroxynonenal derivatives (HNE) which in turn covalently bind to ε-amino group in lysine residues of apoproteins to form MDA-lysine adducts [Steinbrecher, 1987]. Glycation of ε-amino groups in apoprotein moieties by Amadori's reaction results in the formation of advance glycation end product (AGE) [Klein et al., 1995]. Sialic acids are nine carbon monosaccharides that form a protective terminal cap in the oligosaccharide groups that are attached to proteins and lipids [Cohen and Varki, 2010]. Serum sialidase and circulating sialic acid levels are reported to be elevated in patients with diabetes, stroke and cardiovascular diseases (CVD) [Roozbeh et al., 2011; Nanetti et al., 2008; Lindberg et al., 1992; Sönmez et al., 1998]. Increase in serum sialic acid levels are considered as strong predictor for vascular pathology [Lindberg et al., 1992]. Desialylation by microbial or endogenous sialidases can expose the underlying

cryptic glycotopes. Studies also indicate that these sialic acids can undergo oxidative modifications and thereby function as scavengers of reactive oxygen species [Varki et al., 2009a]. The modified epitopes in lipoproteins are categorised as damage or danger-associated molecular patterns (DAMP). In order to prevent deleterious effects of these modified lipoproteins, our immune system has evolved protective mechanisms which enable their removal by pattern recognising receptors (PRR) such as scavenger receptors, toll-like receptors, C-reactive proteins (CRP) and natural antibodies [Miller et al., 2011].

In the course of understanding the nature of modified lipoproteins and their immune response, several studies provided evidence for the presence of antibodies against modified lipoproteins in the serum and atheromatous plaques of humans and experimental animals [Virella et al., 1993; Ylä-Herttuala et al., 1994]. The presence of circulating ICs involving modified lipoproteins and their cognate antibodies was also detected in normal subjects and in patients with hyperlipidemia and coronary heart diseases (CHD) [Beaumont et al., 1988; Boullier et al., 1995; Wang et al., 2003]. Tertov et al. reported the presence of desialylated forms of LDL in circulating IC. Furthermore desialylated forms of LDL were ligands for autoantibodies against LDL [Tertov et al., 1996]. The above mentioned antibodies are components of the humoral immune response, synthesised in the spleen by the B1-cells without any deliberate immunisation, hence called natural antibodies. These antibodies sequester altered self antigens, cell debris, tumour cells and modified plasma components by forming ICs, in turn leading to opsonisation and scavenging by mononuclear phagocytic system [Binder et al., 2005].

Role of lipoprotein immune complex in atherosclerosis

To ascertain the role of lipoprotein IC in vascular pathology epidemiological studies were carried out in patients to find correlation between lipoprotein IC on one hand and vascular disorders, diabetes and myocardial infarction on the other. Antibodies against oxidised LDL have been reported to be elevated in disease conditions like hypertension, peripheral arterial disease, atherosclerosis and CVD [Maggi et al., 1995; Monaco et al., 2001; Bergmark et al., 1995]. Studies by several independent groups have confirmed that serum antibodies against oxidised lipoproteins are independent predictors of the progression of atherosclerosis [Salonen et al., 1992; Puurunen et al., 1994]. Alongside to antibodies LDL-ICs were also higher in patients with CHD [Tertov et al., 1990; Lopes-Virella et al., 1999]. Eight year follow up studies in patients with type I diabetes have shown a positive correlation between LDL-IC levels and development of CHD [Lopes-Virella et al., 1999]. Prospective studies in 1000 type I diabetic patients have shown that increased levels of cholesterol and modified apoB in IC is a positive predictor for internal carotid intima-media thickness [Lopes-Virella et al., 2007]. A 20 year follow up study of circulating IC in 50 year old men has shown circulating IC as an independent risk factor for myocardial infarction [Mustafa et al., 2000]. *In vitro* studies on monocytes and human aortic intimal cells have shown that oxidised LDL-IC and LDL IC have greater tendency to form foam cells than oxidised LDL alone [Griffith et al., 1988; Orekhov et al., 1991]. Further, lipoprotein IC also promotes the release of proinflammatory cytokines leading to plaque rupture [Virella et al., 1995]. However, most of these studies have focused on LDL IC and relatively very little is known about lipoprotein(a) IC.

Lipoprotein(a)

Lipoprotein(a) [Lp(a)] was discovered by Kare Berg in 1963 as a structural variant of LDL unique to humans, apes, old world monkeys and hedgehog. Lp(a) resembles LDL in lipid composition and presence of apoprotein moiety apoB-100 (apoB), but differs from LDL in having a heavily glycosylated polymorphic protein apo(a), covalently attached to apoB by a disulphide bond. The apo(a) gene is located on chromosome 6. Apo(a) synthesis occurs only in the liver but the site of its conjugation to apoB, in the body is still unknown. Apo(a) comprises single copy of kringle IV (type 1, 3-10), multiple copies of kringle IV type 2, kringle V and an inactive protease domain making it homologous to plasminogen. Variation in the number of Kringle IV type 2 repeats has led to apo(a) size (300-800 KDa) heterogeneity and Lp(a) isoforms [Koschinsky and Marcovina, 1997]. Carbohydrates contribute up to 28% by mass of apo(a). O-glycans of core 1 type seen in the inter-kringle regions constitute up to 80% and N-glycans of complex biantennary type seen at the kringle regions constitute up to 20% of the total carbohydrates in apo(a) chain [Koschinsky and Marcovina, 1997; Scanu and Edelstein, 1997]. N-glycans of high mannose, complex and hybrid type constitute 10% by weight of apoB [Harazono et al., 2005; Triplett and Fisher, 1978].

High serum levels of Lp(a) have been correlated with the pathogenesis of stroke, aneurism, CVD, peripheral vascular diseases, migraine and vascular dementia [Jones et al., 2007; Urakami et al., 2000; Cinzia et al., 2009]. Atherosclerotic plaques contain more apo(a) than apoB [Pepin et al., 1991] and recent studies show that apo(a) level in plaques increases with plaque progression while apoB content

remains unaltered [Dijk et al., 2012]. Lp(a) ICs have also been detected in normal subjects and its levels are reported to be elevated in patients with CHD [Wang et al., 2003]. Although Lp(a) has been associated with vast number of vascular pathology its underlying pathological mechanisms still remains unknown. Interaction of Lp(a) with proteoglycans of extracellular matrix being the only explanation for tissue deposition of Lp(a) [Gustafsson and Borén, 2004], it is imperative to elucidate the mechanism by which Lp(a) may be deposited on extravascular tissues.

HYPOTHESIS

Serum sialic acid and sialidase levels are reported to be elevated in cardiovascular disease. *In vitro* studies have demonstrated that desialylated Lp(a) and LDL induce lipid accumulation in human aortic intimal cells. Lp(a) from coronary artery disease (CAD) patients are 2.5 fold more desialylated than from normal subjects. Humoral immune response to desialylated Lp(a) and its mechanism of tissue deposition remains unexplored.

Desialylation of Lp(a) by microbial or endogenous sialidases will expose epitopes which are otherwise cryptic, namely Thomsen-Friedenreich (TF or T) antigen (Galβ1-3GalNAc) in O-glycans and LacNAc groups (Galβ1-4GlcNAc) in N-glycans. Lactose binding antibody (LIg) that recognizes LacNAc is a candidate anti-carbohydrate antibody to form IC with plasma Lp(a).

When an IC is formed between multivalent antigens and antibodies, the complex extends three dimensionally to form a lattice leaving some antigen binding sites on the antibody unoccupied due to steric hindrance or due to binding stochiometry driven by concentrations of participating components. The unoccupied binding site may be available to accommodate a different antigen containing same cognate epitope in different spatial arrangements. Desialylated Lp(a) which contains dozens of exposed LacNAc and T-antigen moieties is a potential candidate antigen for formation of such IC with circulating anti-carbohydrate antibodies such as LIg and anti-T. Multiple valency of Lp(a) offers scope for unoccupied binding sites on these ICs capable of anchoring on to desialylated cell surface. This may be a plausible mechanism of Lp(a) deposition on to vessel wall.

OBJECTIVES

1. Determine the immunoglobulin composition of Lp(a) IC in circulation.
2. Determine the desialylation status of Lp(a) in circulating IC.
3. Elucidate specificity of plasma lactose binding antibody.
4. Examine desialylation-dependent recognition of Lp(a) by lactose binding antibody to form IC.
5. Elucidate possible mechanism of tissue deposition of desialylated Lp(a) IC.

REVIEW OF LITERATURE

2.1. Glycoconjugates

Carbohydrate moieties covalently attached to proteins or lipids are termed glycoconjugates which can be further categorized into glycoproteins, proteoglycans and glycolipids. The carbohydrate content in glycoproteins varies from less than 1% to 90% of the total weight. This class of compounds includes enzymes, hormones, immunoglobulins, lectins, toxins, carrier proteins and structural proteins. On the other hand, proteoglycans which form the ground substance for extracellular matrix, are composed of glycans containing alternating units of aminosugar and uronic acid attached to a protein core. Carbohydrates contribute to more than 90% of the total weight in proteoglycans [Murray, 1996]. In glycolipids glucose or galactose moieties are attached to the terminal hydroxyl group of ceramide which is composed of sphingosine and a fatty acid. The addition of glycans occurs both at the co-translational and post-translational levels in the endoplasmic reticulum and Golgi complex. It is however, not a template driven process i.e. the order in which glycans are added is not encoded in the genome, but is rather determined by a multitude of factors such as expression and intracellular levels of enzyme glycosyltransferase, accessibility of glycosylation sites in the protein, concentration of nucleotide sugar donors and subcellular microenvironment in addition to the presence of appropriate amino acid in the protein chain [Varki et al., 2009b]. The expression of glycosyltransferase is often characteristic of a particular cell type and can vary widely between different cell types and can change significantly during development or stimulation of a cell, resulting in temporal and spatial variation of glycans. Examination of 650 completely sequenced organisms listed in the CAZy database (*c*arbohydrate-*a*ctive en*z*ymes) suggests that 5% of the genome accounts for enzymes

necessary for glycan synthesis, degradation or recognition. About 2% of the genome codes for glycosyltransferase and less than 1% for glycoside hydrolases. Hence a substantial portion of the genome encodes for enzymes necessary for glycosylation [Varki et al., 2009c].

Glycosylation types in glycoproteins

Five major types of glycosylation occur in eukaryotes, namely N- and O-glycosylation, C-mannosylation, phosphoglycosylation and glypiation. In N-glycosylation the sugar is attached to the amide group of an asparagine (Asn), while in O-glycosylation the sugar is bound to the hydroxyl group of serine (Ser) or threonine (Thr). In C-mannosylation α-mannose residue is attached to C-2 of tryptophan by carbon-carbon bond. In phosphoglycosylation the carbohydrate moiety is attached to the hydroxyl group of Ser by phosphodiester bond. Glypiation involves only two sugars i.e. mannose or glucosamine in glycosidic linkage. Mannose is covalently linked to phosphoethanolamine which in turn is attached to the terminal carboxyl group of protein while glucosamine is covalently attached to phosphatidyl-inositol present in the lipid bilayer [Spiro, 2002].

N-glycosylation

N-glycosylation involves the formation of a β-glycosylamine linkage between glucosamine (GlcNAc) and amide nitrogen of asparagine. It is the most widely distributed carbohydrate-peptide bond in nature and is conserved in plants, yeasts and metazoans. The addition of N-glycans occurs at consensus sequences Asn-X-Ser/Thr called *'sequon'* where X can be any amino acid other than proline

while acidic amino acids decrease the tendency for glycosylation. Assembly of N-glycans takes place in three stages. Initially a 14 sugar oligosaccharide chain $Glc_3Man_9GlcNAc_2$ is synthesized on poly-isoprenoid lipid dolichol which is then transferred *en bloc* to nascent polypeptide chain co-translationally by a multienzyme complex oligosaccharyl transferase. After attachment to the protein, the oligosaccharides are processed by trimming glucose and mannose residues, followed by elongation at α1-3 and α1-6 Man branch points. The final step is marked by the addition of a terminal sialic acid and a fucose or glucosamine to the core. All N-glycans contain a common pentasaccharide core sequence ($Man_3GlcNAc_2$). Based on the composition of oligosaccharides attached to α1-3 and α1-6 Man branch points in the core, N-glycans are classified in to 3 types: high-mannose, complex and hybrid type (Figure.1).

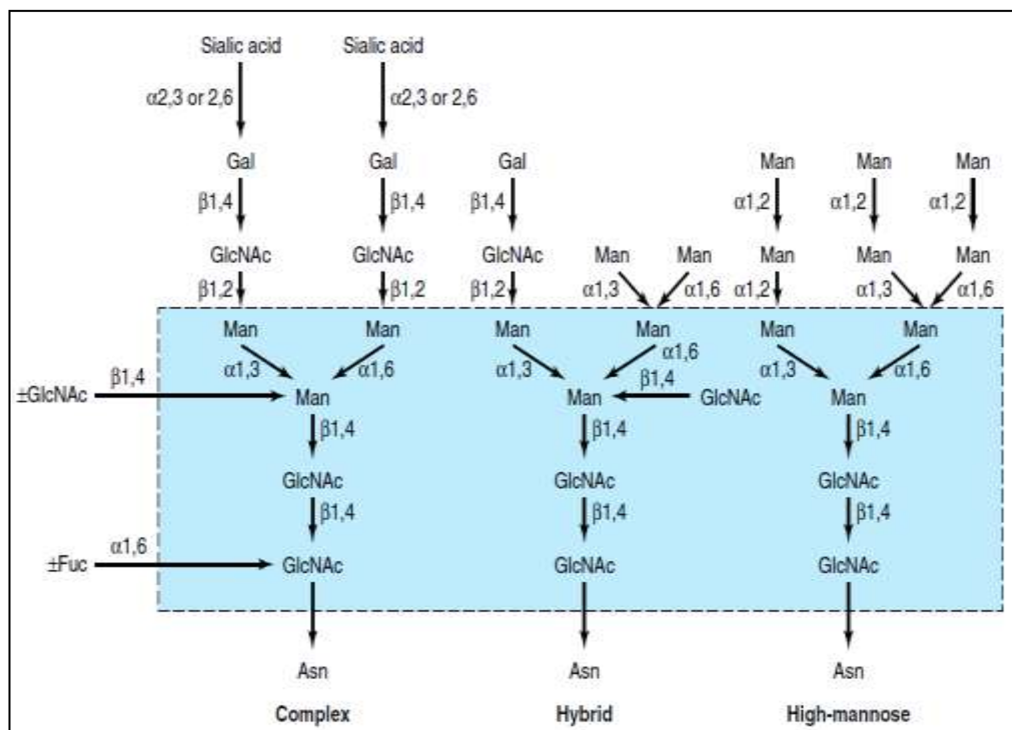


Figure 1. Structure of major aspargine linked oligosaccharides.

In high-mannose only mannose residues are attached to α1-3 and α1-6 Man residues, whereas the complex type involves N-acetyl lactosamine [LacNAc; Gal β1-3 GlcNAc (Type I) or Gal β1-4 GlcNAc (Type II)] linked to α1-3 and α1-6 Man residues. In hybrid type, mannose residues are attached to Man α1-6 arm while LacNAc residues are β1-4 linked to α1-3 Man arm. The total number of mannose residues in high mannose type ranges from 6 to 12 and the chains are often branched. Complex type N-glycans exhibit enormous diversity in the number of side chains attached to Man residues and it can vary between one to five resulting in the formation of mono-, bi-, tri-, tetra- and penta- antennary complex types. Although consensus sequence occurs frequently in proteins only one third of the potential Asn-X-Ser/Thr sites are actually glycosylated, with the most probable explanation being that certain protein conformations make glycosylation sites inaccessible for glycosyltransferase. Presence of acidic aminoacids in X of sequon is another reason. Therefore theoretical predictions of N-glycan sites based on the amino acid sequence may prove unreliable [Varki et al., 2009d].

O-glycosylation

The other major type of carbohydrate-peptide linkage is the O-glycosidic linkage where the carbohydrate moieties are attached to hydroxyl groups of amino acids. O-glycans are broadly classified in to two - mucin and non-mucin type. In mucin type, N-acetyl galactosamine (GalNAc) is α-linked to the hydroxyl group of Ser or Thr while in non-mucin type α-linked fucose, β-linked xylose, α-linked mannose, β-linked *N*-acetylglucosamine, α- or β-linked galactose and α- or β-linked glucose

are attached to the hydroxyl group of either serine, threonine, hydroxyproline, hydroxylysine or tyrosine [Varki et al., 2009e].

Table 1.

Mucin type O-glycans.

Core types	Structure
Tn antigen	GalNAcαSer/Thr
Core 1 or T antigen	Galβ1-3GalNAcαSer/Thr
Core 2	GlcNAcβ1-6(Galβ1-3)GalNAcαSer/Thr
Core 3	GlcNAcβ1-3GalNAcαSer/Thr
Core 4	GlcNAcβ1-6(GlcNAcβ1-3)GalNAcαSer/Thr

Mucin type O-glycans

Mucin type O-glycans are seen at regions where amino acids Ser, Thr and Pro sequences are repeated in tandem [Variable Number Tandem Repeats (VNTR)]. Although no consensus sequence was found preponderance of proline residue was noted at -1 and +3 position at the site of glycosylation. Proline being a helix breaker, it favours the formation of β-turns and β-sheets that facilitate O-glycosylation. Depending on carbohydrate moieties subsequently attached to the protein-linked GalNAc residue, mucin-type O-glycans are divided into four major subtypes (Table.1). Core 1 and 2 structures are widely distributed while the core 3 and core 4 structures are less common and their expression has been mostly associated with mucin producing tissue of the digestive tract. The core 2 and the core 4 branches are

elongated with one or multiple lactosamine structures (Gal β1-4GlcNAc). Usually mucin-type O-glycans are capped with terminal sialic acid residues which render the underlying epitopes cryptic alongside imparting high negative charge density to the protein [Varki et al., 1999].

Thomsen-Friedenerich (TF) or T antigen

T or TF antigen is chemically defined as Gal β1-3GalNAc α-linked to Ser/Thr. T or TF antigens form the core 1 type of O-linked oligosaccharide and are a major constituent of mucin. T antigens are usually covered by sialic acid thereby imparting high negative charge density. T antigen is the immediate precursor of human blood group MN antigen and was discovered about 85 years ago by Thomsen and Friedenreich. The phenomenon of panagglutinability acquired by bacterial contaminated human red blood cells led to the discovery of T-antigen. The sialidase-producing microbes unmasked the cryptic T-antigen on stored human red blood cells thereby leading to panagglutination by complementary anti-T antibodies present in human sera [Friedenreich, 1928]. Both T- and Tn-antigens (Table.1) are tumor-associated carbohydrate antigens expressed during malignant transformation of cells. Since these antigens remain cryptic in normal post-fetal tissues and are expressed only in carcinomas, they are considered as “general pancarcinoma” antigens [Springer et al., 1990; Hanisch and Baldus, 1997]. Incomplete or abnormal glycosylation in malignancies is due to alterations in the gene encoding for glycosyltransferase [Recchi et al., 1998; Kemmner et al., 2003].

Biological role of glycans

In the early part of the 20th century studies on carbohydrates were focused only on the structure and metabolism, hence glycans were thought to be associated only with energy metabolism. With advancement in molecular techniques the biological role of oligosaccharides appears to span an entire spectrum of functions including cell-cell and cell-matrix interaction, growth, adhesion and fertilization. Glycans have been demonstrated to affect the physicochemical properties of proteins like charge, mass, size, hydrophobicity, conformation and stability. They functions as ligands for receptors, mediate protein folding, protein targeting and protect underlying polypeptide chains form proteolysis, microbial recognition and immune response. Incorrectly glycosylated proteins fail to attain proper conformation and are not targeted to the corresponding subcellular compartment but are instead degraded. The dense array of glycans on cell surfaces (glycocalyx) has been demonstrated to play crucial role in mediating cell-cell interaction and cell signaling. Perhaps the best-documented example is that of the selectin family of receptor proteins that mediate the adhesion of leukocytes and platelets to vascular endothelium. The carbohydrate ligands involved in recognition appear to be sialylated fucosylated sugar chains, such as sialyl Lewis^x and sialyl Lewis^a (Figure.2) [Varki, 1993].

Figure 2. Structure of sialyl-Lewis antigen.

Glycans also serve as 'traitorous' signposts for microbial and immune attack on the one hand, while on the other they act as 'decoys' to abrogate these detrimental reactions as well. Glycans act as specific receptors or ligands for viruses, bacteria, parasites, toxins, antibodies and cells of the immune system [Varki, 1993]. In most of these instances, there is exquisite specificity for the oligosaccharide sequence. Influenza A virus initiates infection in humans by binding to α2-6 linked cell-surface sialic acids. However, addition of O-acetyl ester to the 9-position of terminal sialic acid residues abrogates binding of influenza A virus. Influenza-C virus, in contrast, binds exclusively to glycoproteins and glycolipids containing 9-O-acetylated *N*-acetylneuraminic acid. Similarly ganglioside GM1 (Gal β1-3 GalNAc β1-4 [Sia α2-3] Gal β1-4 Glcβ-Cer) is a receptor for cholera toxin, whereas the extension of the oligosaccharide chain of GM1 would prevent binding of cholera toxin [Varki et al., 2009f]. Several studies have demonstrated that pathogens evade the host immune system by either synthesizing or acquiring sialic acid from the host. Alterations in cell surface glycans as seen in cancer confer abnormal properties such as avoidance of immunological destruction, invasiveness and metastatic spread [Bhavanandan, 1991]. Several diseases have been associated with alteration of glycans on biological macromolecules or cell surfaces e.g. I-cell disease, rheumatoid arthritis, IgA nephropathy, muscular dystrophies, paroxysomal nocturnal hemoglobinuria etc [Varki et al., 2009g]. Oligosaccharide sequences on soluble glycoconjugates such as the mucins act as 'decoys' for microorganisms and parasites. The pathogenic organisms attempting to gain access to mucosal membranes might first encounter their cognate oligosaccharide ligands attached to soluble mucins and upon binding to these sequences, be swept away by ciliary action thus leaving the

mucosal cells untouched [Varki, 1993]. Human milk has been reported to contain 200 different oligosaccharides [German et al., 2008]. Human milk oligosaccharide mimics the pathogen binding sites and prevent adherence of pathogens to cell surfaces. This phenomenon is described as ‘immune exclusion’ where the immune system does not have to rely on anti-microbial response towards pathogen, at the same time prevents the development of resistance to immune system by the pathogen [Peterson et al., 2013]. Given the rapid evolution of pathogenic organisms and their frequent use of glycans as targets for host recognition, it seems likely that a significant portion of the overall diversity in vertebrate glycan might have occurred due to pathogen mediated selection process. The glycan expression patterns of a given organism represent a compromise between evading pathogens and preserving intrinsic functions. Van Valen and Hamilton compared this phenomenon to ‘red queen effect’ wherein the host glycans must keep changing in order to prevent deleterious effect of invading pathogens. This is achieved by enormous diversity in the composition, linkage, spatial arrangement and covalent modification like acetylation, acylation, sulphation, methylation and phosphorylation of glycans [Varki, 2006] .

2.2. Glycosylation pattern in glycoproteins

Since the carbohydrate structure and composition of fetuin and thyroglobulin are well characterised, they are routinely used in experiments on molecular recognition.

Bovine Thyroglobulin (Tg)

Thyroglobulin, a 660-690 kDa glycoprotein, is a major secretory product of the follicular cells of thyroid gland. Bovine thyroglobulin is a tetrameric protein composed of subunits with molecular weight of 165 kDa. Thyroglobulins undergo extensive N-linked glycosylation, which varies in type and amount between species. Initial experiments by R.Spiro revealed the presence of two distinct types of N-glycans, one consisting of mannose and N-acetyl glucosamine (high mannose) and the other containing mannose, galactose, N-acetyl glucosamine, N-acetyl neuraminic acid and fucose (complex or hybrid type), but did not throw any light on the number and location of the glycosylation sites [Spiro, 1965]. Recent studies on the cDNA of bovine thyroglobulin suggest 14 potential N-glycosylation sites. However, tryptic digestion and glycan analysis of bovine thyroglobulin reveal that only 13 sites are N-glycosylated out of which 9 are complex or hybrid type and 4 are high mannose type [Rawitch et al., 1993].

Comparative study of thyroglobulin obtained from different mammals (calf, rabbit, dog, pig, guinea pig, rat and man) using *Griffonia simplicifolia* isolectin B4 (GS I-B4; an α-galactoside specific lectin) demonstrated that except humans all other species contain α-galactoside residues in their complex type carbohydrate units. Characterization of complex type oligosaccharides obtained from calf thyroglobulin

revealed that α-galactoside residues were located in the termini of oligosaccharide branches (Gal α1-3 Gal β1-4 GlcNAc). Such terminal α-galactoside (TAG) moieties were unevenly distributed because most of the LacNAc branches terminate in sialic acid. TAG groups account for only 11 mol/mol protein or 17 nmol/mg protein contributing to 23% of total galactose present in complex type glycans [Spiro and Bhoyroo, 1984]. The addition of TAG moieties is limited to lower mammals such as non-primates, prosimians and new world monkeys and absent in glycoconjugates of old world monkeys, apes and humans which produce natural anti-Gal antibody that specifically bind to TAG epitopes. The absence of TAG moieties in humans and its presence in lower animals is a milestone in human evolution which may be due to the suppression of enzyme α1-3 galactosyl transferase that might have occurred about 28 million years ago. However this enzyme is active in non-primate mammals, prosimians, and New World monkeys and catalyzes the addition of TAG groups in glycoconjugates. TAG moieties form an alternative to sialylation of N-glycans [Macher and Galili, 2008]. There are no reports available regarding the presence of O-glycans in thyroglobulin from any species. However in humans, M.J. Spiro has reported the presence of third type of oligosaccharide chain composed of glucuronic acid, galactosamine, xylose and galactose attached to serine residue [Spiro, 1977].

Fetuin

Fetuin, a 48 kDa monomeric α_1-glycoprotein, is isolated form foetal bovine serum. Fetuin contains six oligosaccharide moieties, three N-glyans and three O-glycans. Carbohydrates constitute 23% by mass of fetuin out of which N-glycans account for 80% of the carbohydrate while O-glycans account for only 20%. Structural analysis of N-glycans by Takasaki et al. and E.D.Green et al. have demonstrated that majority of the N-glycans are complex triantennary type (80%). However a minor fraction containing complex biantennary type (8% to 17%) has also been reported (Figure.3). Furthermore, microheterogenity is observed in LacNAc residues and number of sialic acid residues attached to pentasaccharide core. A small fraction (9%) of the triantennary structure contains type I LacNAc (Gal β1-3 GlcNAc), while majority of triantennary structure is composed of type II LacNAc (Gal β1-4 GlcNAc).

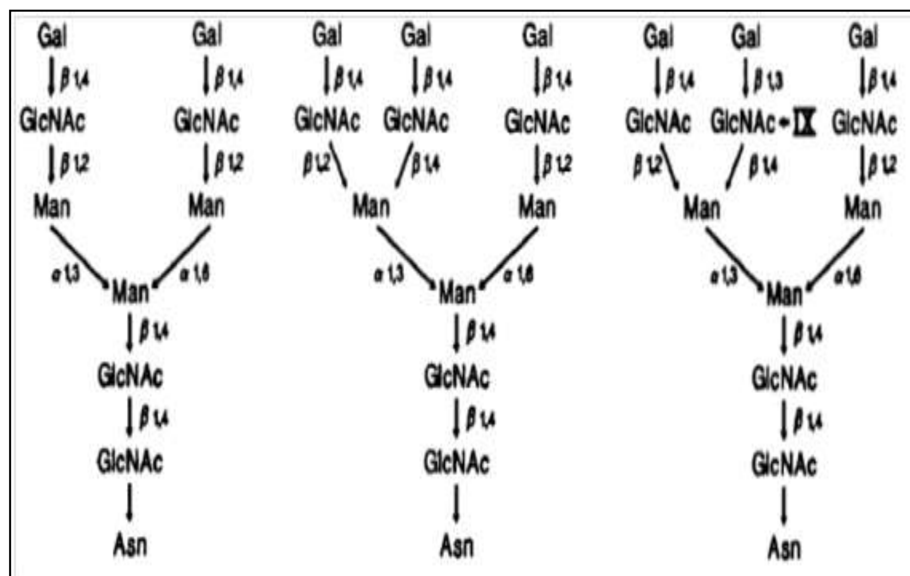


Figure 3. N-glycan pattern in fetuin.

The sialic acid composition in N-glycans is also reported to vary resulting in the formation of monosialylated (3%), disialylated (35%), trisialylated (54%) and tetrasialylated (8%) triantennary structure [Takasaki and Kobata, 1986; Green et al., 1988]. R.Spiro et al. have demonstrated the presence of three O-linked oligosaccharides per molecule of fetuin. O-glycans in fetuin are composed of mono or disialylated core-1 type O-glycans i.e. Sia α2-3 Gal β1-3 GalNAc α-Ser/Thr or Sia α2-3 Gal β1-3 (Sia α2-3) GalNAc α-Ser/Thr [Spiro and Bhoyroo, 1974]. Despite its non-primate origin presence of TAG groups in bovine fetuin has not been reported.

2.3. Anti-carbohydrate antibodies

Recognition of polysaccharide by antibodies began with the observation that when pneumococci were grown in fluid media there was a substance in the culture fluid which was precipitated specifically with anti-sera against pneumococcus [Dochez and Avery, 1917]. Later the substance was shown to be a polysaccharide derived from bacterial surface and not a protein [Heidelberger and Avery, 1924]. The antigenic nature of carbohydrates also became evident by the presence of antibodies against ABO blood group antigens which are carbohydrates. Anti-carbohydrate antibodies form a repertoire of both natural and acquired antibodies that confer immunity against invading pathogens, tumor cells, blood group antigens and xenoantigens. They form the humoral component of the innate immune system [Huflejt et al., 2009]. Since they are produced without any deliberate sensitisation they belong to the class of natural antibodies [Shoenfeld and Isenberg, 1989]. Based

on the serum content, ligand specificity and binding properties, N.V Bovin has classified natural antibodies against glycans into three groups *conservative, allo- and plastic antibodies.* Conservative anti-carbohydrate antibodies remain the same in all healthy individuals with respect to epitope specificity and serum levels. The allo-antibodies occur naturally against foreign tissues of the same species e.g. anti-blood group antibodies. Plastic anti-carbohydrate antibodies vary among individuals depending on the disease states and serve as potential markers for disease conditions [Bovin, 2013].

Biosynthesis of anti-carbohydrate antibodies

In 1969 G.F.Springer proposed the *bacterial paradigm* theory based on the observation that anti-blood group A and B antibodies could be produced by the immunological stimulus via natural routes using appropriate antigens. When *E.coli* O strains were fed to individuals with blood group O and A there was significant increase in anti-B antibodies while they were synthesized de novo in infants following sensitization although it was found lacking in the first few weeks of life. Several studies have also shown the synthesis of anti-carbohydrate antibodies in response to O-antigens and lipopolysaccharide antigens of gastrointestinal bacteria [Springer and Horton, 1969]. Dietary food components may also be a potential stimulant for the production of anti-carbohydrate antibodies e.g. immunoglobulins of IgM type is reported to be produced against milk lactoferrin [Y.P. Venkatesh and V. Prasanna Kumar, 2013]. Human milk oligosaccharides which are composed of over 200 different oligosaccharide moieties might also play a role in the synthesis of anti-carbohydrate antibodies but no evidence of this phenomenon has been reported

so far. Subsequently naturally occurring antibodies of the IgA, IgM and IgG type were found to occur in normal individuals, cod blood and antigen-free mice and this led A.Coutinho et al. in 1995, to propose the *germ line hypothesis* to explain the presence of these antibodies suggesting that these antibodies might be encoded in the germline [Coutinho et al., 1995].

B cell response to polysaccharide antigens

The B cell recognizes and internalises protein antigens via membrane bound Ig receptors followed by the presentation of antigen-derived peptides by class II MHC molecules. T_H cell recognizes the antigenic peptide displayed by the class II MHC molecule to form T-B conjugate thereby stimulating the release of cytokines. The signals produced by cytokine receptor interaction stimulate B-cell proliferation, differentiation into plasma cells and memory cells, affinity maturation and class switching. Since activation of B cells is mediated by T cells this process is termed thymus-dependent immune response. However, production of antibodies in response to polysaccharide antigens and not protein antigens in athymic mice and T cell defective mouse models led to the concept of thymus-independent immune response. Further studies revealed that polysaccharide components in microorganisms were able to initiate antibody production even in the absence of T cells and hence these antigens were designated as T-independent antigens. *In vivo* studies have revealed that antibodies synthesised in response to carbohydrate antigens are produced from a subset of B cells, the $CD5^+$ B1 cells. In humans and mice $CD5^+$ B1 cells are produced from embryonic stem cells during foetal life and constitute only 5% of the total B cell population. B1 cells are a minor fraction among B cell population in

spleen and secondary tissues such as lymph nodes but they form the majority of B cells in peritoneum and pleural cavity. Properties unique to B1 cells that distinguish it from B2 cells are self-renewal, expression of CD 5^{+} receptors, lack of affinity maturation and little proclivity to class switching and differentiation into memory cells. Due to the lack of class switching, cell surface of B1 cells bear IgM instead of IgG and therefore IgM forms the major immunoglobulin type in natural antibodies. However, in humans, immunoglobulins of IgG and IgA class have been observed as minor fractions [Kindt et al., 2007a]. Genetic analysis of VJC gene segments of B1 cells suggest that these segments had appeared about 430 million years ago and remain conserved within and between species [Bovin, 2013]. This may be achieved by the restricted diversity in their variable region due to lack of hypermutations of these Ig genes which in turn is because millions of VDJC rearranged segments already exist in germline or near-germline configuration.

Ligand binding properties of anti-carbohydrate antibodies

Classical examples of anti-carbohydrate antibodies include anti-blood group, anti-Forssman, anti-α-galactoside, anti-T, anti-mannan, anti-β-glucan, dextran binding, lactose binding antibodies etc. The theoretical and experimental advancements in the form of glycan array have led to the identification and characterization of several new anti-carbohydrate antibodies in circulation. Most of the anti-carbohydrate antibodies that make up the front-line defence system of our body show multiple specificity or polyreactivity. S.Avrameas suggested that presence of Lys and Arg residues in the hypervariable region may facilitate formation of salt bridges with negatively charged groups expressed on the surface of

antigens [Avrameas, 1991]. The similarities and differences in charge distribution may be responsible for immunological cross reactions with diverse unrelated antigens. According to A.L.Notkins these polyreactive antibodies bind to antigens with low affinity as compared to monoreactive antibodies. The affinity of the polyreactive antibodies towards various antigens may differ by as much as a 1000 fold. Contrary to classical lock and key hypothesis of antigen antibody interaction, the antigen-binding pocket of polyreactive antibodies are believed to be more flexible accommodating different antigens [Notkins, 2004].

Lactose binding immunoglobulin (LIg)

Matsuda T et al. demonstrated the immunogenic nature of lactose (Gal β1-4Glc) when antibodies specific to lactose were produced upon injection of lactose bound to carrier protein, into mice. Compared to protein bound melibiose (Gal α1-6 Glc) and cellobiose (Glc β1-4 Glc), lactose was found to be most immunogenic [Matsuda et al., 1987]. Dong et al. subjected human serum to affinity chromatography on lactose-Sepharose matrix followed by melibiose-Sepharose matrix and finally protein A-Sepharose to specifically isolate IgG fraction of LIg while excluding subfractions with preferential affinity towards α-galactosides [Dong et al., 1997]. Due to its affinity for lactose, N-acetyl lactosamine (Gal β1-4 GlcNAc or LacNAc) has been suggested to be a ligand for LIg in glycoconjugates. LIg has been shown to recognize desialylated forms of glycoproteins such as fetuin but direct evidence for LacNAc specificity is lacking [Gupta et al., 1996; Dong et al., 1997]. Cross-linking studies comparing binding of IgG fraction of LIg and lectins *Ricinus communis* agglutinin 1 and *Viscum album*

agglutinin with asialofetuin (AF) reveal that LIg binds to AF in the ratio 3:1 i.e. one LIg molecule per N-glycans, while the lectins bind to AF in the ratio 9:1 i.e. three lectin molecules per N-glycan. This result suggests that despite being divalent, IgG of LIg forms only one stochiometric cross-linkage with AF leaving one binding site unoccupied. This type of cross-linking complexes between LIg and antigens may have immunological relevance [Gupta et al., 1996]. Although LIg is found in circulation, its biological role remains unexplored. Dong et al. reported that LIg levels were elevated in patients of small lung carcinoma and metastatic lesions furthermore LIg bound to cancerous tissue in a sugar inhibitable manner [Dong et al., 1997]. Mandal et al. developed monoclonal antibody having specificity for lactose. They described a method for the analysis of relative specificity of the monoclonal anti-lactose IgM and IgG antibodies using structural variants of lactoside epitope and demonstrated that although both IgM and IgG was raised by specific interaction with hexosides of lactose the binding constant of IgG was 100 fold greater than IgM. These differences may be relevant in biological interactions where multivalency and functional affinity govern complex formation [Mandal et al., 1984].

Anti-α-galactoside antibody (anti-Gal)

Anti-α-galactoside antibody (anti-Gal) in human plasma is a naturally occurring immunoglobulin that recognizes both terminal α1-3 and α1-6 linked galactose moieties in glycoconjugates. It comprises approximately 1% of circulating IgG. As many as 1% of B cells in humans are capable of producing anti-Gal but most of them remain in quiescent state as memory cells and only those along the gastrointestinal tract continuously produce it. Studies show that more than 80% of

anti-blood group B activity in A and O individuals are due to anti-Gal and these antibodies bind to α-gal epitopes despite the presence of fucose in B group antigen. However, in blood group B and AB containing B antigen, anti-gal does not recognize the B antigen. Analyses of isotypes of anti-Gal isolated by affinity chromatography reveal that most of this naturally occurring antibody is of IgG class followed by IgM. Anti-Gal IgA is the least prevalent in circulation but large amounts are seen in secretory fluids like saliva, milk, colostrum and bile [Macher and Galili, 2008]. Anti-Gal is a notable exception among anti-carbohydrate antibodies in that it has IgG as the predominant type and is synthesised by B cells in a T-cell dependent mechanism [Tanemura et al., 2000]. Owing to the inverse relation among animals between production of anti-Gal and presence of TAG moieties, anti-Gal forms a unique immunological barrier preventing transplantation of organs from animals (xenotransplantation) [Galili, 1993]. Studies have reported that anti-Gal titre is elevated in Chagas disease and cutaneous leishmaniasis [Avila et al., 1989]. Castronovo V et al. have demonstrated the specificity of anti-Gal to cancerous cells [Castronovo et al., 1989]. Likewise it was found that anti-Gal could also bind to tissue sections of brain [Jaison et al., 1993]. This was an unexpected interaction given that TAG moieties are absent in humans. A possible explanation for this phenomenon was that these organs do not come in direct contact with blood so they may have retained enzymes to synthesise TAG moieties. Sandrin et al. have reported that anti-Gal and TAG specific lectin GS I-B4 are able to react with mucin peptides encoded by MUC- 1, 2, 3 genes. These peptides are rich in Ser, Thr and Pro amino acids. Thus naturally occurring anti-Gal antibodies found in all human serum can react with MUC-1 peptides expressed on the cell surface of tumour cells. This

finding suggests that the expression of TAG moieties by human tumor cells, as reported earlier, may be an artefact and that the reaction could be due to the interaction of anti-Gal antibodies with mucin peptides on tumor cells [Sandrin et al., 1997]. Recently our laboratory demonstrated that anti-Gal binds to serine- and threonine-rich peptide sequences (STPS) present in the heavily O-glycosylated regions of apoprotein(a) of lipoprotein(a) to form circulating IC [Mandagini et al., 2013].

Anti-T antibody

Serum of all humans regardless of blood group has preexisting antibodies against T antigen i.e. desialylated core 1 O-glycans (Gal β1-3 GalNAc). Anti-T antibodies are absent in cord blood and they develop only during the first year of life and their mean levels have been found to remain unaltered in healthy individuals form 3 years of age throughout adulthood [Lind and McArthur, 1947]. IgM is the most dominant immunoglobulin type in anti-T while IgA constitutes nearly 30% of total anti-T fraction. Significant quantities of IgG have also been reported [Springer et al., 1976]. Anti-T titre in human sera was determined by haemagglutination assay using desialylated RBC. Desai et al. developed solid phase enzyme immunoassay to quantify anti-T in serum by coating human O group RBC-derived T antigen on polystyrene wells [Desai et al., 1995]. Alternatively, T antigen conjugated to polyacrylamide beads (Gal β1-3 GalNAc-PAA) coated on polystyrene wells is also used to measure serum anti-T levels [Butschak and Karsten, 2002]. T antigen and anti-T antibodies are of promise in elucidating important aspects of cancer diagnosis and pathogenesis [Springer, 1997]. Measurement of circulating anti-T may be

indicative of tumor burden or disease progression in some malignancies and also radiolabeled anti-T antibodies may prove useful in tumor localization studies [Desai et al., 1995]. Anti-T titre in serum was found to decrease with tumor proliferation but was restored rapidly following tumor removal [Springer and Desai, 1975]. Thatcher et al. reported a direct relation between low serum anti-T titre and poor response to chemotherapy and decreased survival in patients with melanocarcinoma [Thatcher et al., 1980]. Lowering of circulating anti-T in certain cancer patients could be explained by the absorption of antibodies by T antigen expressed or shed from the tumor.

2.4. Atherosclerosis

Atherosclerosis is a progressive disease which affects the large and medium-sized arteries. It is the major underlying cause of mortality and morbidity in cerebrovascular and cardiovascular diseases [Galkina and Ley, 2009]. Reports by National Commission on Macroeconomics and Health, India suggests that atherosclerosis will account for one-third of all deaths and death from coronary artery disease is estimated to rise from 1.3 million in 2000 to 3 million by 2015. Death by CVD is predicted to rise by 103% in men and by 90 % in females by 2015 as compared to statistics from 1985 [A. Indrayan. 2005]. CAD, heart failure, ischemic gangrene, aortic aneurysm and stroke constitute the major cause of death in Western countries. World Health Organisation has projected that CVD would be a major killer disease globally by 2020 [Hansson et al., 2006].

Mechanism of atherogenesis

The walls of arteries are composed of three layers: inner tunica intima, middle tunica media and outer tunica adventitia. Tunica intima is composed of a single layer of endothelium resting on a basement membrane. Tunica media contains densely packed smooth muscle cells, collagen, fibronectin and proteoglycans. Tunica adventitia contains fibroblast, smooth muscle cells, elastic fibre, collagens, vasa vasorum and nerves [Tegos et al., 2001]. Atheromatous lesions develop focally in the arterial intima of regions where the arteries bifurcate and blood flow is turbulent with low shear stress. They are composed of lipid laden macrophages (foam cells), calcium deposits, extracellular lipid core, fibrin, antibodies, complement components, peripheral mononuclear cells and increased expression of mononuclear cell adhesion molecules. Although the exact sequence of events leading to atherogenesis is still unclear histochemical analysis and composition of plaques have led to reasonable understanding of the cause of the disease [Stary et al., 1992] .

Response to Injury

According to Ross et al. desquamation of vascular endothelium may be the primary event in atherogenesis. Denudation of vascular endothelium would increase the vascular permeability thereby leading to deposition of lipoproteins and inflammatory response in the subendothelial layer [Ross et al., 1977]. This hypothesis was disproved when several studies demonstrated the presence of an intact endothelium in developing plaque.

Response to Retention

Schwenke and Carew observed that lipoproteins were substantially retained in the lesion prone regions of the arterial wall than in normal artery irrespective of serum lipoprotein levels [Schwenke and Carew, 1989]. Research in this line has shown that the interaction of lipoproteins with extracellular matrix components in the arterial wall lead to lipid retention. The apoprotein moieties in lipoproteins and enzymes of lipid metabolism irrespective of their catalytic activity have been reported to act as 'bridging molecule' between proteoglycans and lipids. The positively charged amino acids in apoB moiety present in LDL, very low density lipoprotein (VLDL) and Lp(a) interact with negatively charged proteoglycans (chondroitin sulphate, dermatan sulphate) on the arterial wall while apoE moiety found in chylomicrons, VLDL, intermediate density lipoprotein (IDL) and high density lipoprotein (HDL) has also been shown to interact with heparin and biglycan. The binding of LDL to proteoglycans induces irreversible change in the conformation of apoB making them more susceptible to modification and reduced efflux. Lp(a) associated with CVD is reported to also interact with fibrin, laminin and fibronectin. Lipoprotein lipases (LPL) facilitate binding of oxidised LDL to preoteoglycans since mild oxidation of LDL leads to hydrolysis of phospholipids which in turn opens more interfaces for LPL binding. The enzyme phospholipid transferase has been reported to co-localize with apoB and apoE on biglycans in atherosclerotic lesions irrespective of its enzymatic activity. The presence of sialic acid in apoprotein moieties or glycolipids in LDL imparts negative charge thereby decreasing its affinity to proteoglycans while absence of the same in diabetics has been reported to be the major factor mediating interaction with proteoglycans [Gustafsson and Borén, 2004].

Oxidation

Lipoprotein oxidation plays a central role in atherogenesis. Oxidation of lipoproteins is believed to occur when the lipoproteins are sequestered within the subendothelial regions of the arterial wall. The oxidative environments in the intimal region and lack of anti-oxidant species promote this process [Van Hinsbergh et al., 1986]. This hypothesis was substantiated by the discovery of oxidised lipoprotein epitopes and antibodies to oxidised lipoprotein in human atherosclerotic lesions. In vitro studies have demonstrated that exposure of LDL to reactive-oxygen species induces lipid peroxidation and modification of apoB moieties thereby leading to formation of MDA-lysine adducts [Steinbrecher, 1987]. Furthermore, these oxidized LDL molecules have been shown to stimulate the expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cell, monocyte chemotactic protein-1 (MCP-1) and macrophage colony stimulating factor (M-CSF) on vascular cells thereby leading to adhesion, migration, proliferation and transformation of monocytes. Within the intima monocytes mature into macrophages and produce reactive-oxygen species, proteolytic enzymes, proinflammatory cytokines and express pattern recognizing receptors like Toll like receptors and scavenger receptors [Hansson et al., 2006]. The oxidatively modified lipoproteins are taken up by macrophages via scavenger receptors ensuing in formation of foam cells (lipid-laden macrophage). As the lesion progresses the macrophages contain relatively few typical cytoplasmic cholesteryl ester droplets, but significant lysosomal deposits of oxidized lipids and ceroids (undegradable lipid-protein complex). This is probably due to the intrinsic resistance of some oxidized components to lysosomal hydrolases, by inactivation of enzymes and/or by inhibition of ATP-binding cassette transporter which export cholesterol

from cells by reverse cholesterol transport [Jessup et al., 2004]. Due to the presence of oxidatively modified lipoproteins and antibodies to oxidised lipoprotein antibodies in circulation, preventive antioxidant therapy was initially proposed for atherogenesis. Recent studies have failed to demonstrate any beneficial effect of anti-oxidant therapy rendering oxidation as an unconvincing pathway in the mechanism of atherosclerosis [Myung et al., 2013].

Inflammation

In the middle of 19th century Rudolf Virchow and Carl Von Rokitansky described the cellular inflammatory changes in atherosclerosis. Rokitansky considered inflammation to be secondary to arterial changes while Virchow proposed the 'cellular pathology' according to which inflammation could be the primary event leading to atherogenesis [Mayerl et al., 2006]. Several groups have provided evidence that atherosclerosis bears similarity to chronic inflammatory diseases. T-cells (regulatory and cytotoxic T-cells), NK cells, mast cells, vascular dendritic cells, platelets, B cells and cytokines, such as tumor necrosis factor (TNF-α), interleukin (IL), interferon (IFN-γ), M-CSF, transforming growth factor (TGF-β1, TGF-β2, and TGF-β3) have been detected within atherosclerotic vessels [Hansson et al., 2006; Ait-Oufella et al., 2011]. In addition to the presence of inflammatory mediators in plaques, several groups have reported upregulation of the markers for systemic inflammation (acute phase proteins) such as CRP, leukocyte count, fibrinogen, serum amyloid A (SAA), secretory nonpancreatic phospholipase 2 - II (sPLA2-II), ferritin, and ceruloplasmin in patients with atherosclerosis and especially in those with an unstable coronary disease [Ahmed et al., 2012]. Similarly

inflammatory markers of vascular origin such as the adhesion molecules (ICAM-1, MCP-1, VCAM-1 and E-selectin) were also elevated in patients with coronary heart disease [Armstrong et al., 2006]. Although circulating markers of inflammation have a role as risk factors for atherosclerosis, it remains unclear whether they are merely markers or if they can contribute to the development and progression of the disease. Seroepidemiological studies have shown correlation between incidence of atherosclerosis and presence of microorganisms like *Chlamydia pneumoniae, Helicobacter pylori, Mycoplasma pneumoniae, Epstein-Barr virus and cytomegalovirus.* Furthermore, antigens from the above mentioned pathogens have been detected in atherosclerotic lesions. Antibody titre against these pathogens has also been reported to be elevated in patients with myocardial infarction. Although the pathological basis for infection and atherosclerosis is not well established a probable mechanism may be that these pathogens might infect cells in arterial wall and stimulate inflammatory process via humoral and cellular immune response [Libby et al., 1997]. This theory is most widely accepted as a probable mechanism in atherosclerosis.

2.5. Role of Lipoprotein immune complex in atherogenesis

Immune complex mediated vascular pathology

Formation of antigen-antibody complexes (immune complex; IC) is one of the primary events in immunological response against soluble antigen. Normally, IC is eliminated from circulation by reticuloendothelial system without having any pathological consequence to the host. However, IC becomes pathological when they leave the intravascular compartment and get deposited on to tissue surfaces or when they are formed directly in tissues where the antigen is formed or trapped. The deposition or formation of immune complex on the vascular wall initiates a type III hypersensitive reaction, where the Fc-region of the antibody binds to effector molecules like complement components and Fc-receptors, which in turn triggers an inflammatory cascade involving mast cell degranulation, neutrophil chemotaxis and release of lytic enzymes leading to vascular injury. Several diseases of infectious origin, serum sickness, atherosclerosis and connective tissue autoimmune diseases have been reported to have IC mediated pathology [Jancar and Crespo, 2005]. Deposition of circulating IC, which begins with the circulating IC and inflammatory cells crossing the endothelial barrier, can be governed by several factors. Hemodynamic factors and anatomical sites are the primary factors that promote deposition of IC. Organs and tissue surfaces which receive high degree of blood flow per unit mass or altered blood flow (low shear stress) have been shown to trap large quantities of IC in their vascular wall making them susceptible to IC-mediated pathology [Theofilopoulos and Dixon, 1980]. Physico-chemical properties like size, stochiometry and the nature of antigen and antibody involved in IC formation are

also important determinants of IC deposition (Table.2). Very large ICs are usually non-pathogenic because they are difficult to diffuse across the endothelium and are effectively cleared by phagocytic cells. Very small ICs are also non-pathogenic despite their ability to diffuse across the endothelium because they fail to activate complement. The most pathogenic are the intermediate IC (Ag2-3:Ab2-6) because they can diffuse across the endothelium and activate complement factors [Virella, 2007]. Animal experiments show that vasoactive amines such as histamine or serotonin cause separation of endothelial cells along the wall of blood vessels thereby promoting entry of IC into the sub-endothelial spaces. Complement factors can play a dual role in turnover of IC. Binding of complement factor (C3b) promotes solubilisation of IC and facilitates its removal by binding to complement receptors on RBC. On the other hand presence of complement factors bound to IC lodged on tissue surface can trigger antibody dependent cellular cytotoxicity (ADCC) thereby bringing about tissue damage [Theofilopoulos and Dixon, 1980].

Table 2. Antigen and antibody characteristics that affect the pathogenicity of IC.

Antibody	**Antigen**
1. Isotype 2. Valency 3. Affinity for Fc receptors 4. Ability to bind and activate complement 5. Affinity 6. Charge 7. Amount	1. Size 2. Valency 3. Chemical composition 4. Charge 5. Amount

Lipoprotein immune complex

Lipoproteins are susceptible to spontaneous modifications like oxidation and glycation. Oxidation affects both lipid and protein components. The reactive products such as MDA and HNE covalently attach to ε-amino groups of lysine in apoB moiety to form MDA-lysine adducts [Steinbrecher, 1987]. Glycation is a process in which carbohydrate moieties are attached to proteins by non-enzymatic process through Amadori reaction [Klein et al., 1995]. In 1984, Steinbrecher at al. demonstrated that oxidatively modified LDL (OxLDL) promotes foam cell formation in macrophages and that it induces a number of proatherosclerotic effects, including endothelial activation and smooth muscle proliferation [Steinbrecher et al., 1984b]. Berliner et al. reported that the OxLDL co-cultured with endothelial cells had stimulatory effects on many types of cells and these effects were due to the oxidized phospholipids generated in OxLDL [Berliner et al., 1990]. Ever since the discovery of immunogenic nature of modified lipoproteins by Steinbrecher et al. in experimental animals, antibodies raised against modified lipoproteins were used as tools for *in vivo* assays [Steinbrecher et al., 1984a]. Compared to healthy subjects plasma levels of OxLDL were reported to be elevated in disease conditions like CHD, cerebral infarction, and chronic renal failure [Toshima et al., 2000; Uno et al., 2003; Holvoet et al., 1996]. Ehara et al. reported that the plasma OxLDL level in patients with acute myocardial infarction was increased by 3.5 fold as compared to healthy subjects. The plasma levels of OxLDL were found to exhibit temporal variation i.e. OxLDL levels raise with disease progression and drops with prognosis of the disease [Ehara et al., 2001]. Furthermore, several groups have reported the

presence of OxLDL in atheromatous lesions of human coronary and carotid artery [Nishi et al., 2002; Sigala et al., 2010].

Several lines of evidence have suggested the presence of antibodies against modified lipoproteins even in human sera and atheromatous plaques [Virella et al., 1993; Ylä-Herttuala et al., 1994; Lopes-Virella et al., 1999]. This led to the hypothesis that circulating lipoprotein IC may have a potential role in atherogenesis. Studies in this line have gathered a plethora of evidence for the IC mediated pathology of atherosclerosis. Tertov et al. reported that LDL isolated from human circulating IC is partially denatured and desialylated. LDL in circulating IC isolated from human serum by polyethylene glycol precipitation was found to be enriched with oxidation specific epitopes such as carboxymethyl lysine and MDA-lysine groups [Tertov et al., 1990]. Klimov et al. demonstrated that incubation of mouse peritoneal macrophages with LDL-IC leads to excessive cholesterol accumulation and foam cell formation [Klimov et al., 1985]. Orekhov et al. showed an increase in cholesterol accumulation in human subendothelial cells when exposed to LDL-IC [Orekhov et al., 1991]. Several other reports suggest that LDL-IC were more potent inducers of foam cells than modified LDL or LDL alone. Furthermore, uptake of LDL-IC by human monocyte-derived macrophages and THP-1 cells via FcγRI receptors was found to induce respiratory bust, release of proinflammatory cytokines and matrix metalloproteinases [Virella et al., 2002]. A prospective study by Mustafa et al. carried out for 20 years in 50 year old men suggests that IC is an independent risk factor for myocardial infarction [Mustafa et al., 2000]. Several groups have reported a significant correlation between soluble LDL-IC and the occurrence of CVD

[Lopes-Virella and Virella, 2010]. Human autoantibodies to oxidized and AGE-modified LDL have been isolated by affinity chromatography and characterized with respect to their isotype distribution and avidity. Data generated by two separate studies using different assay methods to quantify Ig subclasses have shown that 70% to 84% of the OxLDL antibodies purified from human sera are of the IgG isotype. Likewise a study carried out in patients with Type 1 diabetes found that although IgG was the predominant isotype, antibodies of IgM and IgA class were also present. Another study shows that antibodies against OxLDL are mainly IgG2, IgG3 and IgM type and both IgG and IgM antibodies have high affinity for the antigen. The precise role of the antibody types in mediating atherogenesis remains unknown. Several conflicting reports have emerged that indicate both a proatherogenic as well as antiatherogenic role for ICs [Lopes-Virella et al., 2005; Burut et al., 2010].

2.6. Lipoprotein(a)

Lipoprotein(a) [Lp(a)] is one of the most atherogenic lipoproteins in human plasma and hence research on Lp(a) to delineate its role as a cardiovascular risk factor has gained importance.

Structure

Lp(a) was discovered by Kare Berg in 1963 as a structural variant of LDL and is found only in humans, apes, old world monkeys and hedge hog. Lp(a) resembles LDL in lipid composition and presence of apolipoprotein B-100 (apoB) but differs from LDL by the presence of a heavily glycosylated polymorphic apoprotein(a) [apo(a)] which is linked to apoB by a disulfide bond. The density of Lp(a) ranges between 1.04 - 1.125 g/ml and overlaps with those of LDL (1.019 - 1.063 g/ml) and HDL (1.063 - 1.210 g/ml). Apo(a) is homologous to plasminogen and is composed of kringle 4 (74% homology), kringle 5 and inactive protease domains (95% homology) (Figure.4). Kringles are tri-loop structures containing 75-80 amino acid residues stabilized by 3 intrachain disulfide bonds. The interkringle regions contain 28-36 amino acid residues. The apo(a) gene is located on chromosome 6q26-27. Apo(a) gene sequencing and cDNA analysis have revealed that apo(a) contains 10 different types of kringle 4 domains, designated as kringle 4 type 1 to 10. All kringle 4 types are present as a single copy except for kringle 4 type 2 which can vary between 3 - 40 repeats among individuals, creating apo(a) size heterogeneity. Thus molecular weight of an apo(a) subunit in Lp(a) can vary between 300 - 800 kDa [Koschinsky and Marcovina, 1997; Scanu and Edelstein, 1997]. The apo(a) size or number of its isoforms in an individual is determined by the VNTR sequences in kringle 4 type 2

gene segments [Koschinsky et al., 1990], inherited in a co-dominant Mendelian fashion [Gaubatz et al., 1990].

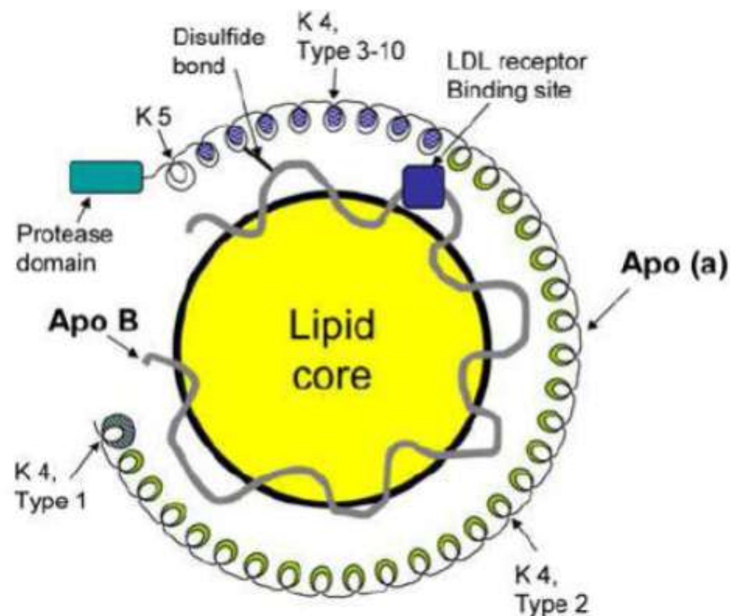


Figure 4. Schematic model of Lp(a)

Apo(a) is the most heavily glycosylated apoprotein. The carbohydrates account for 28% of the molecular weight of apo(a). N-glycans constitute up to 20% of the total carbohydrate content while O-glycans constitute the majority (80%) of glycans in apo(a). Each apo(a) kringle except kringle 5 and inactive protease domain contain one N-glycan of complex biantennary type. The O-glycans (4-6) of mono or disialylated core-1 type are located in the interkringle regions, except interkringle region 4 making it susceptible to enzymatic cleavage. Compared to other interkringle regions in apo(a), interkringle region 7 is highly O-glycosylated region and contains one potential N-glycan site as well. The glycan content in apo(a) increases with

increase in kringle 4 type 2 repeats [Scanu and Edelstein, 1997]. Compared to apo(a), apoB is sparsely glycosylated and carbohydrates constitute 4-10% of apoB [Triplett and Fisher, 1978]. Nineteen potential N-glycosylation sites have been reported in apoB but mass spectrometric analyses show that only 16 sites are actually glycosylated, out of which 5 are high mannose type and 11 are of sialylated complex or hybrid type (Figure.5) [Harazono et al., 2005].

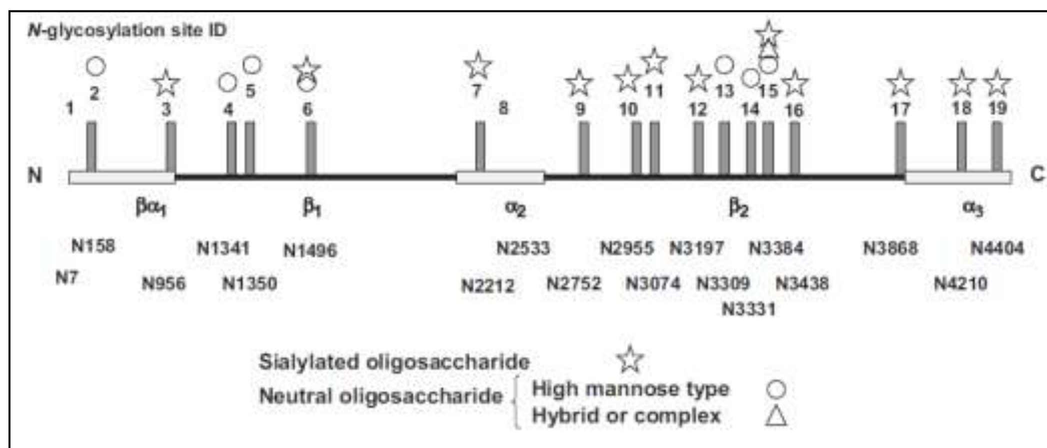


Figure 5. N-glycan distribution in apoB

Biosynthesis, plasma levels and degradation

Liver is the primary site for apo(a) biosynthesis but apo(a) mRNA has been detected in brain and testis as well [Tomlinson et al., 1989]. Although the site of apo(a) synthesis is known its site of conjugation with apoB remains unknown. It is speculated that assembly of Lp(a) may occur at the hepatocyte surface or in circulation. Lp(a) is purely heritable and serum levels are largely determined by apo(a) gene expression, its mRNA stability and post-translational processing of apo(a) moieties but not by its rate of degradation [Hobbs and White, 1999].

Lipid lowering drugs and diet have no effect on serum Lp(a) levels. The size and concentration of Lp(a) in human plasma varies widely within and among individuals. Studies indicate that 69% of the variability in plasma Lp(a) levels is accounted for by apo(a) kringle-4 type 2 repeats, while the remaining is contributed by single nucleotide polymorphisms [Boerwinkle et al., 1992], C/T variation in the promoter region and pentanucleotide ($TTTTA_n$) repeats in the upstream region of apo(a) gene [Valenti et al., 1999]. Population based studies show that 94% of the individuals have 2 Lp(a) isoforms in circulation due to co-dominance of the heterozygous apo(a) alleles. However, null alleles where Lp(a) is totally absent and homozygous apo(a) alleles resulting in only 1 Lp(a) isoform are also observed [Kamboh et al., 1991]. Apo(a) size and plasma Lp(a) levels are inversely related i.e. smaller apo(a) sizes correspond to high plasma Lp(a) levels while larger apo(a) size corresponds to low plasma Lp(a) levels. This is because smaller apo(a) isoforms are synthesised more rapidly than larger apo(a) isoforms [Utermann et al., 1987]. Since Lp(a) levels in plasma vary widely among peoples it has been difficult to assign a normal range for Lp(a). Africans in general have higher serum Lp(a) levels than Asians and Caucasians [Marcovina et al., 1993]. Despite high Lp(a) levels, there is no correlation between Lp(a) levels and CVD in Africans [Moliterno et al., 1995]. However 20 to 30 mg/dl is assigned as a cut-off point and based on this value it has been established that high Lp(a) concentration is a major independent risk factor for CHD and acute myocardial infarction [Rosengren et al., 1990]. Unlike other plasma lipoproteins, very little is known about the degradation of Lp(a). LDL-receptor (LDL-R) plays an important role in the turnover of LDL but has a limited role in degradation of Lp(a) because the LDL-R binding region in apoB of Lp(a) is masked

by the apo(a) subunit. Only 10-25% of plasma Lp(a) is converted to LDL and is degraded via LDL-R mediated endocytosis. In vitro studies have demonstrated that Lp(a) can be degraded through VLDL receptors as well. Kidney may contribute to a minor extent in the excretion of Lp(a). Evidence for this phenomenon is obtained from *in vivo* studies where plasma Lp(a) levels are found to be elevated in patients with renal insufficiency. Furthermore, apo(a) fragments (80-215 kDa) have also been detected in urine of normal individuals yet the underlying mechanism of degradation and glomerular excretion of apo(a) in kidney remains unclear [Hobbs and White, 1999].

Clinical implication

Although Lp(a) is present in varying quantities among individuals its role in lipid metabolism remains enigmatic. Several lines of evidence suggest plausible role in tissue repair and wound healing. *In vitro* and *in vivo* studies have demonstrated a rise in Lp(a) levels in response to acute phase proteins and cytokines (IL-6) where Lp(a) binds to matrix components to augment cell differentiation and proliferation. Furthermore, in the absence of vitamin C, instability of extracellular matrix was improved by large deposition of Lp(a). Based on this observation Linus Pauling and Mathias Rath proposed that Lp(a) may be a surrogate for vitamin C and may play a role in wound healing [Lippi and Guidi, 2000]. High serum Lp(a) levels are positively correlated with CVD, stroke, aneurysm and peripheral vascular disease [Jones et al., 2007]. It has been well established that high serum Lp(a) is an independent risk factor for vascular pathology. Plasma Lp(a) levels greater than 25 mg/dL is a risk factor of atherosclerosis, CHD and ischemic stroke [Marcovina

and Koschinsky, 1998; Ohira et al., 2006]. Lp(a) deposits have been located in atherosclerotic plaques and not in normal arteries. Studies to determine the extent of accumulation of Lp(a) in atheromatous plaques reveal that apo(a) subunits outnumber apoB and accumulation of apo(a) was proportional to their serum levels [Pepin et al., 1991]. Recent studies show that apo(a) content in plaques increases with increase in disease progression [Dijk et al., 2012]. Although Lp(a) is a positive indicator for vascular pathology its mechanism of action remains unknown. In vitro studies suggests that proatherogenic nature of Lp(a) is due to its affinity for extracellular matrix and anti-fibrinolytic property. Since apo(a) is structurally homologous to plasminogen it interferes with the normal fibrinolytic function by competing for the substrate of plasminogen i.e. fibrin and tissue plasminogen activator [Anglés-Cano et al., 2001]. Desialylation of Lp(a) can also favor its aggregation in intimal region. Orekhov and Tetrov reported that Lp(a) in patients with CAD was 2.5 fold more desialylated than normal subjects and desialylated Lp(a) was found to aggregate in human aortic intimal cells [Tertov and Orekhov, 1994]. Furthermore, serum sialic acid and neuraminidase levels have also been reported to be elevated in patients with CAD and diabetes [Sönmez et al., 1998; Roozbeh et al., 2011]. Several studies have found correlation between serum sialic acid levels and occurrence of CAD and may serve as marker for CAD [Lindberg et al., 1992]. Compared to native Lp(a), oxidised-Lp(a) [Ox-Lp(a)] has been reported to play a more potent role in atherosclerosis. In vitro experiments show that uptake of Ox-Lp(a) by monocytes via scavenger receptors leads to foam cell formation [Haberland et al., 1992; Ragab et al., 1996]. Enzyme immunoassays employing monoclonal antibodies against oxidised epitopes in Lp(a) have been developed to

quantify Ox-Lp(a) levels in circulation [Wang et al., 2007]. Morishita R et al. found that Ox-Lp(a) level in CAD patients with diabetes mellitus was significantly higher than in healthy volunteers. Compared to normal subjects hypertensive patients with cardiac complications also showed significant increase in Ox-Lp(a). Moreover, Ox-Lp(a) was detected in the calcified areas of coronary arteries in patients with myocardial infarction [Morishita et al., 2009]. Autoantibodies against Ox-Lp(a) and Lp(a) immune complex [Lp(a)-IC] have been detected in normal individuals and in patients with CVD and it remains uncertain whether these antibodies are atherogenic or atheroprotective. Wang et al. have reported positive correlation between Lp(a)-IgG IC and CHD [Wang et al., 2003]. The plasma levels of Lp(a)-IC was found to vary with plasma concentrations of Lp(a). β-2 glycoprotein I (β-2 GPI or apolipoprotein H) is an anti-phospholipid protein cofactor having affinity for negatively charged phospholipids. Most anti-phospholipid antibodies require β-2 GPI as cofactor for optimum binding [Giles et al., 2003]. Recent studies show that β-2 GPI has high affinity for apo(a) component of Lp(a), thereby leading to formation of β2-GPI-Lp(a) complex [Köchl et al., 1997]. Furthermore, β2-GPI-Lp(a) levels were found increased in patients with acute coronary syndrome (ACS) and stable CAD. Logistic regression analysis of risk factors reveal that the presence of β2-GPI-Lp(a) as well as Ox-Lp(a) or Lp(a) is a strong risk factor for stable CAD, and especially for ACS, suggesting that the β2-GPI-Lp(a) complexes might act as an additional predictor of atherosclerosis [Zhang et al., 2011; Wang et al., 2012].

MATERIALS AND METHODS

3.1. Materials

PEG 6000, soybean trypsin inhibitor, sodium metaperiodate and 1-fluoro-2,4-dinitrobenzene (FDNB) were purchased from Fluka, Buchs, Switzerland. Fetuin, bovine thyroglobulin, guar gum, neuraminidase from *Clostridium perfringens,* ortho-phenylenediamine (OPD), horse radish peroxidise (HRP), jack bean α-mannosidase, O-glycosidase from *Streptococcus pneumoniae*, coffee bean α-galactosidase, divinyl sulphone, cyanogen bromide, lactose, melibiose, cellobiose, galactose, 1-O-methyl α-D-galactopyranoside, 1-O-methyl β-D-galactopyranoside, 1-O-methyl α-D-glucopyranoside, 1-O-methyl β-D-glucopyranoside, *Griffonia simplicifolia* I-B4 (GS I-B4), sulpho NHS-biotin, avidin-HRP, Tween 20, Coomassie brilliant blue R-250 and G-250, sodium cyanoborohydride, potassium borohydride, acrylamide, TEMED, N,N' methylene bis-acrylamide, β-mercaptoethanol, Sigma 7-9 (Tris), fluoroscein isothiocyanate (FITC), mucin from porcine stomach, Sepharose 4B, Sephadex G50, Amicon Ultra centrifugal filter units (MWCO 10KDa) and IgG from human serum reagent grade were purchased from Sigma-Aldrich company, Bangalore, India. Anti-human IgA, anti-human IgG, anti-human IgM raised in goat and anti-apo(a), anti-apoB, anti-apoA1 raised in rabbit were purchased from Dako, Denmark. Barbitone sodium and barbitone was purchased from BDH chemicals, Poole, England. Borate, ammonium persulphate, Triplex (sodium EDTA), hydrogen peroxide, potassium bromide, epichlorohydrin, solvents and buffer components were purchased from Merk, India. Bio Gel P-4 was purchased from Bio-Rad Laboratories, USA. Polystyrene 96 well flat bottom microplates (MAXISORP) were purchased from Nunc, Denmark. Polystyrene 96

well U-bottom well microplates were purchased from Laxbro (Ranbaxy), Delhi, India.

The seeds of *Arachis hypogaea*, *Artocarpus integrifolia* and *Canavalia ensiformis* were obtained locally. Outdated human plasma samples and O-group RBCs were obtained from the Department of Blood Transfusion Services of this Institute with Institutional Ethics Committee approval (IEC-511). Sheep RBC was a gift from Department of Microbiology of this Institute. Rabbit RBC membrane proteins isolated by ultracentrifugation at 100000 g of osmotically lysed rabbit RBC was a gift from Dr.P.L. Jaison of this department.

3.2. Methods

3.2.1. Protein estimation by Bradford's method

Coomassie brilliant blue G-250 dye was prepared as a 0.06% solution in 3% perchloric acid and filtered through Whatman No.1 filter paper before use. The dye and protein solutions were mixed in the ratio 1:1 and the absorbance was measured at 620 nm [Bradford, 1976].

3.2.2. Protein estimation by Lowry's method

a. Alkaline Copper Reagent was prepared fresh by mixing 1 ml of 2% sodium potassium tartrate solution and 1 ml of 1% copper sulphate solution, the mixture was made up to 100 ml with 2% sodium carbonate containing 0.1 N sodium hydroxide.

b. 1 N Folin Ciocalteau reagent.

0.5 ml of protein solution was mixed with 2.5 ml of alkaline copper reagent and incubated at 25^0C for 10 minutes. This was followed by the addition of 0.25 ml of 1 N Folin's reagent and incubation at 25^0C for 30 minutes. Absorbance was measured at 660 nm using bovine serum albumin as protein standard [Lowry et al., 1951].

3.2.3. Preparation of cross-linked guar gum (CLGG)

Soluble guar galactomannan was cross-linked to form an insoluble gel by a modification of the procedure described by Appukuttan et al. [Appukuttan et al., 1977]. Guar gum powder (10 g) was mixed thoroughly with a finely dispersed emulsion of 2 ml epicholorohydrin and 25 ml 3 N NaOH until the mixture became a solid cake. It was then kept at 40°C in a water bath for 24 h and then at 70°C for 10 h. The resulting gel was soaked in distilled water and repeatedly washed with water until pH was neutral. The gel was then equilibrated with PBS and homogenized in a blender to obtain particles of about 300 μm size. Fine particles were discarded by repeated decantation.

3.2.4. Preparation of lactose-Sepharose 4B matrix

Lactose was covalently attached to Sepharose using divinyl sulfone as the cross-linker as described by Dean et al. [Dean and Johnson, 1985]. Sepharose 4B (20 g, moist weight) was washed thoroughly with distilled water by suction over G2 sintered glass funnel and suspended in 20 ml of 1 M Na_2CO_3 pH 11.0. After addition of 4 ml divinylsulfone the suspension was stirred for 1 h at room temperature. Activated gel thus obtained was washed thoroughly in distilled water and its wet

cake obtained by suction over sintered glass funnel was added to 20 ml of 0.5 M lactose in 1 M Na_2CO_3 and stirred overnight at room temperature. The reacted beads were washed successively with

1. 20 ml of 1 M Na_2CO_3 pH 11.0
2. 1000 ml of 0.2 M glycine-HCl, pH 3.0, containing 1 M NaCl to block unreacted activated groups in the gel.
3. 1000 ml of 1 M NaCl.
4. 1000 ml of distilled water.

Finally equilibrated to pH 7.4 with PBS and packed into chromatographic column.

3.2.5. Isolation of jacalin

Jacalin (jack fruit seed agglutinin, JSA) was isolated from the seeds of *Artocarpus integrifolia* (jack fruit) by the procedure described by Suresh kumar et al. [Suresh Kumar et al., 1982]. Thirty gram of jack fruit seeds were dehusked and soaked in PBS 6.5 for 12 h. The seeds were then cut into small pieces, homogenized in 300 ml PBS 6.5 and stirred for 1 h at 4°C. The supernatant of homogenate obtained by centrifugation at 14500 g for 20 min was subjected to 65% saturated ammonium sulphate precipitation by stirring at 4°C for 1 h. The precipitated proteins recovered by centrifugation at 14500 g for 20 min were dissolved in PBS 6.5 and dialysed against PBS 6.5. The retentate was centrifuged at 14500 g for 20 min and the supernatant was loaded on to cross-linked gaur galactomannan (CLGG) column. The column was washed with PBS 6.5 and eluted with 0.15 M galactose in PBS 6.5. Fractions containing proteins were pooled and concentrated using Amicon Ultra centrifugal filter units.

3.2.6. Isolation of peanut agglutinin

Peanut agglutinin (PNA) was isolated from peanut (*Arachis hypogea*) as described by Chacko and Appukuttan [Chacko and Appukuttan, 2001]. Fifty gram dehusked peanut was soaked for 24 h in PBS 6.5, homogenised and stirred for 1 h at 4°C. The supernatant of homogenate obtained by centrifugation at 14500 g for 20 min was subjected to 65% saturated ammonium sulphate precipitation by stirring at 4°C for 1 h. The precipitated proteins recovered by centrifugation at 14500 g for 20 min were dissolved in PBS 6.5 and dialysed against the same buffer. The retentate was centrifuged at 14500 g for 20 min and the supernatant was loaded on to cross-linked gaur galactomannan column. After washing out unbound proteins with PBS 6.5, bound lectin was eluted using 0.15 M lactose in the same buffer. Fraction containing proteins were pooled and concentrated using Amicon Ultra centrifugal filter units.

3.2.7. Isolation of concanavalin A

Concanavalin A (Con A) was isolated from the seeds of *Canavalia ensiformis* (Jack bean) as described by Surolia et al. [Surolia et al., 1973]. Husk was removed from 850 g of jack beans after soaking in distilled water. These seeds were homogenised with 1700 ml of 1 M NaCl-0.01 M Tris HCl pH 7.4. The homogenate was stirred for 24 h at 4°C and filtered through glass wool. The mixture was again stirred for 6 h with 1200 ml of 1 M NaCl-0.01 M Tris HCl pH 7.4 and filtered through glass wool. The filtrate was made to 1 M acetic acid concentration by adding concentrated acetic acid and stirred for 20 min at room temperature. The mixture was then centrifuged at 14500 g for 20 min. the supernatant was dialysed against 1 M NaCl-0.01 M Tris HCl pH 7.4, 1 mM Mg^{2+}, 1 mM Ca^{2+} and 1 mM $Mn^{2+.}$ After

dialysis the pH of the sample was made to 7-7.4 with Tris and NaOH. The sample was centrifuged and the soluble part was passed through Sephadex G-50 column. The column was washed with 1 M NaCl-0.01 M Tris HCl pH 7.4. The column was eluted with 0.1 M dextrose in 1 M NaCl-0.01 M Tris HCl pH 7.4, when the protein content of washing was below 75 µg/ml. Fractions having Con A activity were pooled and dialysed successively against 1 M NaCl in distilled water (3 changes), distilled water containing Mg^{2+}, Ca^{2+} and Mn^{2+} and finally in distilled water. After dialysis insoluble particles were removed by centrifugation and soluble part was lyophilised.

3.2.8. Immobilization of jacalin to Sepharose 4B by cyanogen bromide (CNBr) activation method

Sepharose 4B was activated using cyanogen bromide by the method of March et al. [March et al., 1974]. Sepharose 4B (40 g) was added to 80 ml 2 M Na_2CO_3 followed by 40 ml of distilled water. Cyanogen bromide (1.6 g) dissolved in 1-2 ml dimethyl formamide was also added and the mixture was kept for stirring at 8°C for 5 min. The gel was then washed with ice cold 0.1 M $NaHCO_3$. Jacalin (4 mg/ml gel) in 0.1 M $NaHCO_3$ was added to the activated gel and stirred gently overnight at 4°C, followed by addition of 0.1 M ethanolamine hydrochloride and stirring for 1hr to block the unconjugated activated groups on the gel. The coupled gel was washed successively with 20 times the gel volume using

1. 0.1 M $NaHCO_3$
2. Distilled water

3. Acetate buffer 50 mM, pH 5, containing 1 M NaCl.
4. Distilled water
5. PBS 7.4.

The jacalin-Sepharose 4B affinity matrix was stored at 4°C with 0.02% sodium azide or packed into a column of required size for chromatography.

3.2.9. Isolation of lactose binding immunoglobulin (LIg)

Lactose binding immunogloblulin (LIg) from human plasma was isolated by modification of the procedure described by Dong et al. [Dong et al., 1997] by omitting the protein A-Sepharose chromatography so that non-IgG antibodies were not excluded. Briefly, outdated human plasma (60 ml) dialysed extensively in PBS 7.4 was centrifuged at 14500 g for 20 min; the supernatant was loaded on to lactose-Sepharose 4B column. After washing out unbound proteins with PBS 7.4, bound antibodies were eluted with 0.15 M lactose and dialysed extensively in PBS 7.4 to remove sugar. The sugar free eluate was passed through Sepharose 4B column to remove antibodies that bind to Sepharose. The unbound fractions from Sepharose column were concentrated using Amicon Ultra centrifugal filter units.

3.2.10. Conjugation of horse radish peroxidase (HRP) to lectins

Periodate activation of HRP

Horse radish peroxidise (2 mg) was dissolved in 0.2 ml freshly prepared 0.3 M $NaHCO_3$ and 10 µl flourodinitrobenzene (FDNB; 1% in absolute ethanol) was added, the contents were mixed and incubated for 1 h at room temperature (to prevent self coupling). To create reactive aldehyde groups on HRP the solution was treated with

0.2 ml sodium metaperiodate (0.06 M in water) for 30 min at room temperature, following which 0.2 ml of ethylene glycol (0.32 M in water; 32 µl made up to 1.78 ml with water) was added, mixed and incubated for 1 h at room temperature to remove excess periodic acid. Finally the solution was made up to 1 ml with 0.01 M carbonate buffer pH 9.5 (concentration is 2mg/ml) and dialysed in 0.01 M carbonate buffer pH 9.5.

Conjugation of activated HRP to proteins

Conjugation of HRP to proteins was done according to Heyderman et al [Weir and Herzenberg, 1986]. Briefly, 1 mg of lectin or antibody in 1 ml of 10 mM sodium bicarbonate buffer, pH 9.5 was mixed with 0.67 mg periodate activated HRP in the same buffer and incubated at 25°C for 2 h in dark. Potassium borohydride (1%) solution in distilled water was added to a final concentration of 0.1% (to block unconjugated aldehyde groups). After 30 min incubation, the mixture was dialyzed against PBS with one change overnight. The labeled lectin was stored at 4°C with 30 % glycerol v/v.

3.2.11. Biotin labeling of antibodies/lectins

Griffonia simplicifolia I-B4 and LIg (1 mg/ml) were dialysed in 0.1 M Na_2HPO_4 buffer pH 8. Lectin and antibody were pre-incubated with appropriate disaccharide sugar (50 mM) for 1 h at 4°C to prevent biotinylation at the carbohydrate binding site. Sulfo NHS-biotin (0.002 M) was added and mixture incubated for 2 h at 4^oC. The biotinylated proteins were dialysed extensively in pH 7.4 to remove the sugar, unreacted biotin and sulfo NHS. The labeled compound was stored at 4°C with 30 % glycerol v/v [Paul et al., 2011].

3.2.12. Fluorescein isothiocyanate (FITC) labeling of antibodies

FITC was conjugated to antibodies as described in Hudson and Hay [Hudson and Hay, 1980a]. LIg or IgG from human serum (1 mg/ml) was dialysed in 0.25 M sodium carbonate/bicarbonate buffer. Prior to fluorescence labelling LIg was pre-incubated with 50 mM lactose for 1 h at 4ºC to block sugar binding sites. FITC-celite mixture was added to obtain FITC concentration of 150 µg per mg protein and mixture incubated overnight at 4°C for conjugation. The conjugated antibody was separated from the unreacted fluorochrome by gel filtration chromatography on Bio Gel P-4 column. The protein fractions were pooled and concentrated using Amicon Ultra centrifugal filter units. FITC conjugated antibodies were dialysed extensively in dark (to remove sugar) and stored in amber coloured bottles at 4°C.

3.2.13. Preparation of neoglycoconjugates

Lactose and melibiose were conjugated to protein soybean trypsin inhibitor by reductive amination using sodium cyanoborohydride. Soya bean trypsin inhibitor, melibiose/lactose, sodium cyanoborohydrate were taken in the ratio 1 : 2 : 4 by weight in 2 ml 0.2 M trisodium phosphate-phosphoric acid buffer pH 9.0. The contents were incubated at 25°C for 14 days. The neoglycoconjugates thus formed were dialysed in PBS 7.4 and stored at -20°C [Baues and Gray, 1977].

3.2.14. Preparation of asialofetuin and asialomucin by acid hydrolysis

Asialofetuin (AF) and asialomucin were prepared by hydrolysis of fetuin and mucin (1 mg) with 0.1 N H_2SO_4 (1 ml) for 1 h at 80°C. The samples were cooled in ice-bath and pH was neutralised with NaOH solution following which the contents were dialysed against PBS.

3.2.15. Desialylation of human erythrocytes

Whole blood was collected in the presence of anticoagulant. The packed cell layer containing RBC was washed thrice using ice cold PBS by centrifugation at 400 g for 10 min and supernatant was discarded. Packed cell suspension (100 µl) was added to 1.9 ml PBS containing neuraminidase (50 mU/ml), the contents were mixed and incubated at 37°C for 1 h with occasional mixing. The cells were washed thrice using cold PBS by centrifugation at 400 g for 10 min and the supernatant was discarded. Finally volume was made up to 2 ml with PBS to make 5% cell suspension.

3.2.16. Isolation of immune complex (IC) from human serum/plasma

IC was isolated by precipitation with polyethylene glycol (PEG) 6000 as described in Hudson and Hay [Hudson and Hay, 1980b].

Reagents:

a) Vernol buffered saline (VBS) stock solution contains 2 mM sodium barbitone, 3 mM barbituric acid and 140 mM NaCl pH 7.4 and 5 times dilute solution was used as working VBS buffer.
b) Polyethylene glycol 6000 (20% w/v) was prepared in working VBS buffer.
c) 0.2 M EDTA was adjusted to pH 7.6 with 0.1 N NaOH.

d) Working PEG solution (12%) was prepared by mixing 6 ml of (b), 3 ml of (c) and 1 ml of working VBS buffer.

e) Washing solution was prepared in working VBS buffer containing 2% or 3.5% PEG and 20 times dilute solution (c).

To 1 ml serum/plasma appropriate volume of working solution of PEG solution was added in drops to attain final concentration of 2% or 3.5%, the contents were mixed well and incubated at 37°C for 1 h and then overnight at 4°C. Precipitated IC was collected by centrifugation at 2000 g for 20 min. Pellet was washed twice with washing solution and redissolved in 250 µl PBS 7.4 by incubation at 37°C for 1 h.

3.2.17. Isolation of Lp(a) from plasma

Lp(a) was prepared by affinity precipitation of plasma proteins with lectin jacalin and Tris-Borate-EDTA (TBE) electrophoresis and electroelution as described below [Kalaivani.V and Appukuttan.P.S., communicated].

Isolation of jacalin-binding plasma lipoproteins

Plasma (2 ml) was diluted 1:1 with PBS 7.4 containing 0.8 mg/ml jacalin and incubated overnight at 4°C. Precipitated jacalin binding plasma proteins were collected by centrifugation at 100000 g for 45 min at 4°C. Pellet was redissolved in 1 ml of 150 mM 1-O-methyl α-galactopyranoside by incubation at 4°C for 2 h with occasional mixing to dissociate jacalin from proteins. The density of the solution was made up to 1.24 g/cc with potassium bromide and subjected to ultracentrifugation at

535000 g for 4 h at 4°C. Top 20% layer containing jacalin binding lipoproteins (JL1) was collected and dialysed in 10 times dilute TBE buffer pH 8.7.

Purification of Lp(a) from JL1 by tris-borate EDTA (TBE) electrophoresis and electroelution

Lp(a) and the associated LDL(adduct) were separated by non-denaturing 3.75% polyacrylamide disc gel electrophoresis.

Reagents:

a) TBE buffer containing 50 mM tris, 25 mM borate and 3 mM EDTA pH 8.7 was prepared in deionised water.
b) Acrylamide/bisacrylamide (19:1) 15% solution was prepared by dissolving 14.25 g acrylamide and 0.725 g bisacrylamide in 100 ml distilled water and filtered using Whatmann No.1 filter paper. Stored at 4°C in amber coloured bottle.
c) Acrylamide (20 g) and bisacrylamide (5 g) were dissolved in 100 ml distilled water and filtered.
d) Riboflavin (4 mg) riboflavin in 100 ml water.
e) Ammonium per sulphate (0.2%) dissolved in distilled water.
f) Coomassie brilliant blue R-250 (120 mg) dissolved in 100 ml solution containing 44 ml methanol, 12 ml acetic acid and 44 ml water.

Resolving gel:

Mixture of 2 ml of (a), 2.5 ml of (b), 5.5 ml of (e) and 10 µl TEMED.

Stacking gel:

Mixture of 1 ml of 1:3 dilute solution of (a) containing 14 µl of TEMED, 1 ml of (c), 1 ml of (d) and 5 ml deionised water. The gel was allowed to polymerise under intense fluorescent light.

Sample loading and disc gel electrophoresis:

JL1 (50 µg) containing 10% glycerol and bromophenol blue (tracking dye) was loaded per tube and run was carried out at current of 3 mA per tube till dye came out and continued for 50% more time. The bands were located by staining with Coomassie brilliant blue R-250 and destained with methanol: acetic acid: water (1:1.5:17.5, v/v).

Electroelution:

The stained gel served as reference to cut the protein bands from other tube gels. The corresponding position in unstained gel was cut, minced well and transferred to dialysis bag containing 2 ml elution buffer (10 mM Tris-HCl buffer pH 8.7). Electroelution was carried out in 10 mM Tris-HCl buffer pH 8.7 at 4°C, under constant voltage (100 V) for 3 h in horizontal electrophoresis chamber. The current was reversed for 10 min to detach the protein adhered to the sides of the dialysis bag. Eluted samples were recovered by filtering through glass wool. Electroeluted samples were coated on polystyrene wells and probed with anti-apo(a) and anti-apoB HRP. Band(s) containing both apo(a) and apoB was identified as Lp(a) and bands containing only apoB as LDL.

3.2.18. Lp(a) assay by jacalin-based enzyme immunoassay

Lp(a) in plasma, lipoprotein layer (L1) or TBE eluted Lp(a) sample were assayed by jacalin based enzyme immunoassay as described by Sreekumar et al. [Sreekumar et al., 2013]. Jacalin was coated on polystyrene wells by incubating the wells with 1 µg jacalin in 200 µl PBS for 3 h at 37°C. The wells were washed with 0.05% Tween-20 in PBS (PBS-T) and blocked with 0.5% Tween-20 in PBS for 30 min at 37°C and again washed with PBS-T. Appropriate dilution of TBE-eluted Lp(a), lipoprotein layer (L1) or 500 times dilution of plasma in 200 µl ice-cold PBS-T was added to jacalin coated wells. After incubation for 2 h at 4°C the wells were washed with ice-cold PBS-T and probed with 200 µl HRP-conjugated anti-apo(a) (1.5 µg per ml PBS-T; previously treated with jacalin-Sepharose to remove any jacalin-binding components). Wells were then incubated for 15 min at 25°C with 200 µl orthophenylene diamine (0.5 mg per mL) in 0.1 M citrate-phosphate buffer pH 5.0 containing 0.03% H_2O_2 followed by addition of 50 µl 12.5% H_2SO_4 to stop the reaction. Bound HRP activity was measured as absorbance at 490 nm in a BIOTEK (USA) ELISA reader.

3.2.19. Preparation of de-O-glycosylated asialofetuin (AF)

Fetuin (39 µg) in 17 µl 60 mM sodium phosphate buffer, pH 5, was mixed with 2 µl neuraminidase (500 mU per ml) in PBS. After incubation at 37 ºC for 1 h, 2 µl O-glycanase (0.04 U) was added and the contents incubated for 3 h at 37ºC, AF-free buffer treated as above was taken as enzyme blank.

3.2.20. Preparation of LacNAc-free fetuin

LacNAc-free fetuin was prepared by treating fetuin (1 mg) with jack bean α-mannosidase (0.05 U per ml) in 0.2 M citrate phosphate buffer, pH 4, containing 2 mM zinc acetate for 24 h at 37ºC.

3.2.21. To determine the immunoglobulin distribution in lipoprotein IC

Polystyrene wells were coated with anti-apo(a), anti-apoB or anti-apoA1 antibodies (1 µg in 200 µl PBS) for 3 h at 37°C. The wells were washed with 0.05% Tween-20 in PBS (PBS-T), blocked with 0.5% Tween-20 in PBS for 30 min at 37°C and again washed with PBS-T. Circulating immune complex (IC) isolated from human plasma by 2% and 3.5% PEG precipitation (Section 3.2.16) diluted 10 times in PBS-T was added and well incubated for 2 h at 4°C. After washing thrice with PBS-T the immunoglobulin type distribution in lipoprotein IC was determined by incubation with HRP-conjugated anti-human IgA, IgG, IgM separately (1.5 µg antibody per ml; 200 µl) for 2 h at 4°C and bound HRP assayed as described earlier. Lipoprotein fraction obtained in top 20% layer (L1) following ultracentrifugation of plasma at 535000 g (4 µg in 200 µl PBS-T) was used as control instead of IC to assay non-specific interaction. After washing thrice with PBS-T, bound HRP was assayed by incubating with 200 µl OPD (0.5 mg per ml) in 0.1 M citrate-phosphate buffer, pH 5.0, containing 0.03% H_2O_2 for 15 minutes, followed by addition of 50 µl 12.5% H_2SO_4 and the absorbance read at 490 nm in BIOTEK (USA) microplate reader.

3.2.22. Preparation of IC devoid of free lipoproteins

The density of plasma samples (1 ml) was adjusted to 1.24 gm/cc with potassium bromide (KBr). The samples were then subjected to ultracentrifugaion at 535000 g for 4 h at 4°C following which top 20% layer containing free lipoproteins mixture was collected as lipoprotein layer (L1) remaining bottom 80% layer was dialysed extensively to remove KBr. Immune complex present in bottom 80% layer was isolated by 3.5% PEG precipitation as described earlier (Section 3.2.16).

3.2.23. Dissociation of IC

Circulating immune complex from human plasma (2 ml) was isolated by 2% PEG precipitation as described in section 3.2.16. The immune complex thus obtained was dissociated by treatment with 8 M urea (250 µl) in PBS for 1 h at 37°C followed by incubation at 60°C for 1 h. The contents were made up to 1 ml with PBS, density adjusted to 1.24 g/cc with KBr following which the samples were subjected to ultracentrifugation at 535000 g for 4 h at 4°C. Urea-dissociated lipoproteins from samples were collected in top 20% layer and dialysed extensively to remove urea and KBr. IC obtained by 2% PEG precipitation subjected to the same treatment without addition of 8 M urea was taken as control.

3.2.24. To assay the desialylation status of Lp(a) in IC

TBE eluted Lp(a) or lipoproteins obtained in the top 20% layer with or without prior treatment with 8 M urea treatment of IC was coated on polystyrene wells (1µg per well), blocked and probed with 200 µl HRP-conjugates of anti-apo(a), anti-apoB (1.5 µg per ml in PBS-T) and anti-human IgA, IgG, IgM separately (1.5 µg antibody

per ml in PBS-T). The desialylation status of Lp(a) was assayed using 200 µl HRP-conjugated PNA (15 µg per ml in PBS-T).The bound HRP was assayed with OPD as chromogen as described above.

3.2.25. Immunoglobulin distribution in LIg

LIg (50 ng) was coated on polystyrene wells and wells washed as described earlier. The wells were then probed with 200 µl HRP-conjugated anti-human IgA, IgG, IgM separately (1.5 µg per ml in PBS-T) for 2 h at 4°C. Bound HRP was assayed as described earlier.

3.2.26. Haemagglutination and haemagglutination inhibition assay to determine sugar specificity of LIg

Polystyrene U bottom wells were first blocked with 0.05% Tween 20 in PBS for 30 min at 25°C. Serial two-fold dilutions of LIg (100 µl in PBS-T) followed by 25 µl of desialylated human O group RBC or unmodified sheep RBC suspension (5% v/v) were added to wells. After mixing and incubation for 1 h at 25°C the contents were again mixed and settling of RBCs within 2 minutes taken as positive agglutination. For haemagglutination inhibition minimum agglutinating quantity of LIg was first determined. LIg containing this quantity in 25 µl PBS-T was incubated with inhibitors (two-fold serial dilutions in 75 µl PBS-T) in U-bottom wells for 1 h at 4ºC, following which 25 µl of 5% desialylated human RBC cell suspension was added and incubated for 1 h at 25ºC. Scoring was done as described above.

3.2.27. LIg and lectin binding to glycoconjugates

AF, Tg, TIM, TIL, de-O-glycosylated AF or LacNAc-free fetuin was coated on polystyrene wells by incubating the wells with 200 µl PBS containing 2 µg of each protein at 37ºC for 3 h. The wells were washed and blocked as described earlier. Coated LacNAc-free fetuin was desialylated by treatment with 200 µl neuraminidase (50 mU per ml) in PBS-T for 1 h at 37ºC. After washing with PBS-T, LIg (2 µg in 200 µl PBS-T) was added to the glycoconjugate-coated wells. Wells coated with Tg, TIM, TIL and AF were treated alternatively with 250 ng LIg as well. Following incubation at 4ºC for 2 h the wells were washed and treated with 200 µl mixture of HRP-conjugated anti-human IgA, IgG and IgM (1.5 µg of each antibody per ml in PBS-T) for 2 h at 4ºC. Wells coated with AF, de-O-glycosylated AF and LacNAc-free AF were treated alternatively with HRP-labeled PNA (600 ng), HRP-labeled jacalin (10 ng) or ConA (2 µg) in 200 µl PBS-T. After 2 h incubation at 4ºC ConA-treated wells were incubated for 2 h at 4ºC with 2 µg HRP in 200 µl PBS-T. Wells were washed and treated with OPD as described earlier. To study inhibition of LIg binding to AF- and Tg-coated wells the antibody (2 µg and 250 ng respectively) in 200 µl PBS-T pre-incubated for 1 h at 4ºC with or without 25 mM sugar (melibiose, lactose or cellobiose) was used. Following 2 h incubation at 4ºC, wells were washed and bound antibody was assayed as described earlier.

3.2.28. Alpha-galactoside specificity of LIg.

Polystyrene wells were coated with Tg (1 μg) or rabbit RBC membrane proteins (2 μg) in 200 μl PBS, blocked and incubated with or without coffee bean α-galactosidase (0.8 U per ml) in 0.5 M citrate-phosphate buffer, pH 5.2, for 12 h at 37ºC to remove terminal α-galactoside group. Wells were washed and incubated for 2 h at 4ºC with PBS-T containing LIg pre-incubated with or without 25 mM lactose for 1 h at 4ºC (1 μg for Tg and 2 μg for rabbit RBC membrane proteins). Following washing with PBS-T bound antibodies were assayed as described earlier. Removal of terminal alpha-galactoside groups by the enzyme was verified using α-galactoside-specific lectin GS I-B4. Wells coated with Tg or rabbit RBC membrane glycoprotein as above were treated with or without enzyme and probed with 200 μl PBS-T containing biotin-labeled GS I-B4 (50 ng and 1 μg for Tg and rabbit RBC membrane proteins respectively) for 2 h at 4ºC, following which 200 μl HRP-conjugated avidin (500 ng per ml) in PBS-T was added and incubated for 2 h at 4ºC. Bound HRP was assayed as described earlier.

3.2.29. LIg binding to desialylated Lp(a) and LDL

Polystyrene wells were coated with Lp(a) and LDL (500 ng in 200 μl PBS) for 3 h at 37ºC, blocked and incubated with or without 200 μl neuraminidase (50 mU per ml in PBS-T) for 1 h at 37°C. Wells were washed and biotin-labeled LIg (1 μg in 200 μl PBS-T) pre-incubated with or without 50 mM lactose was added and incubated for 2 h at 4ºC. After washing and 2 h incubation at 4ºC with 200 μl HRP-conjugated avidin (500 ng per ml in PBS-T) followed by washing, bound HRP was assayed as described earlier. To ascertain LacNAc specific binding, microplate coated Lp(a)

was desialylated by treatment with neuraminidase as described above and LacNAc moieties selectively removed by treatment with 200 µl jack bean α-mannosidase (0.3 U per ml) in 0.2 M citrate phosphate buffer, pH 4, containing 2 mM zinc acetate for 24 h at 37°C. Wells were washed and incubated with biotin-labeled LIg (1 µg), heat-inactivated biotin-labeled LIg (1 µg) Con A (2 µg) or HRP-labeled jacalin (10 ng) in 200 µl PBS-T for 2 h at 4ºC. Bound antibodies and lectins were assayed as described earlier.

3.2.30. Incorporation of FITC-conjugated LIg into IC fraction.

Seven samples each of plasma belonging to very low Lp(a) (< 3.5 mg/dl) and high Lp(a) (> 25 mg/dl) categories were used to demonstrate IC formation of their Lp(a) with added LIg. LIg was removed from 2 ml plasma by treatment with 200 µl packed cell suspension of desialylated human O-group RBC for 2 h at 25ºC followed by centrifugation at 400 g. The supernatant (900 µl), devoid of LIg, was desialylated by treatment with 100 µl neuraminidase (500 mU per ml in PBS) for 12 h at 20ºC and mixed with 50 µg FITC-conjugated LIg or FITC-conjugated total IgG from human serum in 50 µl PBS. Following overnight incubation in the dark at 4ºC, the resulting immune complex was isolated by 2% PEG precipitation. FITC-conjugated LIg in IC fraction was assayed in terms of fluorescence measured using excitation/emission wavelength of 485 and 521 nm in BIOTEK (USA) microplate reader.

3.2.31. Preparation of desialylated lipoprotein mixture

Plasma samples were ultracentrifuged at 535000 g for 4 h at 4°C after adjusting their density to 1.24 g/cc with KBr. Top 20% layer (L1) collected from 8 ml plasma were pooled and desialylated by treatment with 160 µl of neuraminidase (500 mU per ml) in PBS at 37°C for 2 h. After desialylation the contents were made up to 4 ml with PBS, density adjusted to 1.24 gm/cc with KBr and subjected to ultracentrifugation at 535000 g for 4 h at 4°C to separate enzyme used form desialylated lipoproteins. Top 20% layer (N-L1) containing only desialylated lipoproteins were collected and dialyzed extensively. L1 treated with heat inactivated neuraminidase and collected by ultracentrifugation (NH-L1) was used as control. The Lp(a) contents in N-L1 and NH-L1 were quantified using jacalin based enzyme immunoassay.

3.2.32. Demonstration of free valence site in de novo lipoprotein immune complex by haemagglutination assay.

a) ***To screen plasma samples for high and low polyagglutinin titre.***

Polyagglutinin titre in plasma samples were determined by haemagglutination assay. Polystyrene U bottom wells were blocked and 25 µl aliquots of desialylated O-group RBC (5% v/v) incubated with plasma samples (two-fold serial dilution in 100 µl PBS-T) for 1 h at 25°C. Contents were then mixed and settling of RBCs within 2 minutes was taken as positive agglutination. Samples that agglutinated at or above 1:8 plasma dilution were considered to be of high polyagglutintin titre and those that agglutinated at or below 1:2 dilution as low titre.

b) ***To assay free valence binding site in de novo lipoprotein IC.***

De novo lipoprotein IC was prepared by incubating 20 µl N-L1 or NH-L1 (10 mg per ml in PBS) with plasma samples (1 ml) of varying polyagglutinin titre for 12 h at 20°C. De novo IC formed between desialylated lipoproteins and anti-carbohydrate antibodies were isolated by 2% PEG precipitation. The pellet was redissolved in 250 µl PBS. IC prepared by 2% PEG precipitation without addition of lipoproteins served as control. For haemagglutination assay, 25 µl desialylated human O-group RBC (5% v/v) was incubated with de novo IC (serially diluted in 100 µl PBS-T) for 1 h at 25°C and scoring was done as described above. The ability of de novo IC to agglutinate desialylated human O-group RBC was used to demonstrate the presence of unoccupied binding sites on de novo IC.

3.2.33. To determine sugar specificity of de novo lipoprotein IC by hemagglutination inhibition

Sugar specificity of de novo IC was assayed by haemagglutination inhibition of desialylated RBC. Minimum agglutinating quantity of de novo IC was determined by haemagglutination assay. De novo IC containing this quantity in 25 µl PBS-T was incubated with inhibitors (two-fold serial dilutions in 75 µl PBS-T) in U-bottom wells for 1 h at 4ºC, following which 25 µl of 5% desialylated human RBC cell suspension was added and incubated for 1 h at 25ºC. Scoring was done as described above.

3.2.34. To determine the contribution of Lp(a) in the formation of de novo lipoprotein IC

To remove Lp(a) from N-L1, 200 μl N-L1 (10 mg per ml in PBS) was mixed with equal volumes of jacalin-Sepharose (2 mg jacalin per ml Sepharose) and incubated for 2 h at 4^oC with occasional mixing. The mixture was centrifuged at 400 g for 5 min and the supernatant was collected. N-L1 mixed with equal volume of Sepharose was used as control. De novo IC was prepared by incubating 40 μl jacalin-Sepharose- or Sepharose-treated N-L1 with plasma (1 ml) containing high polyagglutinin titre and the extent of agglutination was determined as described earlier. To check the efficiency of Lp(a) removal, jacalin-Sepharose or Sepharose treated N-L1 was diluted 1000 times and assayed using jacalin-based enzyme immune assay.

3.2.35. Statistical analysis

Statistical analysis was done using Microsoft Excel 2000 version. Comparison between groups was done using Students t-test. P value of < 0.05 was considered significant.

RESULTS AND DISCUSSION

PART – I

Specificity of Lactose Binding Immunoglobulin: Dual specificity of human plasma lactose-binding immunoglobulin to anomers of terminal galactose enables recognition of desialylated lipoprotein(a) and xenoantigens

Introduction

A property common among natural anti-carbohydrate antibody is multiple specificity for which examples include terminal α-galactoside and MUC-1 peptide sequence for anti-Gal [Sandrin et al., 1997], α-galactoside and β-glucan specificity of dextran binding immunoglobulin [Paul et al., 2009] and human IgM anti-streptococcal antibodies that react with human cardiac myosin as well as streptococcal M proteins [Adderson et al., 1998]. Lactose binding immunoglobulin (LIg) isolated from human plasma by affinity chromatography on lactose-Sepharose matrix forms a repertoire of naturally occurring anti-carbohydrate antibodies. Studies to determine the ligand binding properties of LIg have demonstrated that LIg can bind to glycodendrimers of lactose and desialylated fetuin [André et al., 1999; Gupta et al., 1996]. Due to its affinity for lactose, LacNAc moieties have been considered ligands for LIg although direct evidence for this conclusion in lacking. Nevertheless physiological role of LIg still remains unexplored. Present study examines the immunoglobulin composition and ligand binding properties of LIg as also its contribution to formation of IC with lipoproteins.

RESULTS

A) IgG is the most dominant immunoglobulin type in LIg

Immunoglobulin composition of LIg was assayed by measuring the anti-immunoglobulin response to plate-coated antibody. Results show that IgG is the most dominant antibody type, while IgA and IgM responses were about one-fourth of that due to IgG (Figure.6). Earlier studies had employed lactose-Sepharose and protein A-Sepharose columns to isolate LIg from human plasma so that the antibody

type was restricted to IgG alone. Since the present method of preparation of LIg does not involve the use of protein A-Sepharose results indicate the presence of IgA and IgM type as well in LIg.

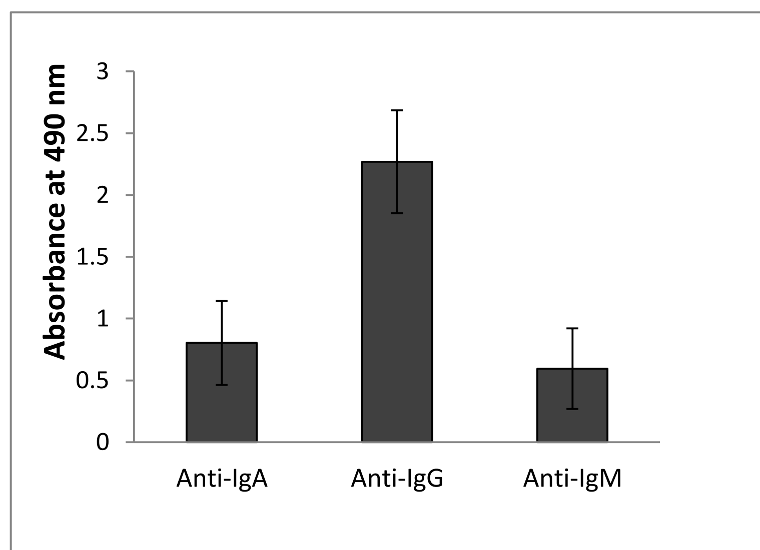


Figure 6. Immunoglobulin distribution in LIg.
LIg prepared by affinity chromatography was coated on polystyrene wells (50 ng in 200 µl PBS), treated with 200 µl HRP-conjugated anti-human IgA, IgG or IgM (1.5 µg antibody per ml) and bound HRP assayed. Details are in 'Methods'. Values are mean ± S.D of 7 samples of LIg.

B) LIg displays overlapping specificity towards terminal α- and β -galactoside residues.

Ligand binding properties of LIg was assayed by haemagglutination assay and by ELISA using synthetic glycoproteins and enzyme-treated natural glycoconjugates. Haemagglutination assay shows that LIg agglutinates human RBC only after desialylation of the cells, minimum agglutinating amount being 12 ± 7 µg (n=7) in the reaction volume employed (125 µl) (Figure.7). Group O human RBC was employed to rule out contribution of A and B group determinants if any to

agglutination. Desialylation of RBC facilitates binding of LIg to their surface glycans which otherwise remain inaccessible due to the presence of terminal sialic acid in both N- and O-glycans.

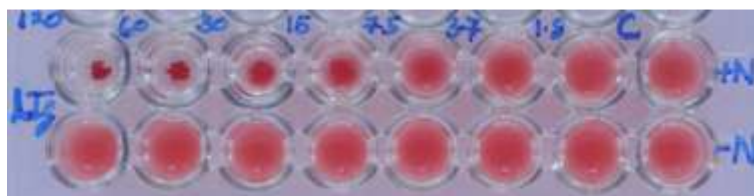


Figure 7. Haemagglutination of human O-group RBC by LIg.
The indicated amounts of LIg per well were used for agglutination of desialylated O group RBC (+N) or non-desialylated RBC (-N) as described under 'Methods' section 3.2.26.

In haemagglutination inhibition assay sugar specificity is expressed in terms of minimum concentration of sugar or its derivative required to inhibit agglutination of desialylated O-group RBC by minimum amount of LIg. Results show that lactose (Gal β1-4 Glc) was the most efficient ligand followed by 1-O-methyl derivatives of α- and β-D-galactopyranoside whereas corresponding derivatives of glucopyranosides were ineffective, as was cellobiose (Glc β1-4 Glc). The α-galactoside-containing disaccharide melibiose was close to the methyl derivatives of α-galactose, inhibiting at 75 mM (Table.3). This shows that LIg can accommodate α-linked galactose as an alternate ligand at its binding site, despite its isolation by binding to Sepharose-immobilized lactose and elution with lactose (a β-galactoside).

Table 3.

Inhibition of LIg-mediated agglutination of desialylated RBC by sugars

Sugar	Minimum inhibitory concentration (mM)*
1-O-Methyl-α-D-galactopyranoside	50 ± 35
1-O-Methyl-β-D-galactopyranoside	43 ± 37
Lactose	12 ± 0.25
Melibiose	75 ± 30
1-O-methyl-α-D-glucopyranoside	> 100
1-O-methyl-β-D-glucopyranoside	> 100
Cellobiose	> 100

* Desialylated O-group human RBC used; mean ± SD of results with 3 LIg samples listed

Alternatively when galactose derivatives were used as inhibitors of LIg binding to plate-coated glycoproteins 25 mM melibose could significantly inhibit LIg binding to either the α-galactoside-bearing Tg or to the β-galactoside-bearing AF while cellobiose was hardly inhibitory, showing again the dual specificity of LIg to galactoside anomers (Figure.8).

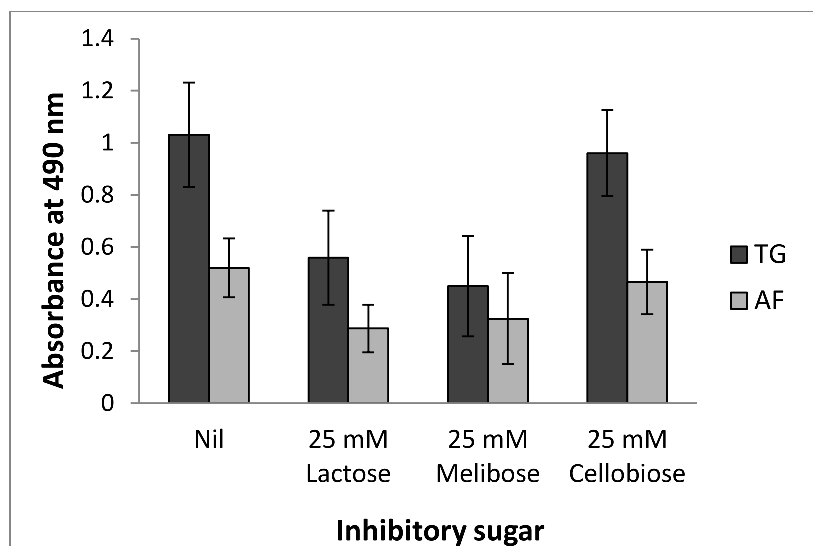


Figure 8. Inhibition ELISA to demonstrate the sugar specificity of LIg.
LIg (250 ng and 2 µg) pre-incubated with 25 mM sugar (lactose, melibiose or cellobiose) for 1 h at 4°C was added to Tg- or AF-coated wells (2 µg) respectively. Bound antibody was assayed by probing with a mixture of HRP-conjugates of anti-human IgA, IgG and IgM (1.5 µg antibody per ml) in 200 µl PBS-T as described in 'Methods'. Values are mean ± SD of 6 LIg samples. P values for inhibition of LIg binding: Tg coating; lactose = 0.0006, melibiose = 0.007. AF coating; lactose = 0.0005, melibiose = 0.032.

C) Terminal α-linked galactose residues are better than terminal LacNAc as ligands in glycoconjugates for LIg binding.

Anomer specificity of LIg was also examined using polystyrene well-coated natural and synthetic glycoproteins bearing terminal galactoside moieties. From either of two widely differing concentrations of LIg more antibody bound to the α-galactose-containing glycoproteins Tg and TIM than to the same amount of AF or TIL which contain β-linked galactose termini. With the same carbohydrate content (2% w/w) TIM was about 5 times more efficient than TIL in capturing LIg. The 660 kDa molecule of bovine thyroglobulin has 11 estimated TAG moieties distributed far between [Spiro and Bhoyroo, 1984]. On the other hand AF of size 48 kDa and

containing three N-linked glycans which are mostly (83%) triantennary complex oligosaccharide could offer about 8.5 terminal LacNAc groups on an average [Green et al., 1988]. Thus the ligand density per unit coated protein is much larger for LacNAc in AF than for TAG in Tg. The remarkably greater binding to Tg than to AF therefore underline the efficiency of LIg to recognize bacterial or non-primate molecules bearing TAG (Figure.9).

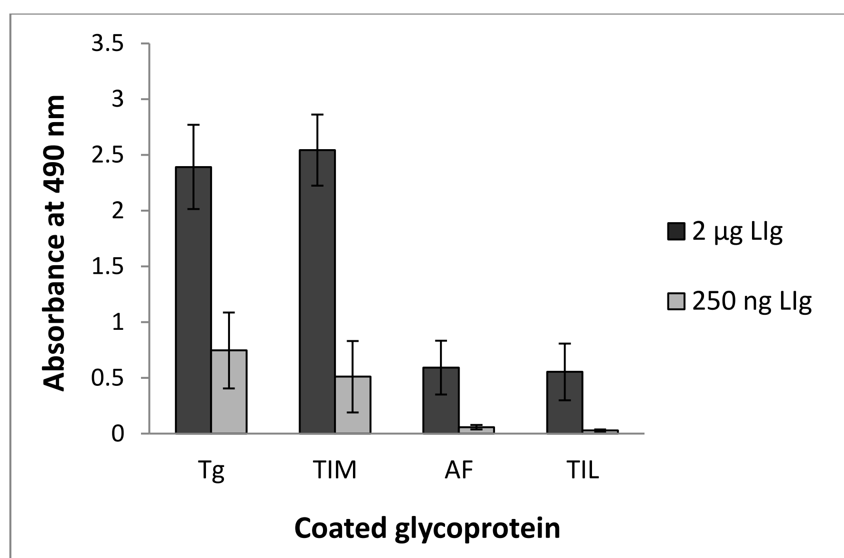


Figure 9. LIg binding to α- and β-anomers of galactose.
Two sets of polystyrene wells were coated with Tg, TIM, AF or TIL (2 µg) in 200 µl PBS, blocked and two different concentrations of LIg (2 µg in one set and 250 ng in the other) in 200 µl PBS-T were added, bound antibody was probed with 200 µl of mixture of HRP-conjugates of anti-human IgA, IgG and IgM (1.5 µg each per ml). Values are mean ± S.D of 6 samples of LIg.

To assess the affinity of LIg towards α-galactoside moiety relative to its β-anomer in the same naturally occurring biomolecule Tg and rabbit RBC membrane proteins, both rich in N-linked glycans containing terminal α-linked galactose attached to penultimate β-galactose moieties of N-acetyl lactosamine unit (Gal α1-3 Gal β1-4 GlcNAc), were used as ligands in polystyrene well-coated form. LIg binding to both

the glycoproteins were inhibitable by 25 mM lactose underlining its dual specificity (Figure.10). Moreover removal of the terminal α-linked galactose (TAG) moiety by coffee bean α-galactosidase treatment substantially reduced binding of LIg to either glycoprotein. Removal of TAG by the enzyme was verified by the loss of binding of the TAG-specific lectin GS I-B4 to the enzyme-treated proteins (Figure.10). Result shows that in glycoconjugates terminal LacNAc is a much poorer ligand for LIg than is TAG.

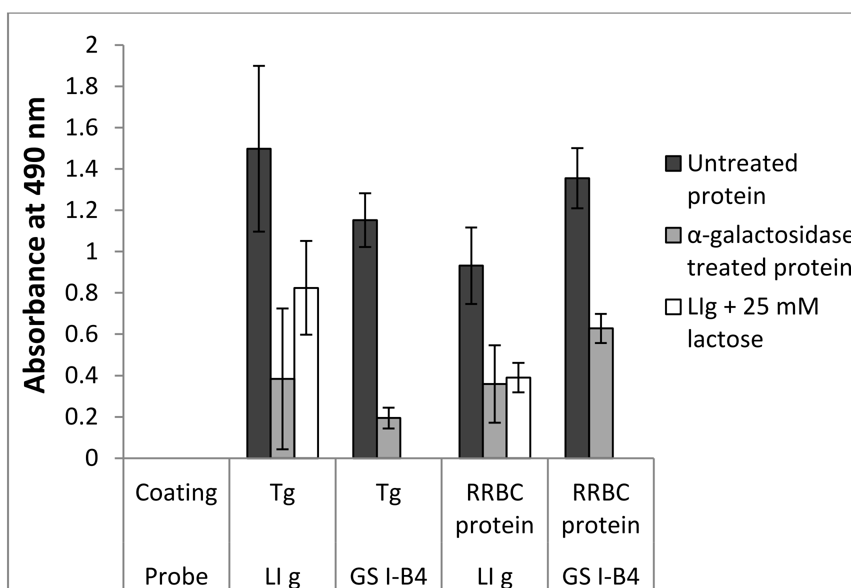


Figure 10. Effect of removal of TAG on LIg recognition of glycoconjugates.
Polystyrene wells were coated with TG (1 μg) or rabbit RBC membrane protein mixture (RRBC protein; 2 μg) in 200 μl PBS, blocked and treated with or without coffee bean α-galactosidase (0.8 U per ml) for 12 h at 37°C. Wells were washed and treated with LIg pre-incubated for 1 h at 4ºC with or without 25 mM lactose in PBS-T (1 μg for TG and 2 μg for RRBC protein) and biotin-labeled GS I-B4 lectin (50 ng in 200 μl PBS-T for TG and 1 μg in 200 μl PBS-T for RRBC protein) for 2 h at 4ºC. Bound antibodies and lectin assayed as described in methods. Values are mean ± S.D of 7 LIg samples. P value for LIg binding to Tg = 0.0002. P value for LIg binding to RRBC protein = 0.006.

Furthermore the TAG specificity of LIg was also verified by agglutination of sheep RBC. Results show that LIg agglutinates unmodified sheep RBC at a minimum agglutinating amount of 10 $\pm$ 7 μg (n=7) (Figure.11). This result also confirms the α-galactoside specificity of the LIg, since these cells contained no other analogue of terminal galactose moiety.

Figure 11. Agglutination of unmodified human and sheep RBC by LIg. *The indicated amounts of LIg per well used for agglutination of unmodified human O-group RBC (Hu) or unmodified sheep RBC (Sheep) as described under 'Methods' section 3.2.26.*

D) Terminal LacNAc groups are ligands for LIg in desialylated glycoconjugates

Since TAG moieties are not synthesised in man, host tissue galactose-terminating glycans that could be candidates for recognition by LIg are desialylated derivatives of N- and/or O-linked oligosaccharides in glycoconjugates. Since fetuin is a TAG-free glycoprotein containing fully characterized N- and O-glycan chains, its desialylated version (AF) was used as a prototype to study the efficiency of these chains as LIg ligands. Results (Figure.12) show that while selective removal of O-glycans drastically reduced recognition of AF by PNA and jacalin as expected LIg binding remained unaffected. Since de-O-glycosylation protocol employed did not affect N-glycans at all as evidenced by undiminished binding of ConA, results show that LacNAc groups in terminal position are responsible for LIg binding to AF. On the other hand removal of peripheral sugar groups of N-glycans by jack bean

α-mannosidase treatment drastically reduced recognition by both LIg and ConA, while leaving jacalin binding unaffected (Figure.12). Together these results suggest that in the absence of TAG moieties LIg recognition of desialylated glycoconjugates is mediated by terminal LacNAc groups.

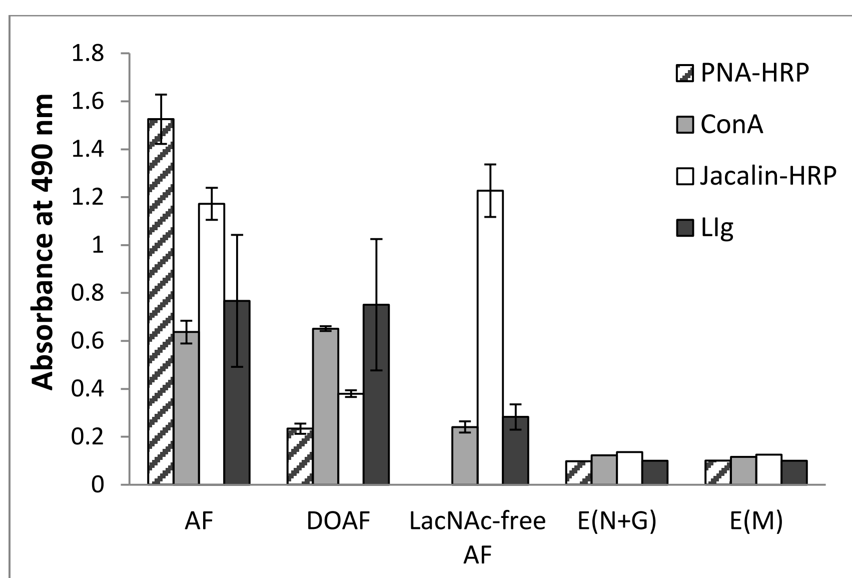


Figure 12. Effect of deletion of N- and O-glycans of AF on LIg recognition.

AF, de-O-glycosylated AF (DOAF), LacNAc-free fetuin (2 µg) and appropriate dilution of AF-free enzyme blank [E(N+G) and E(M)] were coated on polystyrene wells. LacNAc-free fetuin coated on wells were desialylated by treatment with neuraminidase as described in methods. Wells were washed and incubated with LIg (2 µg), HRP-labeled PNA (600 ng), HRP-labeled jacalin (10 ng) or ConA (2 µg) in 200 µl PBS-T for 2 h at 4ºC. Bound antibody and lectins were assayed as described section 3.2.27. E(N+G): neuraminidase and O-glycanase, E(M): α-mannosidase. Values are mean $\pm$ S.D of 3 samples. P value for binding to LacNAc-free AF vs AF: ConA = 0.006, LIg = 0.0269.

E) LIg forms LacNAc-dependent immune complexes with desialylated human plasma Lp(a)

Plasma lipoprotein(a) is unique to advanced mammals such as man and apes and consists of an LDL molecule to which an apo(a) subunit is attached by a disulfide bond. Recent investigations in our laboratory have revealed that Lp(a) accounts for most of the lipoproteins in circulating IC and that Lp(a) in IC are more desialylated than free Lp(a). Each of the 10 kringle IV types except type 2 in apo(a) subunit of Lp(a) contain one biantennary complex N-glycan terminating in sialylated LacNAc on average. Since kringle IV type 2 repeats 3 to 40 times among Lp(a) samples of individuals and each of these kringles contain one biantennary N-glycan on an average the number of biantennary complex N-glycans in apo(a) may vary between 12 and 49 [Scanu and Edelstein, 1997]. These along with about eleven complex or hybrid N-glycans of apoB [Harazono et al., 2005] offer an unusually vast array of potential LIg ligands in desialylated Lp(a). Biotin-labeled LIg binding to plasma Lp(a) purified by lectin affinity precipitation and electrophoresis and coated on polystyrene plates was followed using HRP-labeled avidin. Results (Figure.13) show that the limited binding of LIg to unmodified Lp(a) was significantly enhanced in a lactose-inhibitable manner following desialylation of the lipoprotein. Further, removal of LacNAc groups from desialylated Lp(a) by α-mannosidase treatment significantly reduced LIg binding in proportion to the extent of truncation of N-glycan chains as indicated by ConA binding. The response of LIg to native as well as desialylated LDL however was no more than that of heat-inactivated or sugar-inhibited LIg to desialylated Lp(a). This indicated that the N-glycans that are few and far between in the 500 kDa apoB chain of Lp(a) contributes little towards

LIg binding and that apo(a) N-glycans are responsible for LIg recognition of desialylated Lp(a). The enzyme treatment did not affect O-glycans of Lp(a) since jacalin binding remained undiminished.

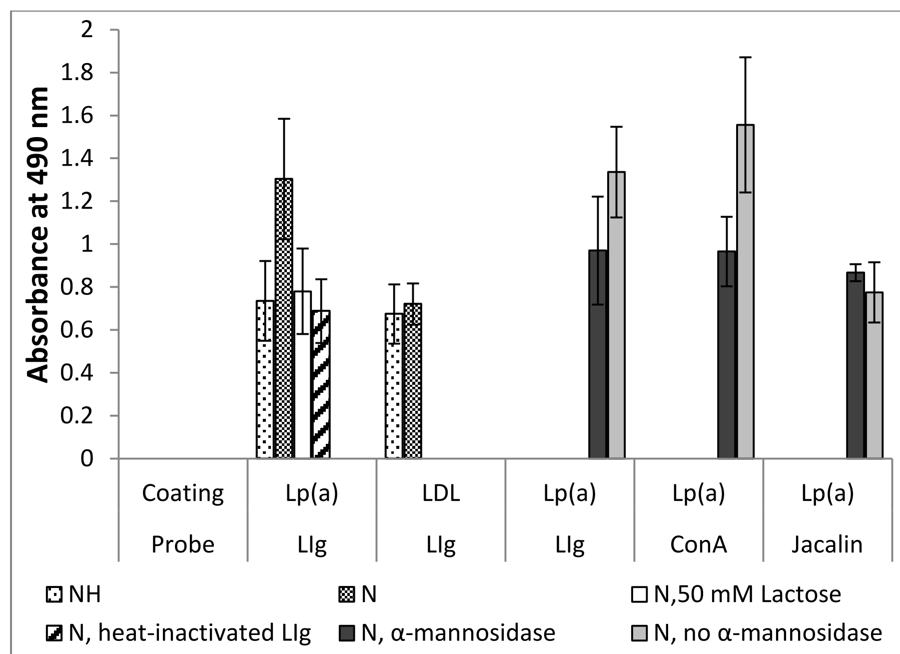


Figure 13. LIg binding to desialylated Lp(a) and LDL.

Polystyrene wells coated with Lp(a) and LDL (500 ng) were blocked and incubated with active (N) or heat-inactivated (NH) neuraminidase (200 μl containing 50 mU per ml in PBS-T) for 1 h at 37°C. To ascertain LacNAc specificity a pair of neuraminidase-treated plate-coated Lp(a) was treated with or without jack bean α-mannosidase (0.3 U per ml) for 24 h at 37ºC. The wells were washed and incubated with PBS-T containing biotin labeled LIg (1 μg) pre-incubated with or without 50 mM lactose, heat-inactivated biotin labeled LIg (1 μg), ConA (2 μg) or HRP-conjugated jacalin (10 ng) for 2 h at 4ºC. Bound antibody and lectins were assayed as described in methods. P values for LIg binding were: desialylated (N) vs native (NH) Lp(a) = 0.000 (n=9); lactose inhibition of binding to Lp(a) (N) = 0.000 (n=9); α-mannosidase-treated desialylated Lp(a) vs desialylated Lp(a) = 0.019 (n=3). P value for ConA binding to α-mannosidase treated desialylated Lp(a) vs desialylated Lp(a) = 0.033.

Efficiency of LIg in forming IC with desialylated Lp(a) from total plasma was followed by the addition of FITC-LIg to plasma which had been treated successively with a) desialylated RBC to remove antibodies reactive to desialylated glycans and b) neuraminidase for desialylation of its lipoproteins. Results in Figure.14 show that desialylated Lp(a) forms significantly more IC than does native Lp(a) and that the extent of IC formation was positively correlated with the Lp(a) concentration in plasma. The limited incorporation of non-specific human IgG into IC, however, was not desialylation-dependent.

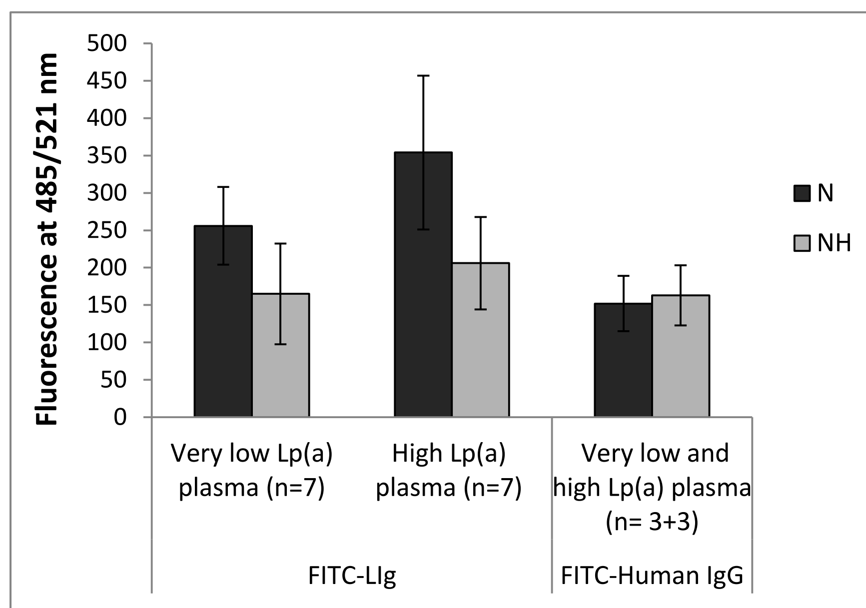


Figure 14. Incorporation of FITC-conjugated LIg into IC.
Plasma samples (900 µl) containing very low or high Lp(a) and freed of LIg using desialylated RBC as described in methods were treated with active (N) or heat-inactivated (NH) neuraminidase (100 µl containing 500 mU per ml in PBS) for 12 h at 20ºC, following which 50 µg FITC-conjugated LIg or FITC-conjugated human IgG in 50 µl PBS was added and the mixture was incubated overnight in the dark at 4ºC. The resulting immune complex formed was isolated by 2% PEG precipitation and fluorescence assayed at 485 and 521 nm (excitation/emission). P values (N vs NH) for FITC-LIg incorporation in IC were 0.003 and 0.002 for very low Lp(a) and high Lp(a) (n=7) plasma respectively.

DISCUSSION

Previous studies on LIg have reported the existence of two different populations of IgG, one specific for α-galactoside and the other for β-galactoside sugars [Dong et al., 1997]. The present results indicate that the binding site of the same LIg molecule can accommodate either α- or β-anomer of galactose, since lactose as well as melibiose could inhibit LIg binding to glycoconjugates containing either TAG or LacNAc. Among the circulating anti-carbohydrate antibodies, anti-Gal is the most widely studied owing mainly to its role in xenograft rejection [Galili, 1993]. Dominance of IgG type antibodies and specificity towards α-galactoside group are features common to LIg and anti-Gal. Presence of anti-Gal in the LIg preparation is ruled out by the inability of lactose to bind to affinity-purified anti-Gal [Galili et al., 1984; Jaison and Appukuttan, 1992]. Elution of LIg from lactose-Sepharose by lactose and removal of Sepharose-binding antibodies by subsequent treatment with Sepharose alone precluded the presence of anti-Gal in the LIg sample used. Inhibition of LIg-mediated agglutination of desialylated human RBC, which is devoid of TAG moieties, by α- and β-anomers of galactose to the same extent and of AF recognition of LIg by melibiose show that the LacNAc-binding site of the antibody can alternatively be occupied by α-linked galactose moieties. Anti-Gal had been shown to accommodate on its binding site the serine and threonine-rich peptide sequence (STPS) in MUC-1 type peptides irrespective of presence of oligosaccharides as surrogate antigens to α-galactosides [Sandrin et al., 1997]. Recent investigations in our laboratory indicate that like anti-Gal, LIg also recognizes the STPS peptide sequences regardless of the presence LacNAc moieties (unpublished observation). Since xenograft rejection due to α-galactoside recognition

and anti-tumor defence due to STPS reactivity are proposed physiological functions of anti-Gal [Galili, 1993; Sandrin et al., 1997], present results tend to implicate LIg as well in these processes.

High serum level of Lp(a) has been correlated to vascular pathology like stroke, aneurysm, CAD and peripheral vascular disorders [Jones et al., 2007]. While serum contains several fold higher concentration of LDL than Lp(a), atherosclerotic plaques have been reported to contain more apo(a) than apoB [Pepin et al., 1991] with increase in levels of apo(a) during plaque progression [Dijk et al., 2012]. While serum neuraminidase levels are elevated in patients with CAD [Sönmez et al., 1998], studies also reveal that Lp(a) from such patients are 2.5 fold more desialylated than from normal subjects and that desialylated Lp(a) forms aggregates in human aortic intimal cells [Tertov and Orekhov, 1994]. However, response of circulating immunoglobulins to desialylated Lp(a) and mechanism of tissue deposition of the lipoprotein remains unexplored. Present results suggest that LIg may form the first line of antibodies to recognize desialylated Lp(a). Binding of the large Lp(a) particle to one binding site of IgG molecule has been found to leave the other binding site free to bind to smaller epitopes in the case of anti-Gal [Mandagini et al., 2013]. Since present results suggest the possibility that desialylation of vascular surface glycoconjugates could also offer epitopes capable of occupying the unoccupied binding site of Lp(a)-LIg IC, formation of the latter could be a potential route for lipoprotein transport to perivascular compartments. Furthermore, detection of antibodies against α-galactoside groups in atherosclerotic plaques [Mayr et al., 2009] supports the involvement of LIg or anti-Gal in atherosclerosis. Since ICs of large

antigens are more likely to get deposited on vessel walls [Virella, 2007] these observations along with our present results suggest a role for ICs of Lp(a) with LIg in atherosclerosis. Alternatively desialylation and subsequent immune complex formation may be a mechanism of scavenging Lp(a) for degradation by the reticuloendothelial system. Since the biological role of LIg is not well studied the present results open new vistas for investigation on the potential physiological and pathological roles of this antibody.

PART – II

Characterization of Lipoprotein Immune Complexes in Plasma

Introduction

Circulating lipoprotein ICs have attracted much interest in atherosclerosis research ever since their discovery in atheromatous plaques. Several epidemiological studies have found correlation between circulating lipoprotein IC and vascular pathology [Tertov et al., 1990; Lopes-Virella et al., 1999]. Studies so far have focused mainly on LDL IC with oxidation as the central theme and very little is known about the ICs of other lipoproteins. Since Lp(a) has also been correlated with vascular pathology we examined the immunoglobulin type distribution and desialylation status of Lp(a) in IC.

RESULTS

A) Immunoglobulin type distribution in lipoprotein IC

Circulating immune complexes were isolated by polyethylene glycol (PEG) precipitation. Two different concentration of PEG 2% and 3.5% were employed to isolate circulating IC from plasma samples. Conventionally 2% PEG was used to isolte circulating IC in general [Hudson and Hay, 1980b] but recent studies have employed 3.5% PEG to isolate LDL IC [Lopes-Virella et al., 1999] Hence both these concentrations of PEG (2% and 3.5%) were tried to preceipitate IC in order to determine an optimum concentration of PEG for isolation of circulating lipoprotein IC. The lipoprotein IC was assayed by sandwich ELISA with antibodies to apoprotein moieties as the capture antibody, followed by addition of IC and finally using HRP-conjugated anti-human immunoglobulins. Antibodies against three different apolipoprotein moieties were employed to capture lipoprotein IC these

included anti-apo(a) specific to Lp(a) because no other serum lipoprotein contains apo(a) subunit, anti-apoB specific to lipoproteins such as VLDL, LDL and Lp(a) because all these lipoproteins contain apoB subunit and anti-apoA1 specific to HDL as apoA1 is present only in HDL.

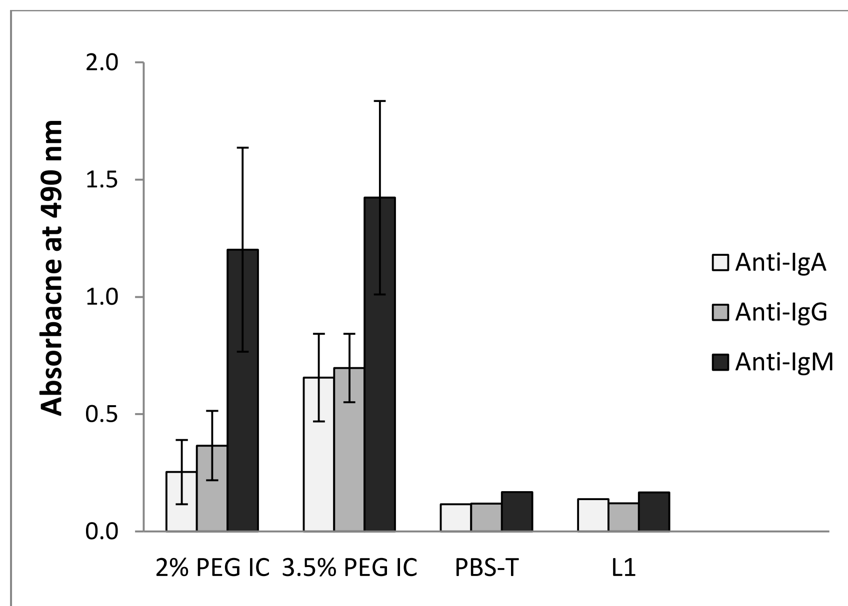


Figure 15. Immunoglobulin distribution in Lp(a) IC. *ICs from 1 ml plasma samples were dissolved in 250 µl PBS. Polystyrene wells were coated with anti-apo(a) (1 µg in 200 µl PBS) blocked and to these wells IC samples (10 times dilution in 200 µl PBS-T) were added. After 2 h incubation at 4°C wells were washed and probed with 200 µl HRP-conjugated anti-human IgA, IgG and IgM separately (1.5 µg antibody per ml in PBS-T). Bound HRP-conjugates were assayed as described (Methods). PBS-T (200 µl) and L1 (Methods, Section 3.2.22; 4 µg in 200 µl PBS-T) were used as controls. Percentage increases in immunoglobulin levels from 2% PEG IC to 3.5% PEG IC were IgA=190%, IgG=90%, IgM=20%. Values are mean ± S.D of 10 samples.*

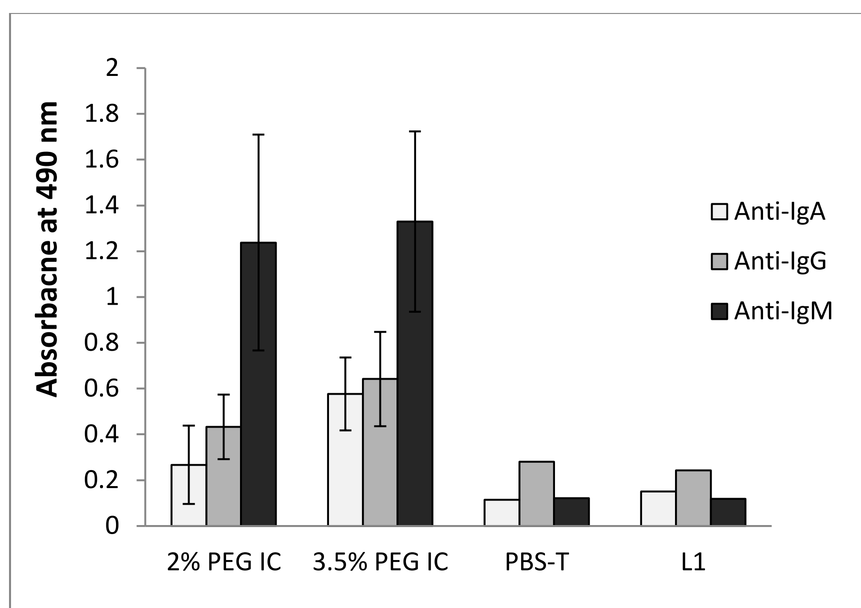


Figure 16. Immunoglobulin distribution in apoB IC. *ICs from 1 ml plasma samples were dissolved in 250 µl PBS. Polystyrene wells were coated with anti-apoB (1 µg in 200 µl PBS) blocked and to these wells IC samples (10 times dilution in 200 µl PBS-T) were added. After 2 h incubation at 4°C wells were washed and probed with 200 µl HRP-conjugated anti-human IgA, IgG and IgM separately (1.5 µg antibody per ml in PBS-T). Bound HRP-conjugates were assayed as described (Methods). PBS-T (200 µl) and L1 (Methods, Section 3.2.22; 4 µg in 200 µl PBS-T) were used as controls. Percentage increases in immunoglobulin levels from 2% PEG IC to 3.5% PEG IC were IgA=116%, IgG=50%, IgM=8%. Values are mean ± S.D of 10 samples.*

Results (Figure.15 and 16) show that both in Lp(a) IC and apoB IC compared to 2% PEG precipitation 3.5% PEG precipitates more IC as evidenced by the percentage increase in HRP-conjugated anti-human IgA, IgG and IgM binding to captured IC. IgM-containing Lp(a) and apoB ICs were precipitated more or less to the same extent both in 2% and 3.5% PEG concentration while majority of IgA and IgG containing Lp(a) and apoB ICs were precipitated largely at 3.5% PEG concentration. Probable explanation for the above observation is that IgM being the largest immunoglobulin

among the above, IgM IC were relatively much larger and could get precipitated even at low PEG concentration. However remarkable increase in IgA-containing IC of Lp(a) and apoB from 2% PEG to 3.5% PEG concentration remains unexplained. By and large IgM is the most dominant immunoglobulin type in both Lp(a) and apoB IC followed by IgG and IgA type antibodies which contribute more or less equally to Lp(a) and apoB IC formation. Recent studies in our laboratory have ascertained that lipoprotein layer obtained in the top 20% layer (L1) following ultracentrifugation of plasma at 535000 g is devoid of antibodies i.e. IC lipoproteins are absent in this layer [Geetha. M et al. communicated]. Hence to determine non-specific response in the present method PBS-T and L1 (lipoprotein layer) obtained from plasma were used as controls. Results (Figure.15 and 16) show that HRP conjugates employed are non-responsive to the coated material and that pure lipoprotein does not contribute to anti-human IgA, IgG or IgM response in the above protocol.

Comparison of immunoglobulin distribution profile between Lp(a) IC and apoB IC isolated by 3.5% PEG precipitation shows that immunoglobulin content of Lp(a) IC was comparable to that of apoB IC (Figure.15 and 16). ApoB IC is contributed by VLDL, LDL and Lp(a) whereas Lp(a) IC is contributed by Lp(a) alone. Present results suggest that although apoB-containing lipoproteins are the most abundant in circulation [usually about 10 times more than Lp(a)] Lp(a) has far greater tendency to associate with antibodies to form IC since antibody response contributed by all apoB-containing ICs in ELISA is little more than that contributed by Lp(a) ICs.

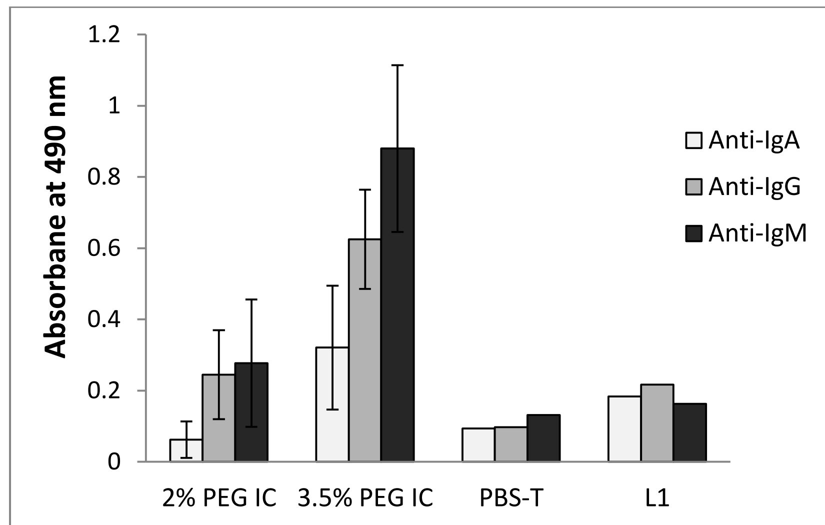


Figure 17. Immunoglobulin distribution in apoA1 IC. *ICs from 1 ml plasma samples were dissolved in 250 µl PBS. Polystyrene wells were coated with anti-apoA1 (1 µg in 200 µl PBS) blocked and to these wells IC samples (10 times dilution in 200 µl PBS-T) were added. After 2 h incubation at 4°C wells were washed and probed with 200 µl HRP-conjugated anti-human IgA, IgG and IgM separately (1.5 µg antibody per ml in PBS-T). Bound HRP-conjugates were assayed as described (Methods). PBS-T (200 µl) and L1 (Methods, Section 3.2.22; 4 µg in 200 µl PBS-T) were used as controls. Percentage increases in immunoglobulin levels from 2% PEG IC to 3.5% PEG IC were IgA=417%, IgG=154%, IgM=218%. Values are mean ± S.D of 10 samples.*

Results in Figure.17 show that besides LDL and Lp(a), HDL also forms IC in circulation. Compared to 2% PEG precipitation HDL IC isolated by 3.5% PEG protocol was remarkably higher as evidenced by the percentage increase in anti-human IgA, IgG and IgM binding. High concentration of PEG (3.5%) required to precipitate most of the HDL-IC is probably due to the smaller size of HDL compared to other plasma lipoproteins. Parallel to Lp(a) IC and apoB IC, HDL IC also contained IgM as the most predominant antibody type but unlike other ICs HDL IC contained IgG as the second most predominant antibody type followed by IgA. Both PBS-T and L1 did not contribute to non-specific binding to HRP conjugates indicating that the protocol is specific for HDL IC.

B) Lp(a) is the most dominant lipoprotein in IC

Although Lp(a) contains both apo(a) and apoB moieties in the ratio 1:1 the anti-apo(a) response to apo(a) subunit of Lp(a) coated on polystyrene plate is higher than of anti-apoB response to apoB subunit. This phenomenon is due to the masking of apoB subunit by apo(a) subunit. Hence anti-apo(a) / anti-apoB response for purified Lp(a) is always >1 and approaches zero for other lipoproteins due to the absence of apo(a) subunit [Kalaivani.V and Appukuttan.P.S, communicated]. It follows that the ratio of response of anti-apo(a) to that of anti-apoB towards a microplate-coated lipid-containing sample would be an index of its Lp(a) content. This principle was used to ascertain the relative Lp(a) content of IC samples.

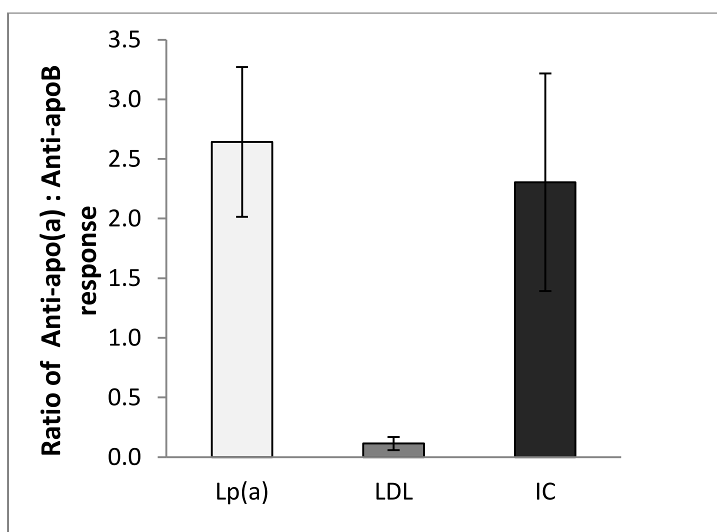


Figure 18. ***Dominance of Lp(a) in IC.*** *Polystyrene wells were coated with Lp(a), LDL and IC devoid of free lipoproteins prepared using 3.5% PEG precipitation from plasma samples from which free lipoproteins were removed in advance by ultracentrifugation at 535000 g (Section 3.2.22; 1 µg in 200 µl PBS) prepared from the same plasma sample, blocked and probed with 200 µl HRP-conjugated anti-apo(a) and anti-apoB for 2 h at 4°C. Bound HRP was assayed as described in methods. Ratio of anti-apo(a) response to anti-apoB response was calculated. Values are mean ± S.D of 8 samples.*

The ratio of anti-apo(a) response to anti-apoB response per unit protein in IC (devoid of free lipoproteins and adduct) was compared with that of purified Lp(a) and LDL from the same sample. Results show that anti-apo(a) / anti-apoB response was 2.6, 2.3 and 0.114 for IC, Lp(a) and LDL respectively (Figure.18). Since anti-apo(a) / anti-apoB response of IC fraction is close to that of Lp(a) and far away from that of LDL, this result suggests that Lp(a) is the most dominant lipoprotein in IC.

C) Lp(a) in IC is desialylated

Circulating IC obtained by PEG precipitation was dissociated using 8 M Urea and liberated lipoproteins were collected by ultracentrifugation in the top 20% layer (protocol described in methods). The efficiency of urea in dissociating lipoprotein IC would reflect in the amount of free lipoprotein released to top layer during ultracentrifugation. The latter was assayed after microplate coating using HRP-conjugated anti-apo(a) and anti-apoB antibodies. Since Lp(a) is the only O-glycosylated plasma lipoprotein PNA response to coated liberated lipoproteins indicated desialylation status of IC Lp(a) since this lectin is specific for desialylated core 1 O-glycans. Results show that 8 M urea treatment did not affect the physical property (density) of lipoproteins as they get sequestered in the top 20% layer following ultracentrifugation. Similarly the integrity of apo(a) subunit was also assayed in terms of anti-apo(a) response. The results show that anti-apo(a) response was more or less equal in both urea treated and untreated samples. However the antigenicity of apoB was marginally reduced after urea treatment (Figure.19). Results thus show that urea treatment of IC releases intact Lp(a).

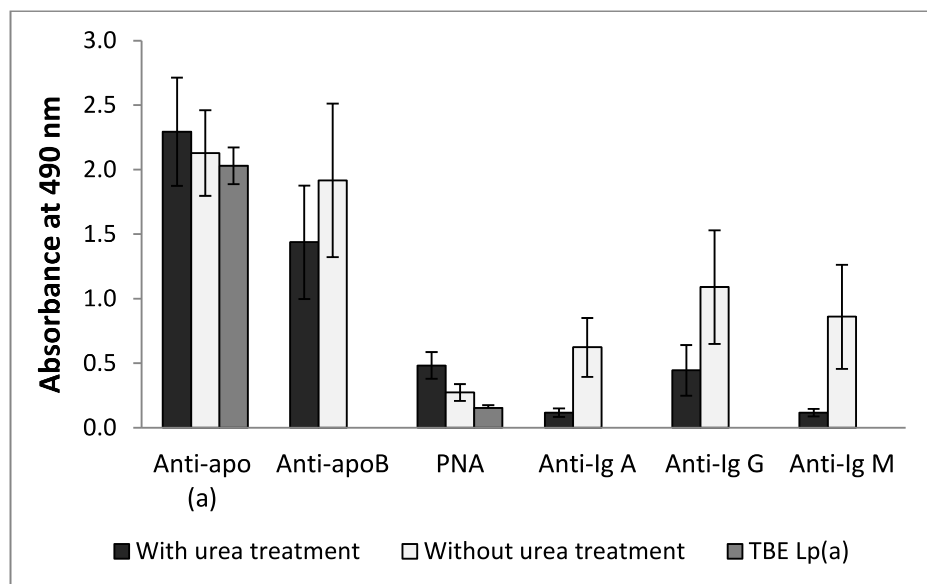


Figure 19. To assay the desialylation status of Lp(a) in IC. *Top 20% layer of 2% IC fraction obtained with or without urea treatment along with TBE-eluted Lp(a) were coated on polystyrene wells (1 µg in 200 µl PBS) blocked and probed with 200 µl HRP-conjugates of anti-apo(a), anti-apoB and anti-Human IgA, IgG and IgM respectively (1.5 µg antibody per ml in PBS-T). To assay desialylation status wells were probed with 200 µl HRP-conjugated PNA (15 µg PNA per ml in PBS-T). After incubation for 2 h at 4°C wells were washed and bound HRP assayed as described earlier. Values are mean ± S.D of 9 IC and 3 Lp(a) samples. P-value for PNA response between urea-treated and untreated samples = 0.000.*

Table 4.

PNA response per unit anti-apo(a) response for IC with or without 8 M Urea treatment and pure Lp(a)

PNA/ anti-apo(a) response ^{x} 100 ± S.D		
Pure Lp(a) (n=3)	IC Lp(a) [with urea treatment] (n=9)	IC Lp(a) [without urea treatment] (n=9)
2.7 ± 0.98	17.9 ± 5.8	8.4 ± 2.1

Top 20% layer after ultracentrifugation of urea treated IC contained lipoproteins without immunoglobulins as detected using anti-immunoglobulin, indicating that treatment with 8 M Urea was effective in dislodging antibodies from lipoproteins. Furthermore, removal of antibodies by urea treatment resulted in concomitant increase in PNA response while TBE purified Lp(a) was non-response to PNA (Figure.19). The PNA binding to IC samples and purified Lp(a) expressed as percentage of anti-apo(a) response shows that PNA response per unit anti-apo(a) response was 2 fold higher in urea treated samples than in untreated samples while the ratio was 6 fold higher in urea treated IC than in TBE Lp(a) (Table 4). These results suggest that Lp(a) in IC is desialylated and that a fraction of antibodies involved in IC formation with Lp(a) is contributed by anti-carbohydrate antibodies that recognizes desialylated glycans in Lp(a).

DISCUSSION

So far only IgG-Lp(a) IC has been detected in circulation [Wang et al., 2003]. Their approach was to use anti-human IgG as the capture antibody and track Lp(a) IC using anti-apo(a) antibody. This approach may not be accurate because other IgG-containing IC can compete for binding to the coated material. Furthermore this protocol cannot delineate the immunoglobulin type distribution in Lp(a). The present protocol employs anti-apo(a) as the capture antibody thereby making it specific for Lp(a) and at the same time enabling the detection of antibodies in Lp(a) IC. In serum IgG forms the most dominant immunoglobulin type followed by IgA and IgM [Kindt et al., 2007b]. Present results show that in Lp(a) IC IgM is the most dominant type of

followed by IgG and IgA, probably because antibodies involved in the formation of IC belong to the natural antibody repertoire in which IgM is the most dominant immunoglobulin [Binder et al., 2005]. Several groups have reported that IgM lipoprotein IC are efficiently removed by complement factors and are atheroprotective in nature whereas IgG- and IgA-containing lipoprotein IC are sequestered less fast by complement factors and may get deposited on the tissue surfaces [Burut et al., 2010]. Atheromatous plaques have been shown to contain more apo(a) than apoB [Pepin et al., 1991]. Furthermore apo(a) levels are reported to increase with plaque progression [Dijk et al., 2012]. While mechanism of tissue deposition of Lp(a) remains unknown the relative abundance of Lp(a) in IC points out that formation of Lp(a) IC may be a probable route by which Lp(a) gets deposited on to vessel wall.

Tertov et al. demonstrated the presence of desialylated LDL in IC and found that desialylated versions of LDL were efficient ligands for auto-antibodies against LDL [Tertov et al., 1990; Kacharava et al., 1993]. Several groups have correlated high serum sialic acid and neuraminidase levels with vascular pathology [Lindberg et al., 1992; Nanetti et al., 2008]. Furthermore *in vitro* experiments have demonstrated that desialylated and native Lp(a) and LDL have been shown to aggregate in aortic intimal cells [Orekhov et al., 1991; Tertov and Orekhov, 1994]. Lp(a) being a profusely O-glycosylated lipoprotein desialylation can lead to formation of strongly bonded IC with anti-carbohydrate antibodies. Since the mechanism of degradation of Lp(a) is unexplained, desialylation and subsequent IC formation is a possible mechanism by which Lp(a) may be removed from circulation. Present results suggest

that in conditions like diabetes and CVD where serum sialidase levels are elevated Lp(a) IC formation may be more pronounced.

Studies so far have not reported the presence of HDL IC in circulation though presence of auto-antibodies of IgG type against apoA1 moieties of HDL has been reported in both man and mouse [Montecucco et al., 2011]. Moreover these antibodies are also reported to be predictors of cardiovascular events [Vuilleumier et al., 2010; Vuilleumier et al., 2013]. While LDL and Lp(a) ICs have been implicated in CVD, besides these lipoproteins HDL has also been detected in atherosclerotic plaques [Huang et al., 2014]. The present study suggests that along with LDL and Lp(a), HDL also forms circulating IC. Characterization of HDL IC, its role in CVD and mechanism of tissue deposition await investigation.

PART – III

Immune complex formed between desialylated Lp(a) and anti-carbohydrate antibodies retain sugar binding sites that enable attachment of the complex to desialylated host cells

Introduction

Although high serum levels of Lp(a) and its IC have been correlated with vascular pathology its mechanism of tissue deposition still remains an enigma. Increase in serum neuraminidase and sialic acid levels in patients with CVD and stroke suggest a role of desialylation in mediating vascular pathology [Lindberg et al., 1992; Nanetti et al., 2008]. Recent studies in our laboratory reveal that anti-Gal can accommodate Lp(a) in one binding site while the other binding site can anchor on affinity matrix [Mandagini et al., 2013]. Since desialylated Lp(a) is a ligand for anti-carbohydrate antibodies such as LIg [Results and Discussion in part I] and anti-T [Anuradha Ph.D Thesis] IC between desialylated Lp(a) and anti-carbohydrate antibodies were analysed for possible presence of balance binding sites on IC antibodies that facilitate their attachment to cells bearing appropriate ligands.

RESULTS

A) De novo lipoprotein IC that agglutinate desialylated O group RBCs are formed by desialylated lipoproteins, but not by their native versions.

De novo IC was prepared by incubating desialylated lipoproteins with plasma as the antibody source. IC thus formed was isolated by 2% PEG precipitation because free immunoglobulins do not precipitate at this concentration [Hudson and Hay, 1980b]. The ability de novo IC to agglutinate desialylated human O group RBC was taken as a measure of unoccupied binding sites on antibody molecules involved in IC formation.

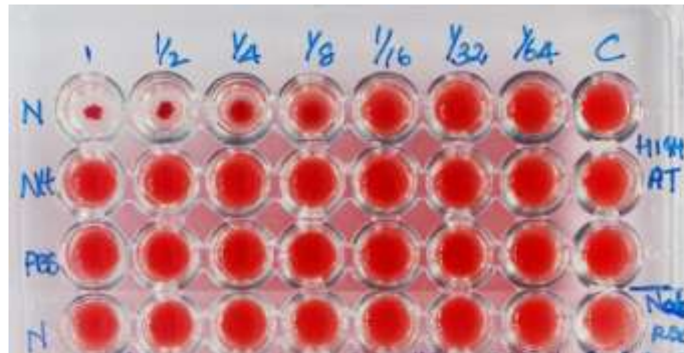


Figure 20. Haemagglutination assay of de novo IC.
Desialylated lipoproteins (N-L1) or non-desialylated lipoproteins (NH-L1) (Methods section 3.2.31) 20 µl (10 mg per ml PBS) was incubated with 1 ml plasma overnight. De novo IC formed was isolated by 2% PEG precipitation, dissolved in 250 µl PBS and serial dilutions checked for agglutination (Section 3.2.32b).
Row 1-3 from top: desialylated RBC with N-L1vs plasma IC (N), NH-L1 vs plasma IC (NH) and natural IC in serum (PBS). Row 4: Native RBC with N-L1 vs serum IC (N)

The results show that de novo IC formed between desialylated lipoproteins (N-L1) and plasma anti-carbohydrate antibodies were able to agglutinate desialylated RBC up to 1:8 dilution whereas native RBC (non-desialylated) was not agglutinated by this complex. Likewise IC obtained by addition of non-desialylated lipoproteins (NH-L1) or of PBS only (native IC) did not agglutinate desialylated RBC (Figure.20). These results suggest that IC formed between desialylated lipoproteins and anti-carbohydrate antibodies retain free binding sites of the antibodies so that the IC is capable of recognizing desialylated glycans on RBC to cause agglutination. IC formation and agglutination were desialylation-dependent because both desialylated RBC and desialylated lipoprotein were necessary for agglutination. The inability of native IC to agglutinate desialylated RBC confirms that antibodies in native IC are not involved in agglutination and that free plasma antibodies that are capable of

agglutinating desialylated RBC do not precipitate with 2% PEG used to prepare IC. On the other hand it is also possible that naturally occurring ICs involving desialylated lipoproteins are too few to casue agglutination. Out of 7 random plasma samples that were used as an antibody source to prepare de novo IC by adding the same amount of desialylated lipoproteins, only 4 samples (60%) aggutinated beyond 1:4 titre (Figure.21). The variation in agglutination titre may be attributed to the difference in anti-carbohydrate antibody titre in plasma samples used and was verified in later experiments.

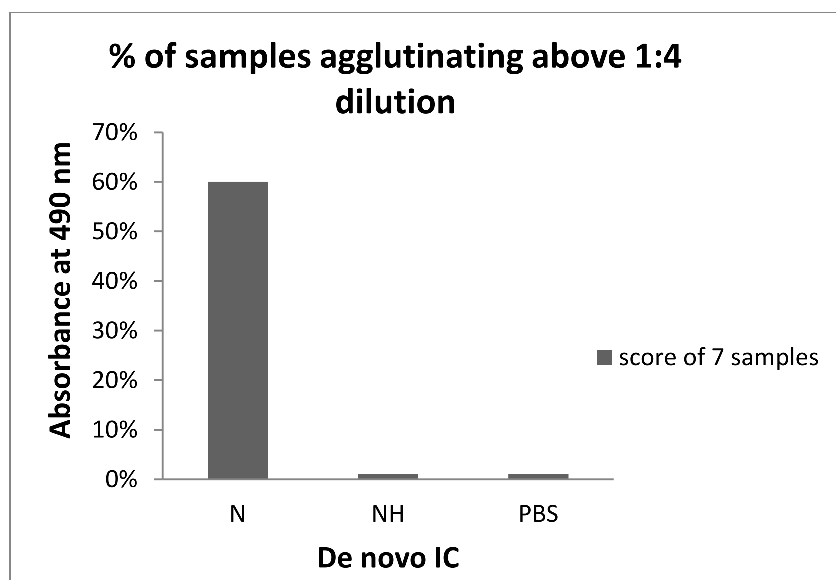
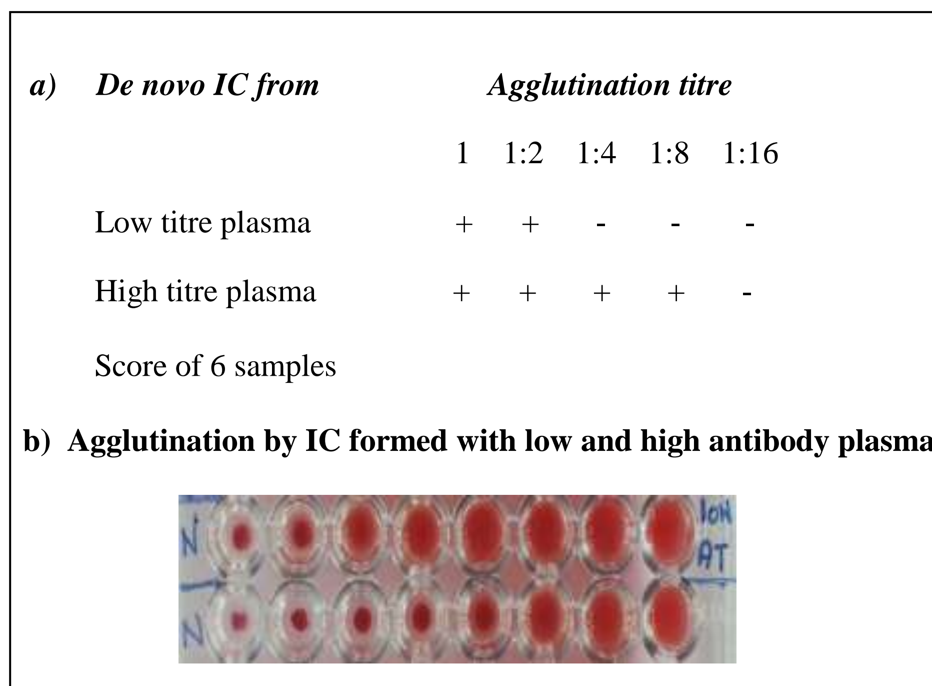


Figure 21. Agglutination by de novo IC from different plasma samples. *De novo IC was prepared by incubating same quantity of desialylated lipoproteins with plasma samples selected at random (described in section 3.2.32b). Number of samples that agglutinate above 1:4 titre is expressed as percentage.*

B) Antibodies specific to desialylated N- and O-glycans are involved in IC formation.

Since anti-carbohydrate antibodies occur naturally, their serum concentrations vary amongst individuals depending on the intestinal microbial flora, diet and pathogen exposure [Springer and Horton, 1969; Bos et al., 1989]. Titre of plasma anti-glycan antibodies specific to desialylated glycoconjugates was assayed by polyagglutination. Polyagglutination is a phenomenon in which serum of normal individuals agglutinate human RBC irrespective of their blood group due to the exposure of cryptic antigens by bacterial enzymes. Polyagglutination is brought about by recognition of altered erythrocyte membrane antigens by agglutinins normally present in human sera [Schenkel-Brunner, 1995]. Plasma samples were screened for polyagglutinins against desialylated glycoconjugates by haemagglutination assay using desialylated RBC (as described in methods). The plasma samples that agglutinate only up to 1:2 were categorised as low antibody titre plasma and those that agglutinate up to 1:8 or above were categorised as high antibody titre plasma samples. De novo IC was formed by adding same quantity of lipoprotein (as N-L1) to low and high antibody titre plasma. The effect of antibody concentration in formation of agglutinating IC was assayed by haemagglutination of desialylated RBC. Agglutinability of de novo IC was proportional to the polyagglutinin titre in plasma (Figure.22). This result provides evidence that the antibody titre in plasma dictates the extent of formation of IC which in turn influences agglutanibility. This result also explains the previous observation that agglutination of IC varied among plasma samples (Figure.21).



a) *De novo IC from*	*Agglutination titre*				
	1	1:2	1:4	1:8	1:16
Low titre plasma	+	+	-	-	-
High titre plasma	+	+	+	+	-
Score of 6 samples					

b) Agglutination by IC formed with low and high antibody plasma

Figure 22. Effect of polyagglutinin titre in formation of agglutinating IC. *a) Agglutination scores of de novo IC formed with plasma containing varying antibody titre and same lipoprotein concentration protocol described in 'Methods section 3.2.32'. b) Image of agglutinating de novo IC formed with plasma samples of varying antibody titre.*

Furthermore, nature of anti-carbohydrate antibodies involved in de novo IC formation was determined by haemagglutination inhibition using monosaccharide or disaccharide sugars and desialylated glycoconjugate (asialomucin) as the inhibitors. Lactose was the most efficient inhibitor with Minimum Inhibitory Concentration (MIC) of 26 mM, α-anomers of galactose viz. melibiose (Gal α1-6 Glc) and 1-O-methyl β-D-galactopyranoside were able to inhibit equally at 58 mM while 1-O-methyl α-D-galactopyranoside was inhibitory at 83 mM whereas 1-O-methyl derivatives of glucopyranosides did not inhibit agglutination even at 100 mM concentration. Glycoconjugate asialomucin obtained by acid hydrolysis of mucin from porcine stomach is rich in TAG groups as well as T-antigens. Asialomucin was

inhibitory at a concentration of 14 μg (Table.5). Since LIg has dual specificity towards both anomers of galactose, inhibition of agglutination by either anomers of galactose suggest involvement of LIg in de novo IC. Monosaccharide 1-O-methyl-β-D-galactopyranoside and T-antigen (Gal β1-3 GalNAc) are efficient ligands for anti-T [Balu. K. Chacko Ph.D Thesis] and their ability to inhibit agglutination of de novo IC indicates the involvement of anti-T antibodies as well in IC formation. Results from haemagglutination inhibition experiments confirm the involvement of LIg and anti-T antibodies in the formation of agglutinating de novo IC.

Table 5.

Agglutination inhibition of de novo IC to demonstrate the involvement of anti-carbohydrate antibodies.

	Minimum inhibitory concentration (MIC; mM) Mean ± SD of 3 samples
1) 1-O-Methyl α-D-galactopyranoside	83 ± 28
2) 1-O-Methyl β-D-galactopyranoside	58 ± 38
3 1-O-Methyl α-D-glucopyranoside	>100
4) 1-O-Methyl β-D-glucopyranoside	>100
5) Melibiose	58 ± 38
6) Lactose	23 ± 19
7) Asialomucin	14 ± 8 μg/well

C) Lp(a) is the major antigen in agglutinating IC.

Lp(a) is the most profusely glycosylated lipoprotein in plasma and contains both N- and O-glycans [Scanu and Edelstein, 1997]. Results in this thesis and earlier work from this laboratory [Anuradha, PhD thesis] have shown Lp(a) to be a ligand for both LIg and anti-T antibodies whereas other lipoproteins which contain much fewer and widely spaced N-glycans are not recognized by these antibodies. To demonstrate the role of Lp(a) in forming agglutinating de novo IC, Lp(a) was selectively removed by incubating N-L1 with jacalin-Sepharose. Jacalin is a lectin specific for core 1 O-glycans so that only Lp(a) binds to jacalin-Sepharose while all other lipoproteins remain unbound in the supernatant. N-L1 treated with Sepharose was taken as positive control. The efficiency of jacalin-Sepharose to remove Lp(a) from the lipoprotein mixture was verified using jacalin based enzyme immunoassay [Sreekumar et al., 2013]. Unlike Sepharose, jacalin-Sepharose removed most of the Lp(a) from N-L1 (Figure.23). Lp(a) content in Sepharose treated sample was about 1.2 mg per ml and 60 µg per ml in jacalin-Sepharose-treated sample.

De novo IC was prepared by the addition of jacalin-Sepharose treated or Sepharose treated N-L1 to plasma with high anti-carbohydrate antibody titre. IC thus formed was assayed by haemagglutination. There was 4 to 8 fold reduction in agglutination of IC when Lp(a) was selectively removed (Figure.24) indicating the cardinal contribution of Lp(a) among plasma lipoproteins towards IC formation with plasma antibodies.

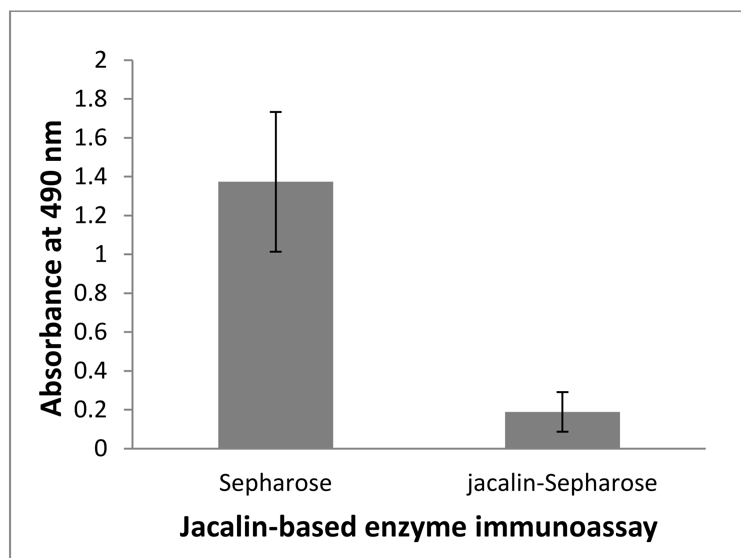


Figure 23. Extent of Lp(a) removal from N-L1. *Supernatant of jacalin-Sepharose- or Sepharose-treated N-L1 (Method described in Section 3.2.34) diluted 1000 times were used for jacalin-based enzyme immunoassay (Methods described in Section 3.2.18). Values are mean ± S.D of 6 samples.*

Sample	***Agglutination titre***				
	1	1:2	1:4	1:8	1:16
Jacalin-Sepharose treated N-L1	+	+/-	-	-	-
Sepharose treated N-L1	+	+	+	+	-
Mean score of 6 samples					

Figure 24. Contribution of Lp(a) in agglutinating IC. *Haemagglination assay of de novo IC formed between jacalin-Sepharose-treated N-L1 and Sepharose-treated N-L1.*

D) Effect of Lp(a) isoform size on formation of agglutinating IC.

The extent of glycosylation in Lp(a) varies widely among plasma samples due to variation in kringle 4 type II repeats. The number of N-glycans can range between 13 – 49 while the number of O-glycans can range between 80 – 240 [Scanu and Edelstein, 1997] depending on apo(a) size which decides Lp(a) molecular weight. Since IC formation is glycosylation specific, the molecular size of Lp(a) (number of N- and O-glycans) may have profound effect on the formation of agglutinating IC. The effect of molecular size of Lp(a) on the formation of agglutinating IC was studied.

Since serum Lp(a) levels and molecular size of Lp(a) molecules are inversely related [Utermann et al., 1987], plasma samples were assayed for their Lp(a) levels by jacalin-based enzyme immunoassay [Sreekumar et al., 2013]. Plasma samples containing mean Lp(a) concentration of 16.6 $\pm$ 4.2 mg/dL were taken as high molecular weight Lp(a) group and those with mean concentration of 51.5 $\pm$ 7.5 mg/dL as low molecular weight Lp(a) (Figure.25). De novo IC was prepared using N-L1 containing the same amount of Lp(a) from plasma samples of different Lp(a) size and the same plasma as immunoglobulin source. Agglutination assay of IC revealed that low molecular weight Lp(a) produced IC which were 2 fold more agglutinating than IC of high molecular weight Lp(a) (Figure.26)

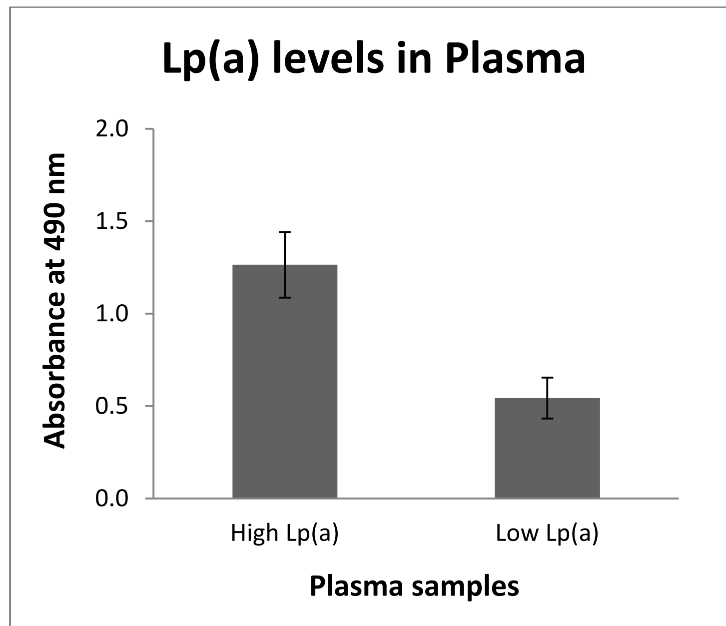


Figure 25. Jacalin-based enzyme immunoassay to determine Lp(a) levels in plasma. *Plasma samples were diluted 500 times, added to jacalin coated wells and probed with HRP-conjugated anti-apo(a) as described in section 3.2.18. Values are Mean ± S.D of 5 samples.*

De novo IC from	*Agglutination titre*[b]				
	1	1:2	1:4	1:8	1:16
High MW[a] Lp(a)	+	+	-	-	-
Low MW[a] Lp(a)	+	+	+	-	-

Figure 26. Effect of Lp(a) size on agglutination by lipoprotein IC. *Agglutination assay of de novo IC prepared with desialylated lipoprotein (N-L1) of differing Lp(a) size and the same plasma used as antibody source. a: molecular weight; b: mean of 5 samples from each group. L(a) from plasma samples with high or low Lp(a) titre (Figure.25) was taken as low or high MW Lp(a) respectively.*

DISCUSSION

Several studies have correlated high plasma levels of Lp(a) and sialidase to vascular pathology. However the mechanism of this pathology still remains obscure. The present study demonstrates that desialylation of Lp(a) results in exposure of underlying cryptic epitopes such as LacNAc and T-antigens which form IC with plasma antibodies such as LIg and anti-T respectively. More significantly de novo IC formed between desialylated Lp(a) and plasma anti-glycan antibodies can bind to desialylated glycoconjugates on surfaces of host cells such as RBC. RBC was used as a prototype of host cell as it could be easily obtained, desialylated and IC binding demonstrated in terms of agglutination. ICs formed between several multivalent antigen molecules on one hand and multivalent antibody molecules on the other retain some of the binding sites on the antibody unoccupied due to steric hindrance [Kindt et al., 2007b]. These unoccupied binding sites can recognize the same ligand presented in different spatial configuration and this may possibly explain the observed haemagglutination by de novo IC. The same phenomenon is also observed in anti-Gal-Lp(a) IC that exist in all human plasma samples [Mandagini et al., 2013]. Although desialylated Lp(a) could be detected in native plasma IC [Results and discussion Part II] its concentration is nominal so that contribution to host-reactivity is negligible. However the above phenomenon may be operative in disease conditions in which desialylation is rampant as exemplified by diabetes and CVD [Roozbeh et al., 2011; Sönmez et al., 1998]. Inhibition of agglutination by lactose and asialomucin confirms the involvement LIg and anti-T antibodies in IC formed by desialylated Lp(a). These antibodies constitute nearly more than 1 mg/dL in plasma [Dong et al., 1997; Butschak and Karsten, 2002] and form the first line of defence

against desialylated epitopes. Anti-glycan antibodies belong to natural antibodies and their plasma levels vary among individuals depending on antigen exposure. The present study shows that agglutinability of de novo IC is proportional to the polyagglutinin titre (LIg and anti-T) of the individual plasma. Individuals with high serum polyagglutinin may be at disadvantage because they may have greater tendency for IC formation which in turn can lodge on desialylated tissue surface. Lp(a) though a minor fraction among plasma lipoproteins forms the most agglutinating IC and this property is attributed to the enormous glycan density in Lp(a). The glycan content in Lp(a) constitutes up to 28% by mass of apo(a) while apoB moieties contains 4-10% glycans. This suggests that Lp(a) may be the most profusely glycosylated lipoproteins in plasma. Although LDL is the most abundant lipoprotein its low glycan content and absence of O-glycans may explain its poor contribution to desialylation-dependent agglutinating IC. Generally high serum Lp(a) (low molecular weight) has been correlated with vascular pathology and results from the present study offer another reason for the same since low molecular weight Lp(a) IC is capable of agglutinating desialylated RBC to a greater extent than high molecular weight Lp(a) IC. This is probably because high molecular weight Lp(a) has greater tendency to associate with LDL to form Lp(a)-LDL adducts [Kalaivani.V and Appukuttan P.S, communicated]. The attachment of LDL to Lp(a) is mediated by non-covalent interaction between lysine groups in apoB and lysine binding sites in apo(a) moieties [Yashiro et al., 1993]. The negatively charged lysine binding sites provided by apo(a) are sialic acid group present on its glycan moieties. In support of this assumption formation of Lp(a)-LDL adduct masks oligosaccharide moieties of apo(a) making them unavailable for recognition by antibodies. Hence

high molecular weight Lp(a) might have very little glycans to offer thereby resulting in formation of IC with low agglutinability. Lp(a) is the most dominant lipoprotein in atheromatous plaques [Pepin et al., 1991] though its plasma concentration is below that of LDL and HDL. But mechanism of tissue deposition of Lp(a) remains unknown. Furthermore Lp(a) isolated from patients of CVD was found to be more desialylated than Lp(a) of normal individuals [Tertov and Orekhov, 1994]. Notably studies on animal models have demonstrated that lesion prone areas of the arterial wall are desialylated [Görög and Born, 1983]. Such regions could act as anchoring points for lipoprotein ICs containing balance binding sites for desialylated glycans. IC formation between desialylated Lp(a) and serum anti-carbohydrate antibodies may be a mechanism of sequestering Lp(a). Notably rampant desialylation of tissue surfaces like vascular endothelium as well as of lipoproteins can occur during microbial infections, diabetes or CVD that are accompanied by elevated levels of microbial or endogenous sialidase leading to formation of IC capable of anchoring on tissue surfaces. Moreover persistent neuraminidase activity may desialylate vessel wall glycans rendering them susceptible to lipoprotein IC binding. Taken together these results suggest that factors such as Lp(a) concentration, size, desialylation and anti-carbohydrate antibody levels contribute towards formation of tissue binding IC which could be a vehicle for lipoprotein deposition on vascular surface leading to atherosclerosis.

SUMMARY AND CONCLUSION

Studies over the years have identified multiple risk factors (reversible and non-reversible) that may predispose individuals to atherosclerosis. Nevertheless no single factor has been identified as the exclusive cause of the disease. Recent studies have shown that one-third of patients with CVD do not exhibit conventional risk factors of atherogenesis and that mere reduction of serum cholesterol or supplementation with anti-oxidants was insufficient to prevent or contain the occurrence cardiovascular events or vascular pathology. Plasma Lp(a) concentration and phenotype have been shown to be independent risk factors for several vascular disorders including atheroscleoris, stroke, aneurysm and Alzheimer's disease. Plasma Lp(a) is found only in man and other primates and is notable for very high frequency of glycosylation with N- and O-glycans unlike any other lipoprotein. On the other hand human serum is rich in naturally occurring antibodies that recognize desialylated glycan structures of autologous glycoconjugates. Hence it is imperative to investigate the recognition of lipoproteins by natural anti-carbohydrate antibodies to form ICs which hold potential for blood vessel wall deposition. The latter incident can lead to plaque formation and atheroscleorsis. Information on molecular mechanisms of the above recognition may lead to better therapeutic strategies. The present study aims at understanding the role of serum anti-carbohydrate antibodies in the formation of Lp(a) IC following desialylation as well as elucidating a possible mechanism of tissue deposition of these ICs.

Naturally occurring anti-carbohydrate antibodies form a repertoire of innate immune system that performs diverse function like tumor surveillance, anti-blood group reaction, graft rejection etc. LIg is a lactose-inhibitable anti-glycan antibody whose

biological role still remains unexplored. Hence ligand binding properties and potential biological role of LIg was studied. In contrast to previous studies wherein only IgG type LIg molecules had been reported, the present result suggest that immunoglobulins of all three types are present in LIg with IgG being the most dominant where as IgA and IgM contribute one-fourth each to total LIg content. Given that LIg is isolated on lactose-Sepharose affinity matrix LacNAc groups were considered as ligands for LIg but experimental proof for this conclusion is lacking. Results show that selective removal of O-glycans on asialofetuin using O-glycanase did not affect LIg binding while removal of LacNAc groups on asialofetuin using α-mannosidase reduced LIg binding, confirming the LacNAc specificity of LIg. Results from 3 independent experiments point out that in addition to lactose and LacNAc moieties, LIg specificity extends towards terminal alpha-galactosides 1. Agglutination of desialylated human O-group RBC was inhibited equally by 1-O-methly α-galactoside and 1-O-methyl β-galactoside at 50 mM concentration. TAG specificity was further confirmed by the ability of LIg to agglutinate unmodified sheep RBC which contains TAG moieties. 2. Binding of LIg to microplate coated glycoconjugates show that affinity of LIg towards α-galactoside groups is greater than towards its β-anomer when ligands are presented in glycoconjugate form than in solution. However lactose and melibiose were equally inhibitory in LIg binding to bovine thyroglobulin and asialofetuin. 3. Removal of terminal alpha-galactoside group from bovine thyroglobulin and rabbit RBC membrane glycoprotein by α-galactosidase substantially reduced LIg binding showing that the residual LacNAc groups were much less efficient ligands than

α-galactoside moieties. These results suggest that TAG groups are preferred to β-galactosides as ligands for LIg.

Lp(a) being a profusely glycosylated lipoprotein containing both N- and O-glycans desialylated N-glycans terminating in LacNAc moieties on Lp(a) could be candidate ligands for LIg. Results show that LIg recognized Lp(a) only after desialylation of the latter and binding was specific to LacNAc groups on Lp(a) since truncation of LacNAc moieties by α-mannosidase decreased LIg binding while desialylated plate coated LDL was not recognized by LIg. Further, incorporation of fluorescence-tagged LIg into IC fraction was dependent on desialylation of plasma and increased with plasma Lp(a) concentration, while FITC-labeled non specific antibody was not incorporated into IC fraction upon desialylation. These results suggest that LIg can form circulating IC with desialylated Lp(a) and that apart from oxidation desialylation can also be a predisposing factor for IC formation. Mechanism of degradation of Lp(a) remains unknown. Present results suggest that desialylation and subsequent IC formation may be a possible route for Lp(a) sequestration in the system. Although anti-carbohydrate antibodies reactive towards alpha-galactoside moieties have been detected in atheromatous plaques, to date except anti-Gal no other anti-carbohydrate antibody has been demonstrated to form IC with circulating plasma lipoproteins. Present finding suggests the involvement of LIg in the formation of Lp(a) IC which may get deposited on the arterial wall.

Wang et al. in 2003 first reported the presence of Lp(a)-IgG IC in normal controls and in higher amounts in patients with CHD but the composition of antibodies in Lp(a) IC and its relative distribution in IC fraction is not well documented. Hence 2 different concentrations of PEG was tried to isolate lipoprotein IC from plasma and their immunoglobulin composition was assayed by sandwich ELISA. Results show that compared to 2% PEG concentration 3.5% PEG precipitated more Lp(a) and apoB IC, while HDL ICs were precipitated mostly at 3.5% PEG. Antibodies of IgA, IgG and IgM type were involved in formation of IC with apo(a)- and apoB-containing lipoproteins, IgM was the most dominant immunoglobulin type whereas IgG and IgA were more or less equal. On the other hand in HDL IC IgM was the most dominant immunoglobulin type followed by IgG while IgA was a minor constituent. Unlike in serum, dominance of IgM over other immunoglobulin types in lipoprotein IC suggests the presence of natural antibodies in IC formation. Though serum concentration of LDL is several times greater than that of Lp(a), two independent experiments suggest that Lp(a) has greater tendency to form IC: a) apo(a)-ICs outnumber apoB-ICs suggesting that Lp(a) has greater tendency to associate with antibodies than LDL; b) Ratio of response towards anti-apo(a) to that towards anti-apoB in IC prepared form plasma devoid of free lipoproteins was close to the corresponding ratio for purified Lp(a) indicating that Lp(a) is the predominant lipoprotein in plasma IC. In addition to the dominance of Lp(a) in IC, IC Lp(a) was found to be 6 fold more desialylated than non-IC Lp(a). With Lp(a) far exceeding LDL in atherosclerotic plaque and Lp(a) IC being the most dominant in human plasma the present result suggests that Lp(a) IC may be a possible vehicle for lipid deposition on to the arterial walls. However desialylation status of Lp(a) in

atherosclerotic plaques and role of HDL IC in atherosclerosis await further investigations.

A novel mechanism of tissue deposition of desialylated Lp(a) IC is proposed based on the observation that de novo IC formed between desialylated lipoproteins and plasma anti-carbohydrate antibodies can agglutinate desialylated human O-group RBC and not unmodified RBC. This result suggests that de novo IC formed between desialylated lipoproteins and plasma anti-carbohydrate antibodies were able to retain unoccupied binding sites in the antibody capable of binding to desialylated host cells. Agglutination was inhibitable with β-anomers of galactose viz. lactose, 1-O-methyl β-galactoside and asialomucin demonstrating the involvement LIg and anti-T in de novo IC. Here again Lp(a) formed the majority of agglutinating IC since removal of Lp(a) from the lipoprotein mixture reduced agglutination due to the resulting IC by 4 to 8 fold, which again confirms that Lp(a) has greater tendency to form IC upon desialylation. The molecular size of Lp(a) in the lipid layer used was also found to have immediate effect on the formation of agglutinating de novo IC. Given the same number of Lp(a) molecules de novo IC formed using lipid layers containing low molecular weight Lp(a) agglutinated 2 fold higher than high molecular weight Lp(a) apparently due to the glycan-rich kringle 4 type 2 repeat regions of larger Lp(a) molecules in lipid layer being more occupied by LDL in adduct formation.

Taken together these results suggest that IC formed between multivalent antigen like desialylated Lp(a) and anti-carbohydrate antibodies retain some of the biding sites on antibodies unoccupied which can in turn bind to appropriate ligands on cell surfaces. With alpha-galactoside-specific antibodies and apo(a) having been detected in

atheromatous plaques and serum of patients with diabetes and CVD reported to contain elevated serum sialidase and sialic acid levels this study suggests that desialylation and subsequent IC formation may be a mechanism by which Lp(a) can mediate vascular pathology.

FUTURE DIRECTION

1) Translate the present findings to clinical research.

   a) To characterize Lp(a)-IC in patients with CVD/diabetes mellitus

   b) Assay desialylation status of Lp(a) in diabetes mellitus

2) To check if LIg recognizes MUC-1 peptides.

3) Does balance binding site on LIg of LIg IC enable its adhesion to desialylated endothelial cell surface glycoconjugates?

BIBLIOGRAPHY

Adderson, E.E., Shikhman, A.R., Ward, K.E., Cunningham, M.W., 1998. Molecular Analysis of Polyreactive Monoclonal Antibodies from Rheumatic Carditis: Human Anti-N-Acetylglucosamine/Anti-Myosin Antibody V Region Genes. J. Immunol. 161, 2020–2031.

Ahmed, M.S., Jadhav, A.B., Hassan, A., Meng, Q.H., 2012. Acute Phase Reactants as Novel Predictors of Cardiovascular Disease. ISRN Inflamm. 2012, 1–18.

Ait-Oufella, H., Taleb, S., Mallat, Z., Tedgui, A., 2011. Recent Advances on the Role of Cytokines in Atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 31, 969–979.

André, S., Ortega, P.J., Perez, M.A., Roy, R., Gabius, H.J., 1999. Lactose-containing starburst dendrimers: influence of dendrimer generation and binding-site orientation of receptors (plant/animal lectins and immunoglobulins) on binding properties. Glycobiology 9, 1253–1261.

Anglés-Cano, E., de la Peña Díaz, A., Loyau, S., 2001. Inhibition of fibrinolysis by lipoprotein(a). Ann. N. Y. Acad. Sci. 936, 261–275.

Anuradha. (2007) Microbial enzyme-mediated changes in glycosylated biomolecules leading to their recognition by sugar-specific serum and tissue proteins. Ph.D Thesis. Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India; p.106-117.

Appukuttan, P.S., Surolia, A., Bachawat, B.K., 1977. Isolation of two galactose-binding proteins from Ricinus communis by affinity chromatography. Indian J. Biochem. Biophys. 14, 382–384.

Armstrong, E.J., Morrow, D.A., Sabatine, M.S., 2006. Inflammatory Biomarkers in Acute Coronary Syndromes Part II: Acute-Phase Reactants and Biomarkers of Endothelial Cell Activation. Circulation 113, e152–e155.

Avila, J.L., Rojas, M., Galili, U., 1989. Immunogenic Gal alpha 1-3Gal carbohydrate epitopes are present on pathogenic American Trypanosoma and Leishmania. J. Immunol. Baltim. Md 1950 142, 2828–2834.

Avrameas, S., 1991. Natural autoantibodies: from "horror autotoxicus" to "gnothi seauton". Immunol. Today 12, 154–159.

Balu. K. Chacko. (2001) Interactions of carbohydrate binding human immunoglobulins with glycoconjugates bearing corresponding epitopes. Ph.D Thesis. Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India; p.67.

Baues, R.J., Gray, G.R., 1977. Lectin purification on affinity columns containing reductively aminated disaccharides. J. Biol. Chem. 252, 57–60.

Beaumont, J.L., Doucet, F., Vivier, P., Antonucci, M., 1988. Immunoglobulin-bound lipoproteins (Ig-Lp) as markers of familial hypercholesterolemia, xanthomatosis and atherosclerosis. Atherosclerosis 74, 191–201.

Bergmark, C., Wu, R., Faire, U. de, Lefvert, A.K., Swedenborg, J., 1995. Patients With Early-Onset Peripheral Vascular Disease Have Increased Levels of Autoantibodies Against Oxidized LDL. Arterioscler. Thromb. Vasc. Biol. 15, 441–445.

Berliner, J.A., Territo, M.C., Sevanian, A., et al. 1990. Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. J. Clin. Invest. 85, 1260–1266.

Bhavanandan, V.P., 1991. Cancer-associated mucins and mucin-type glycoproteins. Glycobiology 1, 493–503.

Binder, C.J., Shaw, P.X., Chang, M.-K., et al. 2005. Thematic review series: The Immune System and Atherogenesis. The role of natural antibodies in atherogenesis. J. Lipid Res. 46, 1353–1363.

Boerwinkle, E., Leffert, C.C., Lin, J., et al. 1992. Apolipoprotein(a) gene accounts for greater than 90% of the variation in plasma lipoprotein(a) concentrations. J. Clin. Invest. 90, 52–60.

Bos, N.A., Kimura, H., Meeuwsen, C.G., et al. 1989. Serum immunoglobulin levels and naturally occurring antibodies against carbohydrate antigens in germ-free BALB/c mice fed chemically defined ultrafiltered diet. Eur. J. Immunol. 19, 2335–2339.

Boullier, A., Hamon, M., Walters-Laporte, E., et al. 1995. Detection of autoantibodies against oxidized low-density lipoproteins and of IgG-bound low density lipoproteins in patients with coronary artery disease. Clin. Chim. Acta Int. J. Clin. Chem. 238, 1–10.

Bovin, N.V., 2013. Natural antibodies to glycans. Biochem. Biokhimii͡a 78, 786–797.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Burut, D.F.P., Karim, Y., Ferns, G.A.A., 2010. The role of immune complexes in atherogenesis. Angiology 61, 679–689.

Butschak, G., Karsten, U., 2002. Isolation and characterization of thomsen-friedenreich-specific antibodies from human serum. Tumour Biol. J. Int. Soc. Oncodevelopmental Biol. Med. 23, 113–122.

Castronovo, V., Colin, C., Parent, B., et al. 1989. Possible role of human natural anti-Gal antibodies in the natural antitumor defense system. J. Natl. Cancer Inst. 81, 212–216.

Chacko, B.K., Appukuttan, P.S., 2001. Peanut (Arachis Hypogaea) Lectin Recognizes α-Linked Galactose, but Not N-Acetyl Lactosamine in N-Linked Oligosaccharide Terminals. Int. J. Biol. Macromol. 28, 365–371.

Cinzia, F., Daniela, P., Elena, S., et al. 2009. Lipoprotein (a) [Lp(a)]: a possible link between migraine and stroke. Transl. Res. J. Lab. Clin. Med. 153, 44–47.

Cohen, M., Varki, A., 2010. The sialome--far more than the sum of its parts. Omics J. Integr. Biol. 14, 455–464.

Coutinho, A., Kazatchkine, M.D., Avrameas, S., 1995. Natural autoantibodies. Curr. Opin. Immunol. 7, 812–818.

Dean, P.D.G., Johnson, W.S., 1985. Affinity chromatography: a practical approach. Oxford: IRL Press; 1985:65.

Desai, P.R., Ujjainwala, L.H., Carlstedt, S.C., Springer, G.F., 1995. Anti-Thomsen-Friedenreich (T) antibody-based ELISA and its application to human breast carcinoma detection. J. Immunol. Methods 188, 175–185.

Dijk, R.A. van, Kolodgie, F., Ravandi, A., et al. 2012. Differential expression of oxidation-specific epitopes and apolipoprotein(a) in progressing and ruptured human coronary and carotid atherosclerotic lesions. J. Lipid Res. 53, 2773–2790.

Dochez, A.R., Avery, O.T., 1917. the elaboration of specific soluble substance by pneumococcus during growth. J. Exp. Med. 26, 477–493.

Dong, X., Andre, S., Hofer, B., et al. 1997. Disease type-associated increases of the plasma levels and ligand expression for natural alpha- or beta-galactoside-binding immunoglobulin G subfractions in patients with lung cancer. Int. J. Oncol. 10, 709–719.

Ehara, S., Ueda, M., Naruko, T., et al. 2001. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. Circulation 103, 1955–1960.

Friedenreich, V., 1928. Investigations into the Thomsen Hemagglutination Phenomena. Acta Pathol. Microbiol. Scand. 5, 59–101.

Galili, U., 1993. Interaction of the natural anti-Gal antibody with alpha-galactosyl epitopes: a major obstacle for xenotransplantation in humans. Immunol. Today 14, 480–482.

Galili, U., Rachmilewitz, E.A., Peleg, A., Flechner, I., 1984. A unique natural human IgG antibody with anti-alpha-galactosyl specificity. J. Exp. Med. 160, 1519–1531.

Galkina, E., Ley, K., 2009. Immune and Inflammatory Mechanisms of Atherosclerosis. Annu. Rev. Immunol. 27, 165–197.

Gaubatz, J.W., Ghanem, K.I., Guevara, J., et al. 1990. Polymorphic forms of human apolipoprotein[a]: inheritance and relationship of their molecular weights to plasma levels of lipoprotein[a]. J. Lipid Res. 31, 603–613.

German, J.B., Freeman, S.L., Lebrilla, C.B., Mills, D.A., 2008. Human milk oligosaccharides: evolution, structures and bioselectivity as substrates for intestinal bacteria. Nestlé Nutr. Workshop Ser. Paediatr. Programme 62, 205–218; discussion 218–222.

Giles, I.P., Isenberg, D.A., Latchman, D.S., Rahman, A., 2003. How do antiphospholipid antibodies bind β2-glycoprotein I? Arthritis Rheum. 48, 2111–2121.

Görög, P., Born, G.V., 1983. Uneven distribution of sialic acids on the luminal surface of aortic endothelium. Br. J. Exp. Pathol. 64, 418–424.

Green, E.D., Adelt, G., Baenziger, J.U., et al. 1988. The asparagine-linked oligosaccharides on bovine fetuin. Structural analysis of N-glycanase-released oligosaccharides by 500-megahertz 1H NMR spectroscopy. J. Biol. Chem. 263, 18253–18268.

Griffith, R.L., Virella, G.T., Stevenson, H.C., et al. 1988. Low density lipoprotein metabolism by human macrophages activated with low density lipoprotein immune complexes. A possible mechanism of foam cell formation. J. Exp. Med. 168, 1041–1059.

Gupta, D., Kaltner, H., Dong, X., Gabius, H.J., Brewer, C.F., 1996. Comparative cross-linking activities of lactose-specific plant and animal lectins and a natural lactose-binding immunoglobulin G fraction from human serum with asialofetuin. Glycobiology 6, 843–849.

Gustafsson, M., Borén, J., 2004. Mechanism of lipoprotein retention by the extracellular matrix. Curr. Opin. Lipidol. 15, 505–514.

Haberland, M.E., Fless, G.M., Scanu, A.M., Fogelman, A.M., 1992. Malondialdehyde modification of lipoprotein(a) produces avid uptake by human monocyte-macrophages. J. Biol. Chem. 267, 4143–4151.

Hanisch, F.G., Baldus, S.E., 1997. The Thomsen-Friedenreich (TF) antigen: a critical review on the structural, biosynthetic and histochemical aspects of a pancarcinoma-associated antigen. Histol. Histopathol. 12, 263–281.

Hansson, G.K., Robertson, A.-K.L., Söderberg-Nauclér, C., 2006. Inflammation and atherosclerosis. Annu. Rev. Pathol. Mech. Dis. 1, 297–329.

Harazono, A., Kawasaki, N., Kawanishi, T., Hayakawa, T., 2005. Site-specific glycosylation analysis of human apolipoprotein B100 using LC/ESI MS/MS. Glycobiology 15, 447–462.

Heidelberger, M., Avery, O.T., 1924. The soluble specific substance of pneumococcus. J. Exp. Med. 40, 301–317.

Hobbs, H.H., White, A.L., 1999. Lipoprotein(a): intrigues and insights. Curr. Opin. Lipidol. 10, 225–236.

Holvoet, P., Donck, J., et al. 1996. Correlation between oxidized low density lipoproteins and von Willebrand factor in chronic renal failure. Thromb. Haemost. 76, 663–669.

Huang, Y., DiDonato, J.A., Levison, B.S., 2014. An abundant dysfunctional apolipoprotein A1 in human atheroma. Nat. Med. advance online publication. 10.1038/nm.3459.

Hudson, L., Hay, F.C., 1980a. Practical Immunology. Oxford: Blackwell Scientific.1980: p.11-13.

Hudson, L., Hay, F.C., 1980b. Practical Immunology. Oxford: Blackwell Scientific.1980: p.226-228.

Huflejt, M.E., Vuskovic, M., Vasiliu, D., et al. 2009. Anti-carbohydrate antibodies of normal sera: findings, surprises and challenges. Mol. Immunol. 46, 3037–3049.

Hyderman E., Strudly K., Richardson T., Immunohistochemistry in pathology. In: Weir, D.M., Herzenberg, L.A., Handbook of Experimental Immunology: Oxford: Blackwell Scientific Publication, 1986: 129.

Indrayan. A, Forecasting vascular disease cases and associated mortality in India, In: Burden of Diseases in India, 2005. National Commission on Macroeconomics and Health, Government of India. pp.197.

Jaison, P.L., Appukuttan, P.S., 1992. Rapid isolation of human plasma anti-alpha-galactoside antibody using sugar-specific binding to guar galactomannan or agarose. Indian J. Biochem. Biophys. 29, 266–270.

Jaison, P.L., Kannan, V.M., Geetha, M., Appukuttan, P.S., 1993. Epitopes recognized by serum anti-α-galactoside antibody are present on brain glycoproteins in man. J. Biosci. Vol. 18, Number 2, June 1993, pp 187–193.

Jancar, S., Crespo, M.S., 2005. Immune complex-mediated tissue injury: a multistep paradigm. Trends Immunol. 26, 48–55.

Jessup, W., Kritharides, L., Stocker, R., 2004. Lipid oxidation in atherogenesis: an overview. Biochem. Soc. Trans. 32, 134–138.

Jones, G.T., van Rij, A.M., Cole, J., et al. 2007. Plasma lipoprotein(a) indicates risk for 4 distinct forms of vascular disease. Clin. Chem. 53, 679–685.

Kacharava, A.G., Tertov, V.V., Orekhov, A.N., 1993. Autoantibodies against low-density lipoprotein and atherogenic potential of blood. Ann. Med. 25, 551–555.

Kamboh, M.I., Ferrell, R.E., Kottke, B.A., 1991. Expressed hypervariable polymorphism of apolipoprotein (a). Am. J. Hum. Genet. 49, 1063–1074.

Kemmner, W., Roefzaad, C., Haensch, W., Schlag, P.M., 2003. Glycosyltransferase expression in human colonic tissue examined by oligonucleotide arrays. Biochim. Biophys. Acta 1621, 272–279.

Kindt, T.J., Goldsby, R.A., Osborne, B.A., B-cell generation, activation, and differentiation. In: Kuby Immunology. 6ed. New York: W.H. Freeman and company, 2007a: 275-276

Kindt, T.J., Goldsby, R.A., Osborne, B.A., Antigens and antibodies. In: Kuby Immunology. 6ed. New York: W.H. Freeman and company, 2007b: 76-110.

Klein, R.L., Laimins, M., Lopes-Virella, M.F., 1995. Isolation, characterization, and metabolism of the glycated and nonglycated subfractions of low-density lipoproteins isolated from type I diabetic patients and nondiabetic subjects. Diabetes 44, 1093–1098.

Klimov, A.N., Denisenko, A.D., Popov, A.V., et al. 1985. Lipoprotein-antibody immune complexes their catabolism and role in foam cell formation. Atherosclerosis 58, 1–15.

Köchl, S., Fresser, F., Lobentanz, E., et al. 1997. Novel interaction of apolipoprotein(a) with beta-2 glycoprotein I mediated by the kringle IV domain. Blood 90, 1482–1489.

Koschinsky, M.L., Beisiegel, U., Henne-Bruns, D., Eaton, D.L., Lawn, R.M., 1990. Apolipoprotein(a) size heterogeneity is related to variable number of repeat sequences in its mRNA. Biochemistry (Mosc.) 29, 640–644.

Koschinsky, M.L., Marcovina, S.M., 1997. Lipoprotein(a): structural implications for pathophysiology. Int. J. Clin. Lab. Res. 27, 14–23.

Libby, P., Egan, D., Skarlatos, S., 1997. Roles of Infectious Agents in Atherosclerosis and Restenosis An Assessment of the Evidence and Need for Future Research. Circulation 96, 4095–4103.

Libby, P., Ridker, P.M., Maseri, A., 2002. Inflammation and Atherosclerosis. Circulation 105, 1135–1143.

Lind, P.E., Mcarthur, N.R., 1947. The distribution of T agglutinins in human sera. Aust. J. Exp. Biol. Med. Sci. 25, 247–250.

Lindberg, G., Råstam, L., Gullberg, B., Eklund, G.A., 1992. Serum sialic acid concentration predicts both coronary heart disease and stroke mortality: multivariate analysis including 54,385 men and women during 20.5 years follow-up. Int. J. Epidemiol. 21, 253–257.

Lippi, G., Guidi, G., 2000. Lipoprotein(a): from ancestral benefit to modern pathogen? QJM Mon. J. Assoc. Physicians 93, 75–84.

Lopes-Virella, M.F., McHenry, M.B., et al. DCCT/EDIC Research Group, 2007. Immune complexes containing modified lipoproteins are related to the progression of internal carotid intima-media thickness in patients with type 1 diabetes. Atherosclerosis 190, 359–369.

Lopes-Virella, M.F., Thorpe, S.R., Derrick, M.B., Chassereau, C., Virella, G., 2005. The immunogenicity of modified lipoproteins. Ann. N. Y. Acad. Sci. 1043, 367–378.

Lopes-Virella, M.F., Virella, G., 2010. Clinical significance of the humoral immune response to modified LDL. Clin. Immunol. Orlando Fla 134, 55–65.

Lopes-Virella, M.F., Virella, G., Orchard, T.J., et al. 1999. Antibodies to oxidized LDL and LDL-containing immune complexes as risk factors for coronary artery disease in diabetes mellitus. Clin. Immunol. Orlando Fla 90, 165–172.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 193, 265–275.

Macher, B.A., Galili, U., 2008. The Gal α1,3Galβ1,4GlcNAc-R (alpha-Gal) epitope: a carbohydrate of unique evolution and clinical relevance. Biochim. Biophys. Acta 1780, 75–88.

Maggi, E., Marchesi, E., Ravetta, V., et al. 1995. Presence of autoantibodies against oxidatively modified low-density lipoprotein in essential hypertension: a biochemical signature of an enhanced in vivo low-density lipoprotein oxidation. J. Hypertens. 13, 129–138.

Mandagini, G., Subramanian, S.P., Vasantha, K., Appukuttan, P.S., 2013. Human Plasma Anti-α-galactoside Antibody Forms Immune Complex with Autologous Lipoprotein(a). Immunol. Invest. 42, 324–340.

Mandal, C., Mandal, C., Karush, F., 1984. Restriction in IgM expression--V. Fine structure analysis in the anti-lactose system. Mol. Immunol. 21, 895–900.

March, S.C., Parikh, I., Cuatrecasas, P., 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal. Biochem. 60, 149–152.

Marcovina, S.M., Albers, J.J., Jacobs, D.R., et al. 1993. Lipoprotein[a] concentrations and apolipoprotein[a] phenotypes in Caucasians and African

Americans. The CARDIA study. Arterioscler. Thromb. J. Vasc. Biol. Am. Heart Assoc. 13, 1037–1045.

Marcovina, S.M., Koschinsky, M.L., 1998. Lipoprotein(a) as a risk factor for coronary artery disease. Am. J. Cardiol. 82, 57U–66U.

Matsuda, T., Nakashima, I., Kato, Y., Nakamura, R., 1987. Antibody response to haptenic sugar antigen: immunodominancy of protein-bound lactose formed by amino-carbonyl reaction. Mol. Immunol. 24, 421–425.

Mayerl, C., Lukasser, M., Sedivy, R., et al. 2006. Atherosclerosis research from past to present--on the track of two pathologists with opposing views, Carl von Rokitansky and Rudolf Virchow. Virchows Arch. Int. J. Pathol. 449, 96–103.

Mayr, M., Grainger, D., Mayr, U., et al. 2009. Proteomics, metabolomics, and immunomics on microparticles derived from human atherosclerotic plaques. Circ. Cardiovasc. Genet. 2, 379–388.

Miller, Y.I., Choi, S.-H., Wiesner, P., et al. 2011. Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. Circ. Res. 108, 235–248.

Moliterno, D.J., Jokinen, E.V., Miserez, A.R., et al. 1995. No Association Between Plasma Lipoprotein(a) Concentrations and the Presence or Absence of Coronary Atherosclerosis in African-Americans. Arterioscler. Thromb. Vasc. Biol. 15, 850–855.

Monaco, C., Crea, F., Niccoli, G., et al. 2001. Autoantibodies against oxidized low density lipoproteins in patients with stable angina, unstable angina or peripheral vascular disease; pathophysiological implications. Eur. Heart J. 22, 1572–1577.

Montecucco, F., Vuilleumier, N., Pagano, S., et al. 2011. Anti-Apolipoprotein A-1 auto-antibodies are active mediators of atherosclerotic plaque vulnerability. Eur. Heart J. 32, 412–421.

Morishita, R., Ishii, J., Kusumi, Y., et al. 2009. Association of serum oxidized lipoprotein(a) concentration with coronary artery disease: potential role of oxidized lipoprotein(a) in the vasucular wall. J. Atheroscler. Thromb. 16, 410–418.

Murray, R.K., 1996. Harper’s biochemistry. Prentice-Hall International, London.pp.648-685.

Mustafa, A., Nityanand, S., Berglund, L., Lithell, H., Lefvert, A.K., 2000. Circulating Immune Complexes in 50-Year-Old Men as a Strong and Independent Risk Factor for Myocardial Infarction. Circulation 102, 2576–2581.

Myung, S.-K., Ju, W., Cho, B., et al. 2013. Efficacy of vitamin and antioxidant supplements in prevention of cardiovascular disease: systematic review and meta-analysis of randomised controlled trials. BMJ 346, f10.

Nanetti, L., Vignini, A., Raffaelli, F., Taffi, R., Silvestrini, M., Provinciali, L., Mazzanti, L., 2008. Sialic acid and sialidase activity in acute stroke. Dis. Markers 25, 167–173.

Nishi, K., Itabe, H., Uno, M., et al. 2002. Oxidized LDL in carotid plaques and plasma associates with plaque instability. Arterioscler. Thromb. Vasc. Biol. 22, 1649–1654.

Notkins, A.L., 2004. Polyreactivity of antibody molecules. Trends Immunol. 25, 174–179.

Ohira, T., Schreiner, P.J., Morrisett, J.D., et al. 2006. Lipoprotein(a) and incident ischemic stroke: the Atherosclerosis Risk in Communities (ARIC) study. Stroke J. Cereb. Circ. 37, 1407–1412.

Orekhov, A.N., Tertov, V.V., Kabakov, A.E., et al. 1991. Autoantibodies against modified low density lipoprotein. Nonlipid factor of blood plasma that stimulates foam cell formation. Arterioscler. Thromb. Vasc. Biol. 11, 316–326.

Paul, A., Antony, M., Mathai, J., Appukuttan, P.S., 2011. High polymeric IgA content facilitates recognition of microbial polysaccharide-natural serum antibody immune complexes by immobilized human galectin-1. Immunol. Lett. 136, 55–60.

Paul, A., Geetha, M., Chacko, B.K., Appukuttan, P.S., 2009. Multiple specificity of human serum dextran-binding immunoglobulin: α(1-6)- and β(1-3)-linked glucose and α (1-3)-linked galactose in natural glycoconjugates are recognized. Immunol. Invest. 38, 153–164.

Pepin, J.M., O'Neil, J.A., Hoff, H.F., 1991. Quantification of apo[a] and apoB in human atherosclerotic lesions. J. Lipid Res. 32, 317–327.

Peterson, R., Cheah, W.Y., Grinyer, J., Packer, N., 2013. Glycoconjugates in human milk: Protecting infants from disease. Glycobiology 23, 1425–1438.

Puurunen, M., Mänttäri, M., Manninen, V., et al. 1994. Antibody against oxidized low-density lipoprotein predicting myocardial infarction. Arch. Intern. Med. 154, 2605–2609.

Ragab, M.S., Selvaraj, P., Sgoutas, D.S., 1996. Oxidized lipoprotein (a) induces cell adhesion molecule Mac-1 (CD 11b) and enhances adhesion of the monocytic cell line U937 to cultured endothelial cells. Atherosclerosis 123, 103–113.

Rawitch, A.B., Pollock, H.G., Yang, S.X., 1993. Thyroglobulin glycosylation: location and nature of the N-linked oligosaccharide units in bovine thyroglobulin. Arch. Biochem. Biophys. 300, 271–279.

Recchi, M.A., Hebbar, M., Hornez, L., et al. 1998. Multiplex reverse transcription polymerase chain reaction assessment of sialyltransferase expression in human breast cancer. Cancer Res. 58, 4066–4070.

Roozbeh, J., Merat, A., Bodagkhan, F., Afshariani, R., Yarmohammadi, H., 2011. Significance of serum and urine neuraminidase activity and serum and urine level of sialic acid in diabetic nephropathy. Int. Urol. Nephrol. 43, 1143–1148.

Rosengren, A., Wilhelmsen, L., Eriksson, E., Risberg, B., Wedel, H., 1990. Lipoprotein (a) and coronary heart disease: a prospective case-control study in a general population sample of middle aged men. BMJ 301, 1248–1251.

Ross, R., 1999. Atherosclerosis--an inflammatory disease. N. Engl. J. Med. 340, 115–126.

Ross, R., Glomset, J., Harker, L., 1977. Response to injury and atherogenesis. Am. J. Pathol. 86, 675–684.

Salonen, J.T., Korpela, H., Salonen, R., et al. 1992. Autoantibody against oxidised LDL and progression of carotid atherosclerosis. The Lancet 339, 883–887.

Sandrin, M.S., Vaughan, H.A., Xing, P.X., McKenzie, I.F., 1997. Natural human anti-Gal alpha(1,3)Gal antibodies react with human mucin peptides. Glycoconj. J. 14, 97–105.

Scanu, A.M., Edelstein, C., 1997. Learning about the structure and biology of human lipoprotein [a] through dissection by enzymes of the elastase family: facts and speculations. J. Lipid Res. 38, 2193–2206.

Schenkel-Brunner, D.H., 1995. Polyagglutination, in: Human Blood Groups. Springer Vienna, pp. 283–298.

Schwenke, D.C., Carew, T.E., 1989. Initiation of atherosclerotic lesions in cholesterol-fed rabbits. II. Selective retention of LDL vs. selective increases in LDL permeability in susceptible sites of arteries. Arterioscler. Dallas Tex 9, 908–918.

Shoenfeld, Y., Isenberg, D.A., 1989. The mosaic of autoimmunity. Immunol. Today 10, 123–126.

Sigala, F., Kotsinas, A., Savari, P., et al. 2010. Oxidized LDL in human carotid plaques is related to symptomatic carotid disease and lesion instability. J. Vasc. Surg. 52, 704–713.

Sönmez, H., Süer, S., Ulutin, T., Kökoglu, E., Uçişik, N., 1998. The Relationship of Various Factors in the Pathogenesis of Atherosclerosis. Clin. Appl. Thromb. 4, 105–110.

Spiro, M.J., 1977. Presence of a glucuronic acid-containing carbohydrate unit in human thyroglobulin. J. Biol. Chem. 252, 5424–5430.

Spiro, R.G., 1965. The Carbohydrate Units of Thyroglobulin. J. Biol. Chem. 240, 1603–1610.

Spiro, R.G., 2002. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. Glycobiology 12, 43R–56R.

Spiro, R.G., Bhoyroo, V.D., 1974. Structure of the O-Glycosidically Linked Carbohydrate Units of Fetuin. J. Biol. Chem. 249, 5704–5717.

Spiro, R.G., Bhoyroo, V.D., 1984. Occurrence of alpha-D-galactosyl residues in the thyroglobulins from several species. Localization in the saccharide chains of the complex carbohydrate units. J. Biol. Chem. 259, 9858–9866.

Springer, G.F., 1997. Immunoreactive T and Tn epitopes in cancer diagnosis, prognosis, and immunotherapy. J. Mol. Med. Berl. Ger. 75, 594–602.

Springer, G.F., Desai, P.R., 1975. Increase in anti-T titer scores of breast-carcinoma patients following mastectomy. Naturwissenschaften 62, 587.

Springer, G.F., Desai, P.R., Scanlon, E.F., 1976. Blood group MN precursors as human breast carcinoma-associated antigens and “naturally” occurring human cytotoxins against them. Cancer 37, 169–176.

Springer, G.F., Desai, P.R., Wise, W., et al. 1990. Pancarcinoma T and Tn epitopes: autoimmunogens and diagnostic markers that reveal incipient carcinomas and help establish prognosis. Immunol. Ser. 53, 587–612.

Springer, G.F., Horton, R.E., 1969. Blood group isoantibody stimulation in man by feeding blood group-active bacteria. J. Clin. Invest. 48, 1280–1291.

Sreekumar, A., Mandagini, G., Subramanian, S.P., Sankunni, A.P., 2013. apoB-independent enzyme immunoassay for lipoprotein(a) by capture on immobilized lectin (jacalin). J. Immunoassay Immunochem. 34, 166–179.

Stary, H.C., Blankenhorn, D.H., Chandler, A.B., et al. 1992. A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 85, 391–405.

Steinbrecher, U.P., 1987. Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. J. Biol. Chem. 262, 3603–3608.

Steinbrecher, U.P., Fisher, M., Witztum, J.L., Curtiss, L.K., 1984a. Immunogenicity of homologous low density lipoprotein after methylation, ethylation, acetylation, or carbamylation: generation of antibodies specific for derivatized lysine. J. Lipid Res. 25, 1109–1116.

Steinbrecher, U.P., Parthasarathy, S., Leake, D.S., et al. 1984b. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc. Natl. Acad. Sci. U. S. A. 81, 3883–3887.

Suresh Kumar, G., Appukuttan, P.S., Basu, D., 1982. α-D-galactose-specific lectin from jack fruit (Artocarpus integra) seed. J. Biosci. 4, 257–261.

Surolia, A., Prakash, N., Bishayee, S., Bachhawat, B.K., 1973. Isolation and comparative physico-chemical studies of Concanavalin A from Canavalia ensiformis and Canavalia gladiata. Indian J. Biochem. Biophys. 10, 145–148.

Takasaki, S., Kobata, A., 1986. Asparagine-linked sugar chains of fetuin: occurrence of tetrasialyl triantennary sugar chains containing the Gal beta 1-3GlcNAc sequence. Biochemistry (Mosc.) 25, 5709–5715.

Tanemura, M., Yin, D., Chong, A.S., Galili, U., 2000. Differential immune responses to α-gal epitopes on xenografts and allografts: implications for accommodation in xenotransplantation. J. Clin. Invest. 105, 301–310.

Tegos, T.J., Kalodiki, E., Sabetai, M.M., Nicolaides, A.N., 2001. The genesis of atherosclerosis and risk factors: a review. Angiology 52, 89–98.

Tertov, V.V., Orekhov, A.N., 1994. Effect of lipoprotein(a) on lipid metabolism of cultured human intimal aortic cells. Chem. Phys. Lipids 67-68, 161–166.

Tertov, V.V., Orekhov, A.N., Kacharava, A.G., et al. 1990. Low density lipoprotein-containing circulating immune complexes and coronary atherosclerosis. Exp. Mol. Pathol. 52, 300–308.

Tertov, V.V., Sobenin, I.A., Orekhov, A.N., et al. 1996. Characteristics of low density lipoprotein isolated from circulating immune complexes. Atherosclerosis 122, 191–199.

Thatcher, N., Hashmi, K., Chang, J., Swindell, R., Crowther, D., 1980. Anti-T antibody in malignant melanoma patients. Influence of response and survival following chemotherapy-changes in serum levels following C parvum, BCG immunization. Cancer 46, 1378–1382.

Theofilopoulos, A.N., Dixon, F.J., 1980. Immune complexes in human diseases: a review. Am. J. Pathol. 100, 529–594.

Tomlinson, J.E., McLean, J.W., Lawn, R.M., 1989. Rhesus monkey apolipoprotein(a). Sequence, evolution, and sites of synthesis. J. Biol. Chem. 264, 5957–5965.

Toshima, S., Hasegawa, A., Kurabayashi, M., et al. 2000. Circulating oxidized low density lipoprotein levels. A biochemical risk marker for coronary heart disease. Arterioscler. Thromb. Vasc. Biol. 20, 2243–2247.

Triplett, R.B., Fisher, W.R., 1978. Proteolytic digestion in the elucidation of the structure of low density lipoprotein. J. Lipid Res. 19, 478–488.

Uno, M., Kitazato, K.T., Nishi, K., Itabe, H., Nagahiro, S., 2003. Raised plasma oxidised LDL in acute cerebral infarction. J. Neurol. Neurosurg. Psychiatry 74, 312–316.

Urakami, K., Wada-Isoe, K., Wakutani, Y., et al. 2000. Lipoprotein(a) phenotypes in patients with vascular dementia. Dement. Geriatr. Cogn. Disord. 11, 135–138.

Utermann, G., Menzel, H.J., Kraft, H.G., et al. 1987. Lp(a) glycoprotein phenotypes. Inheritance and relation to Lp(a)-lipoprotein concentrations in plasma. J. Clin. Invest. 80, 458–465.

Valenti, K., Aveynier, E., Leauté, S., Laporte, F., Hadjian, A.J., 1999. Contribution of apolipoprotein(a) size, pentanucleotide TTTTA repeat and C/T(+93) polymorphisms of the apo(a) gene to regulation of lipoprotein(a) plasma levels in a population of young European Caucasians. Atherosclerosis 147, 17–24.

Van Hinsbergh, V.W., Scheffer, M., Havekes, L., Kempen, H.J., 1986. Role of endothelial cells and their products in the modification of low-density lipoproteins. Biochim. Biophys. Acta 878, 49–64.

Varki, A., 1993. Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 3, 97–130.

Varki, A., 2006. Nothing in Glycobiology Makes Sense, except in the Light of Evolution. Cell 126, 841–845.

Varki, A., Cummings, R., Esko, J., Freeze, H., Hart, G., Marth, J., O-glycans, In: Essentials of Glycobiology, 1st ed. New York, Cold Spring Harbor Laboratory Press: 1999: p.103

Varki, A., Cummings, R.D., Esko, J.D., et al. 2009a . Essentials of Glycobiology, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).pp.209.

Varki, A., Cummings, R.D., Esko, J.D., et al. 2009b . Essentials of Glycobiology, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).pp.1-22.

Varki, A., Cummings, R.D., Esko, J.D., et al. 2009c . Essentials of Glycobiology, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).pp.89-100.

Varki, A., Cummings, R.D., Esko, J.D., et al. 2009d . Essentials of Glycobiology, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).pp.101-114.

Varki, A., Cummings, R.D., Esko, J.D., et al. 2009e . Essentials of Glycobiology, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).pp.115-128.

Varki, A., Cummings, R.D., Esko, J.D., et al. 2009f. Essentials of Glycobiology, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).pp.489-500.

Varki, A., Cummings, R.D., Esko, J.D., et al. 2009g. Essentials of Glycobiology, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).pp.601-616.

Gabriel Virella and George C. Tsakos. Immune complex Diseases. In: Virella G, 6th ed. Medical Immunology. New York: Informa healthcare, 2007: 323-324.

Virella, G., Atchley, D., Koskinen, S., Zheng, D., Lopes-Virella, M.F., DCCT/EDIC Research Group, 2002. Proatherogenic and proinflammatory properties of immune complexes prepared with purified human oxLDL antibodies and human oxLDL. Clin. Immunol. 105, 81–92.

Virella, G., Muñoz, J.F., Galbraith, G.M., et al. 1995. Activation of human monocyte-derived macrophages by immune complexes containing low-density lipoprotein. Clin. Immunol. Immunopathol. 75, 179–189.

Virella, G., Virella, I., Leman, R.B., Pryor, M.B., Lopes-Virella, M.F., 1993. Anti-oxidized low-density lipoprotein antibodies in patients with coronary heart disease and normal healthy volunteers. Int. J. Clin. Lab. Res. 23, 95–101.

Vuilleumier, N., Bas, S., Pagano, S., et al. 2010. Anti-apolipoprotein A-1 IgG predicts major cardiovascular events in patients with rheumatoid arthritis. Arthritis Rheum. 62, 2640–2650.

Vuilleumier, N., Montecucco, F., Spinella, G., et al. 2013. Serum levels of anti-apolipoprotein A-1 auto-antibodies and myeloperoxidase as predictors of major adverse cardiovascular events after carotid endarterectomy. Thromb. Haemost. 109, 706–715.

Wang, J., Gong, J., Li, H., et al. 2012. Lipoprotein(a) complexes with beta2-glycoprotein I in patients with coronary artery disease. J. Atheroscler. Thromb. 19, 81–89.

Wang, J., Qiang, H., Zhang, C., et al. 2003. Detection of IgG-bound lipoprotein(a) immune complexes in patients with coronary heart disease. Clin. Chim. Acta Int. J. Clin. Chem. 327, 115–122.

Wang, J., Zhang, C., Gong, J., et al. 2007. Development of new enzyme-linked immunosorbent assay for oxidized lipoprotein(a) by using purified human oxidized lipoprotein(a) autoantibodies as capture antibody. Clin. Chim. Acta Int. J. Clin. Chem. 385, 73–78.

Y.P. Venkatesh., V. Prasanna Kumar., 2013. Natural Antibodies to Dietary Components. Global Journal of Immunology and Allergic Diseases. 1, 25-34.

Yashiro, A., O'Neil, J., Hoff, H.F., 1993. Insoluble complex formation of lipoprotein (a) with low density lipoprotein in the presence of calcium ions. J. Biol. Chem. 268, 4709–4715.

Ylä-Herttuala, S., Palinski, W., Butler, S.W., et al. 1994. Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL. Arterioscler. Thromb. J. Vasc. Biol. Am. Heart Assoc. 14, 32–40.

Zhang, C., Li, X., Niu, D., et al. 2011. Increased serum levels of β_2-GPI-Lp(a) complexes and their association with premature atherosclerosis in patients with rheumatoid arthritis. Clin. Chim. Acta Int. J. Clin. Chem. 412, 1332–1336.

LIST OF PUBLICATION

Sabarinath P.S, Appukuttan P.S - Immunopathology of desialylation: human plasma lipoprotein (a) and circulating anti-carbohydrate antibodies form immune complexes that recognize host cells. *Molecular and Cellular Biochemistry*, 2015. 10.1007/s11010-015-2332-3

Sabarinath P.S, Balu K Chacko, Appukuttan.P.S. Dual specificity of human plasma lactose-binding immunoglobulin to anomers of terminal galactose enables recognition of desialylated lipoprotein(a) and xenoantigens. *Scandinavian Journal of Immunology*. 2014. 79(4), 251-259.

Geetha Mandagini, Kalaivani Vasantha, **Sabarinath P.S** & Appukuttan P.S. Plasma anti-α-galactoside antibody binds to serine- and threonine-rich peptide sequence of apo(a) subunit in Lp(a). *Glycoconjugate Journal*. 2014. 31(4), 289-298.

Geetha Mandagini, **Sabarinath P. Subramanian**, Kalaivani Vasantha & Appukuttan P. Sankunni. Human plasma anti-α-galactoside antibody forms immune complex with autologous lipoprotein(a). *Immunological Investigations.* 2013; 42(4):324-340.

Anuradha Sreekumar, Geetha Mandagini, **Sabarinath P. Subramanian** & Appukuttan P. Sankunni. ApoB-independent enzyme immunoassay for lipoprotein(a) by capture on immobilized lectin (jacalin). *Journal of Immunoassay and Immunochemistry.* 2013; 34 (2):166-179