Studies of the pathogenesis of IgA nephropathy and Henoch-Schönlein purpura, with special reference to Streptococcus pyogenes infections and complement

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“Nature is an infinite sphere whose center is everywhere

and whose circumference is nowhere” *Blaise Pascal (1623-1662)*

Table of contents

Page

- List of paper ……..... 4
- Abbreviations …… 5
- Abstract …… 6

1. Introduction
   - 1.1. IgA
     - 1.1.1. IgA …… 7
       - 1.1.1.1. Serum IgA …….. 8
       - 1.1.1.2. Secretory IgA …….. 10
     - 1.1.2. O-glycosylation of the hinge-region of IgA_1 ……... 11
     - 1.1.3. IgA-receptors …….. 13
   - 1.2. The complement system
     - 1.2.1. Activation …… 16
     - 1.2.2. Regulation …… 18
     - 1.2.3. Complement Factor H 20
     - 1.2.4. Complement-mediated renal disease 22
   - 1.3. IgA nephropathy
     - 1.3.1. Clinical features …….. 23
     - 1.3.2. Pathology …… 23
     - 1.3.3. Diagnostic work-up ..…… 24
     - 1.3.4. Epidemiology …….. 24
     - 1.3.5. Outcome and predictors of outcome ……. 25
     - 1.3.6. Pathogenesis
       - 1.3.6.1. Genetic factors …….. 25
       - 1.3.6.2. Infectious agents …….. 26
       - 1.3.6.3. IgA_1 in IgA nephropathy ……. 26
       - 1.3.6.4. IgA-receptors in IgA nephropathy …… 29
       - 1.3.6.5. Cytokines ……... 30
       - 1.3.6.6. Toll-like receptors ……... 31
       - 1.3.6.7. The complement system in IgA nephropathy …… 32
     - 1.3.7. Malignant hypertension and thrombotic microangiopathy associated with IgA nephropathy …….. 34
   - 1.4. Henoch-Schönlein purpura
     - 1.4.1. Clinical features ……... 35
     - 1.4.2. Pathology ……. 36
     - 1.4.3. Definition ……. 36
     - 1.4.4. Epidemiology …… 36
     - 1.4.5. Outcome and predictors of outcome …….. 37
     - 1.4.6. Etiology, Pathogenesis
       - 1.4.6.1. Genetic factors …… 38
       - 1.4.6.2. Infectious and non-infectious agents ...…….. 39
       - 1.4.6.3. Underglycosylated, polymeric IgA_1 ……. 39
       - 1.4.6.4. Mediators of inflammation ……... 39

1.5. Evidence for a common background of IgA nephropathy and Henoch Schönlein purpura ……..... 42
1.6. Group A streptococci
1.6.1. Streptococci ……. 42
1.6.2. Group A streptococcal disease – epidemiology ……. 43
1.6.3. M proteins ……. 43
1.6.4. IgA-binding regions of group A streptococcal M proteins 46
1.6.5. Group A streptococci and renal disease
1.6.5.1. Acute post-streptococcal glomerulonephritis ……. 46
1.6.5.2. Involvement in other forms of glomerulonephritis ….. 47
2. The present investigation
2.1. Aims ……. 48
2.2. Patients and materials
2.2.1. Streptococcal proteins, peptides and antibodies ……. 49
2.2.2. Tissue and blood samples from patients and controls ……. 51
2.2.3. Primary human mesangial cells …….. 52
2.3. Methods and results
2.3.1. Paper I
2.3.1.1. Characterization of the IgA-binding region in different group A streptococcal M proteins…….. 53
2.3.1.2. Detection of IgA-binding M proteins in tissue samples. 53
2.3.1.3. Ultrastructural localization of IgA-binding M proteins and co-localization with IgA …….. 54
2.3.1.4. Mass spectrometry for detection of IgA-binding M proteins in a skin sample …….. 55
2.3.2. Paper II
2.3.2.1. Detection of antibodies to IgA-binding regions of streptococcal M proteins ……. 55
2.3.3. Paper III
2.3.3.1. Binding affinity of IgA-binding proteins to IgA_1 …….. 56
2.3.3.2. Binding of M4 protein to human mesangial cells ……. 57
2.3.3.3. IL-6 synthesis and secretion and C3 secretion from mesangial cells stimulated with M4 and IgA_1 ……. 57
2.3.4. Paper IV
2.3.4.1. Detection of a novel mutation and polymorphisms in a patient with IgA nephropathy …….. 58
2.4. Discussion ……. 59
- Populärvetenskaplig sammanfattning (svenska) …….. 63
- Populärwissenschaftliche Zusammenfassung (deutsch)…… 64
- Acknowledgements ……. 67
- References ……. 69

List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:

PAPER I Tissue deposits of IgA-binding streptococcal M proteins in IgA nephropathy and Henoch-Schönlein purpura. Roland Schmitt, Fredric Carlsson, Matthias Mörgelin, Ramesh Tati, Gunnar Lindahl, Diana Karpman. *Am J Pathol*. 2010;176: 608-618.

PAPER II Antibody response to IgA-binding streptococcal M proteins in children with IgA nephropathy. Roland Schmitt, Gunnar Lindahl, Diana Karpman. *Nephrol Dial Transplant*. 2010;25: 3434-3436 (plus online supplement).

PAPER III Underglycosylated polymeric IgA_1 binds to streptococcal IgA-binding M protein inducing IL-6 and C3 secretion from human mesangial cells: implications for the pathogenesis of IgA nephropathy. Roland Schmitt, Anne-Lie Ståhl, Anders Olin, Ramesh Tati, Ann-Charlotte Kristoffersson, Johan Rebetz, Jan Novak, Gunnar Lindahl, Diana Karpman (manuscript).

PAPER IV IgA nephropathy associated with a novel N-terminal mutation in factor H. Roland Schmitt, Raphael T Krmar, Ann-Charlotte Kristoffersson, Magnus Söderberg, Diana Karpman. *Eur J Pediatr*. 2011; 170: 107-110.

Abbreviations

CFH	Complement factor H
CFI	Complement factor I
C1GalT-1	Core1-galactose transferase 1
Cosmc	Core-1-galactose transferase-specific molecular chaperone
FHL-1	Factor H-like protein 1
GalNAc	N-acetylgalactosamine
GAS	Group A streptococci
HSN	Henoch-Schönlein nephropathy
HSP	Henoch-Schönlein purpura
IgA	Immunoglobulin A
sIgA	secretory IgA,
pIgA	polymeric IgA
IgA-BR	IgA-binding region of streptococcal M protein
IgAN	IgA nephropathy
IL-6	Interleukin-6
MAC	Membrane attack complex
NeuNAc	N-acetylneuraminic acid, sialic acid
PDGF	Platelet-derived growth factor
Sap	Streptococcal IgA-binding peptide
SCR	Short consensus repeat
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TMA	Thrombotic microangiopathy
TNF-α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor

Abstract

IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis and Henoch-Schönlein purpura (HSP) the most common form of vasculitis in childhood. HSP may affect kidneys, a complication termed Henoch-Schönlein nephropathy (HSN). Renal pathology in HSN resembles that seen in IgAN. The pathogenesis of IgAN and HSP is so far unclear. Both are characterized by tissue deposits of underglycosylated polymeric IgA_1 and the debut or exacerbations are regularly preceded by infections usually affecting the respiratory tract and often caused by group A streptococci (GAS). GAS express the surface bound M protein, which varies in sequence between strains and in certain serotypes includes an IgA-binding region (IgA-BR). The complement system, an important part of the innate immune system, is activated during IgAN and HSN reflected by the common finding of mesangial depositions of C3.

Paper I-III investigated whether IgA-binding M proteins are involved in the pathogenesis of IgAN and HSP. In the first study we examined tissue samples from pediatric patients with IgAN and HSP and detected IgA-BR co-localizing in the mesangial region with IgA in most of the kidney samples from patients with IgAN and HSN and skin samples from patients with HSP. In the second study we showed that pediatric patients with IgAN had higher antibody levels to IgA-BR than age-matched controls. The third study showed that the IgA-binding M protein from GAS serotype 4 (M4) had a significantly higher binding affinity for underglycosylated polymeric IgA_1 than for other forms of IgA_1. Mesangial cells stimulated with M4 exhibited increased synthesis and secretion of IL-6. Co-stimulation with both M4 and IgA_1 induced excessive IL-6 secretion. IgA_1 also induced C3 secretion from mesangial cells, which was enhanced when the cells were co-stimulated with M4.

Paper IV identified a novel mutation heterozygous mutation in exon 2 of the factor H gene (*CFH*) in a child with IgAN complicated by thrombotic microangiopathy (TMA) most probably triggered by malignant hypertension. In addition, three heterozygous *CFH* polymorphisms were identified, known to increase the risk for TMA. This genotype may thus have contributed to the combined phenotype of IgAN and TMA.

This thesis provides evidence for the involvement of GAS expressing IgA-binding M proteins in the etiology and pathogenesis of IgAN and HSP. An N terminal mutation in CFH may have influenced the course and pathological findings in IgAN and particularly conferred susceptibility for TMA.

1. Introduction

This thesis addressed the contribution of group A streptococci and the complement system to the pathogenesis of IgA nephropathy and Henoch-Schönlein purpura. In the introduction a short comprehensive overview is given regarding the physiological background of immunoglobulin A and the complement system, the current understanding of IgA nephropathy and Henoch-Schönlein purpura and group A streptococci.

1.1. Immunoglobulin A (IgA)

1.1.1. IgA

IgA is the most abundant immunoglobulin in humans with a synthesis rate that exceeds that of all other immunoglobulin classes combined (66 mg/kg/d)[1]. IgA is synthesized by plasma cells after antibody class switch induced by exposure to transforming growth factor β (TGF-β) and further augmented by interleukin-5[2]. There are two major forms of IgA, secretory IgA (sIgA, present at mucosal surfaces) and serum IgA. The vast majority of IgA is synthesized in mucosa-associated lymphoid tissue (MALT)-located plasma cells and secreted at mucosal sites as polymeric, predominantly dimeric, sIgA[3]. Serum IgA is derived from plasma cells in bone marrow, peripheral lymphoid tissues (such as spleen, tonsils, adenoids) and MALT and prevalent at levels, which are usually about a fifth of those for IgG, the most abundant immunoglobulin in human serum. However, as the catabolism of IgA is five times faster than that of IgG the actual synthesis rate of these two immunoglobulins is thought to be similar[4,5].

Table 1: Forms and distribution of IgA[6-8]

Type of IgA		Origin	Prevalence	IgA_1/ IgA_2	pIgA (%)	Composition (see Figure 1)
Serum IgA	**mIgA**	Bone marrow, secondary lymphoid tissue (tonsils, adenoids, spleen, MALT)	87% of serum IgA	9/1		2x light chains + 2x heavy chains (rarely with a J-chain)
	pIgA	Bone marrow, secondary lymphoid tissue	13% of serum IgA	2.7/1		2-4x m IgA plus 1 x j-chain
IgA in secretions		MALT	Colostrum	2/1	96	pIgA + SC (sIgA)
			Saliva	2/1	96	
			Gastric juice	4/1	NA	
			Jejunal fluid	7/3	95	
			Colonic fluid	1/2	NA	
			Hepatic bile	3/1	65	mIgA (+ j-chain + SC ?)
			Nasal fluid	19/1	NA	
			Bronchial secret	2/1	82	
			Tears	4/1	95	

Abbreviations: mIgA = monomeric IgA, pIgA = polymeric IgA, sIgA=secretory IgA, MALT = mucosa associated lymphoid tissue, SC = secretory component, NA: data not available.

1.1.1.1. *Serum IgA*

Serum IgA is mainly prevalent in monomeric form (87%)[7]. The serum level of IgA increases with age. The relative amount of polymeric IgA in serum is significantly higher until the age of 2 years and decreases gradually until adult levels of IgA are reached[7]. The function of serum IgA is a matter of discussion. Bound to an antigen it may lead to an inflammatory response by binding to the $Fc_{\alpha}R$ on myeloid cells (see chapter IgA-receptors), which induces phagocytosis, oxidative burst and cytokine release from the myeloid cell. Another possible but contradictory function is anti-inflammatory. This is achieved in the absence of antigen by exerting an inhibitory effect on phagocytes inhibiting IgG-mediated phagocytosis, oxidative burst and cytokine release, which together result in an anti-inflammatory effect. As IgA-deficiency usually does not confer major immune incompetency, but instead is related to increased risk for autoimmune and allergic diseases, the anti-inflammatory effect of serum-IgA seems to dominate[9,10].

Human IgA exists in two subclasses: IgA_1 and IgA_2, each a product of a separate gene[1]. The distribution of IgA_1 and IgA_2 in the body is presented in Table 1[7-9].

The principal structure of IgA_1 is shown in Figure 1[1]. The structure of IgA resembles that of other immunoglobulins, but instead of the Y-shape known from IgG it displays a T-shaped structure[11] (Figure 1A). The Fab-segment of IgA is responsible for antigen recognition and

binding, whereas the Fc-segment provides binding-sites for specific Fc_{α}-receptors and streptococcal M proteins[1].

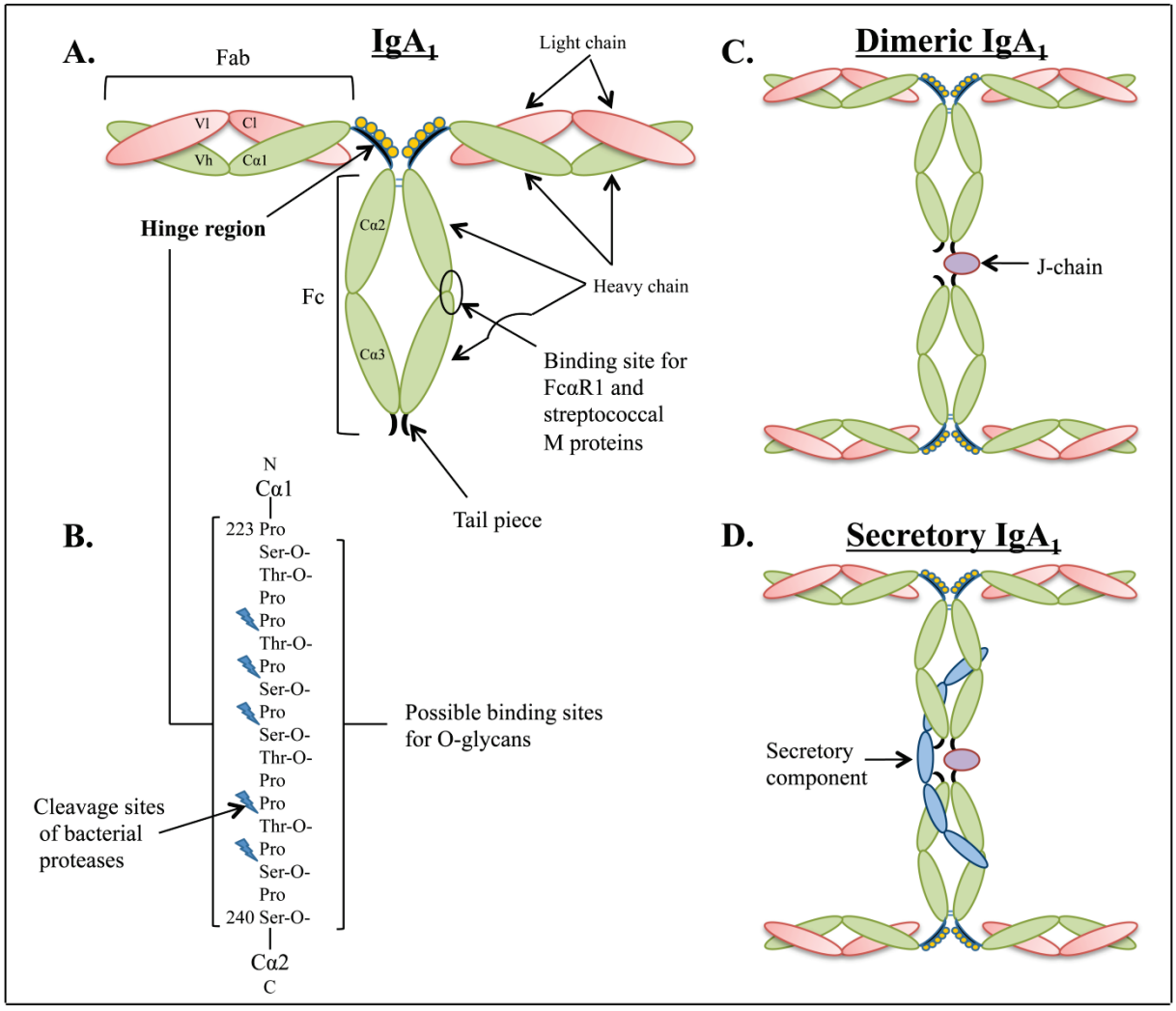

Figure 1: Structural details of IgA_1 (A.) Monomeric IgA consists of two heavy chains (green) and two light chains (pink). The heavy chains include three constant regions ($C_{\alpha1}$-$C_{\alpha3}$) and a variable region (V_h), the light chain consists of a constant region (C_l) and a variable region (V_l). V_h, $C_{\alpha1}$, V_l and C_l form the Fab-segment. The remainder constitutes the Fc-segment. The binding site for Fc_{α}-receptor 1 and streptococcal M proteins are indicated. (B.) The amino acid sequence of the hinge region with possible binding sites for ortho-glycans and cleavage sites of bacterial proteases. (C.) Dimeric IgA_1 consists of two monomeric IgA_1 units convalently joined by the J-chain. (D.) Secretory IgA_1 differs from dimeric IgA_1 by the addition of the secretory component.

IgA_1 differs from IgA_2 mainly due to its hinge region, a proline (Pro)-rich sequence of about 18 amino acids between Val^{222} ($C_{\alpha1}$) and Cys^{241} ($C_{\alpha2}$)[12]. The hinge-region harbors binding-sites for three to five, occasionally up to six, O-linked sugars attached to serine (Ser) or threonine (Thr) residues[1,13] (Figure 1B). Besides these O-glycans both subclasses of IgA contain several N-linked sugars adding to their molecular mass[14].

Due to the presence of Pro-Ser or Pro-Thr amino acid bonds in the hinge region of IgA_1 this form of IgA is susceptible to several bacterial proteases (eg from *Streptococcus pneumonia, S. oralis, S. sanguis, S. mitis, Haemophilus influenza, Neiseria meningitides* and *N. gonnorrhoeae*) which cleave the hinge region and render the IgA dysfunctional (Figure 1B)[1,15].

The development of an elongated hinge-region in IgA_1 is considered to be advantageous as it allows IgA_1 to spread its Fab-segments farther apart and thus reach antigens with considerable space in between[16]. On the other hand, IgA_2 has the advantage of being resistant to bacterial proteases, which may be the reason for its predominance in colonic mucosal secretions (Table 1). Immunoglobulins, which polymerize (IgA, IgM) share a common 18 amino acid elongation of the C-terminal of their heavy chains, the so-called tail piece[17].

Dimeric IgA is assembled in plasma cells by covalently connecting the tail pieces of $C_{\alpha 3}$ of two monomeric IgAs with a 15 kD joining (J)-chain into a dimer (Figure 1C). Occasionally more IgA molecules are connected by J-chains forming larger oligomeric forms. Dimeric and oligomeric forms are designated polymeric IgA (pIgA).

1.1.1.2. *Secretory IgA*

PIgA synthesized in MALT is mainly secreted (e.g. in saliva, gastric juice, jejunal and colonic fluid, hepatic bile, colostrums, nasal fluid, bronchial secret, and tears) and therefore found on mucosal surfaces. It initially binds covalently to the polymeric immunoglobulin receptor (pIgR) on the basolateral side of mucosal epithelial cells and is, while still receptor-bound, actively transported to the luminal side of the epithelial cell. At the mucosal surface the 80 kD extracellular fragment of pIgR is proteolyzed from the rest of pIgR and secreted attached to IgA as the stabilizing secretory component, thus forming sIgA[1] (Figure 1D). sIgA is the most abundant immunoglobulin at mucosal sites, where it serves as a first line of defense against invading pathogens and toxins. This is accomplished by binding to the invading pathogen and inducing immune exclusion including the blocking of binding to mucosal receptors, facilitating the entrapment in mucus and removal from mucosal sites. sIgA may transport bound pathogens within the mucosal layer via the pIgR-shuttle back to the mucosal lumen. Furthermore, sIgA may be important for the maintenance of the intestinal homeostasis and tolerance towards antigens by influencing the intestinal microbiota through manipulation of bacterial virulence factors and promotion of biofilm formation. Presentation of IgA-bound antigens to dendritic cells within the MALT leads to a down-regulation of pro-inflammatory

responses, which usually are generated by the mucosal up-take and presentation of bacterial or other potentially allergenic antigens (reviewed in[18]).

A minor fraction of $sIgA_1$ is found in the circulation. Thus sIgA may be released to the basolateral side of mucosal epithelial cells[19] or be reabsorbed from the mucosal lumen for exemple by M cells (microfold cells, specialized on mucosal up-take of luminal antigen for presentation in the MALT of the upper gastro-intestinal tract) and thus gain access to the circulation[18].

1.1.2. Ortho-glycosylation of the hinge-region of IgA_1

Ortho (O)-glycosylations are common in human membrane-bound proteins, whereas in plasma proteins they are only found in IgA_1, IgD, and complement factor 1 (C1)-inhibitor[20]. O-glycosylation of proteins, which is usually initiated in the Golgi apparatus of cells, influence the properties of the protein in various ways, for example by influencing its structure and thereby its receptor affinity, activity and clearance, aggregability, stability, and antigenic properties. O-glycosylation may function as a neoepitope, promote antigenicity and display molecular mimicry of other similar epitopes (reviewed in[21]).

The hinge region in IgA_1 includes several Ser and Thr residues, which are potential binding-sites for three to five (occasionally six) N-acetylgalactosamine (GalNAc) residues (Figure 1B). This ortho-glycosylation is controlled by a GalNAc-transferase (GalNAc-T, UDP-N-acetyl-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase). Several isotypes of GalNAc-T have been characterized, with varying tissue specificity, and shown to exhibit different primary sequence preferences and thereby substrate specificity. The primary binding site of a specific isotype of GalNAc-T is determined by the isotype itself as well as by which ser/thr is exposed (reviewed in[21], Figure 2).

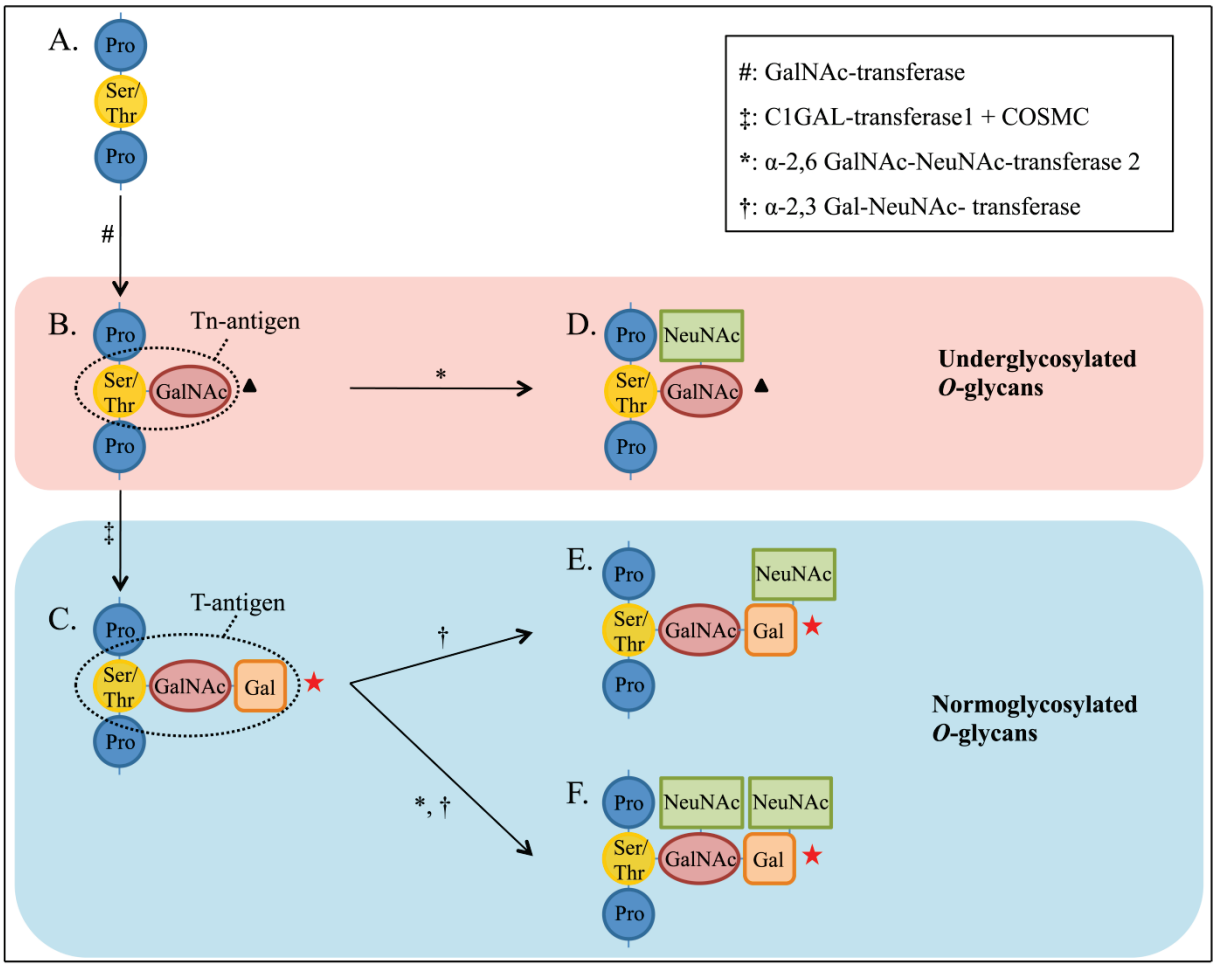

Figure 2: Ortho-glycosylation of the hinge region in IgA_1 Serine or threonine residues of the hinge region of IgA_1 (A.) are O-binding-sites for GalNAc (B.) resulting in the Tn-antigen. Tn-antigen further binds either Gal (to form the T-antigen (C.)) or NeuNAc (which precludes further binding of Gal (D.)) . T-antigen can bind up to two NeuNAc (E-F). Normoglycosylated O-glycans (C, E, F) and underglycosylated forms of O-glycans (B, D.) are indicated. Reactivity with Jacalin is depicted by a red star and to *Helix aspersa* lectin by a black triangle. Abbreviations: Ser: serine, Thr: threonine, GalNAc: N-acetyl galactoseamine, NeuNAc: N-acteyl neuraminic acid, Gal: galactose

The resulting Ser/Thr-GalNAc is termed the Tn-antigen[22]. It can be further extended by a β1,3-binding of galactose (Gal) to GalNAc facilitated by the enzyme C1GAL-transferase 1 (core 1-synthase, T-synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1), which function is dependent on its chaperone Cosmc[23]. The resulting ortho-glycan Ser/Thr-GalNAc-Gal is termed the Thomsen-Friedenreich antigen or T-antigen. Finally, the Tn- or T-antigen may be extended by the addition of highly negatively-charged N-acetylneuraminic acid (NeuNAc, sialic acid) in a α2, 3-linkage to Gal and then in a α2, 6-linkage to GalNAc under the influence of specific NeuNAc-transferases[24]. A premature sialation of the Tn-antigen precludes elongation with Gal and may be one reason for the generation of underglycosylated forms of IgA_1[25] (Figure 2).

The O-glycans in IgA_1 may appear in five different compositions as shown in Figure 2 (B, D, C, E, F). IgA_1 variants lacking galactose bound to GalNAc are referred to as underglycosylated or undergalactosylated IgA_1 (Figure 2). In healthy individuals the carbohydrate composition of serum IgA_1 is heterogeneous and the assembly of O-glycans within one IgA_1 may vary. The most prevalent glycosylation forms may include the T-antigen and its mono– and di-sialylated forms, but underglycosylated variants of IgA_1 can be found in normal serum in minute amounts[26].

1.1.3. IgA receptors

Several structurally unrelated IgA Fc receptors ($Fc_\alpha R$) have been described in humans (Table 2).

Table 2: Human IgA Fc receptors

IgA receptors	Presence	Ligand	Response to IgA binding	Ref
$Fc_\alpha R1$ (CD89)	Myeloid cells	$mIgA_1$, $mIgA_2$ $pIgA_1$, $pIgA_2$	Pro-inflammatory or anti-inflammatory Immunomodulation	27
sCD89	Blood circulation	pIgA	Unknown	28
$Fc_{\alpha/\mu}R$	Secondary lymphoid tissues Mesangial cells	$pIgA_1$, $pIgA_2$ IgA-coated targets IgM	Immunomodulation Absorption of pathogens	29
Polymeric Ig receptor	Mucous membranes Glandular epithelia in liver, breast, lacrimal glands	$pIgA_1$, $pIgA_2$ IgM IgA-immune complexes Intraepithelial/ luminal bacteria and viruses	Mucosal /glandular transport of secretory immunoglobulin Antigen and pathogen excretion Immune exclusion	27,30
Asialo-glycoprotein receptor	Hepatocytes	$IgA_2 > IgA_1$	IgA_2 clearance	31,32
Transferrin receptor (CD71)	Bone marrow stromal cells Activated T and B lymphocytes Macrophages Proliferating cells Mesangial cells	Transferrin $pIgA_1 > mIgA_1$	Pro-inflammatory Mesangial proliferation	33-36
Mannose receptor	Dentritic cells Macrophages Mesangial cells	pIgA	Inflammatory responses Phagocytosis	37,38

mIgA: monomeric IgA, pIgA: polymeric IgA, Ig: immunoglobulin

$Fc_{\alpha}R1$ (CD89) is strictly restricted to cells of the myeloid linage and found on neutrophilic and eosinophilic granulocytes, monocytes, macrophages, dendritic cells, and hepatic Kupffer cells. It binds with high affinity to Fc of both IgA_1 and IgA_2. For signal transduction and cellular responses to IgA binding CD89 is dependent on the association with the signaling unit of the receptor, the FcR γ-chain[39]. When IgA-antigen complex bind the cross-linking of CD89 results in an inflammatory response due to release of proinflammatory cytokines and superoxides from the myeloid cell, antibody-mediated cellular cytotoxicity, and phagocytosis[32]. On the other hand, if IgA is bound without antigen CD89 will mediate antibody recycling and an anti-inflammatory response. This is achieved by the induction of the inhibitory configuration of the FcR γ chain[40] and the subsequent down-regulation of pro-inflammatory cytokines as well as up-regulation of IL-1 receptor-antagonist in monocytes and peripheral blood mononuclear cells[41-44] (reviewed in[27,32]). CD89 shedding from myeloid cells has been described after activation or in response to elevated serum levels of underglycosylated polymeric IgA_1. The extracellular part of CD89 has been detected in varying amounts in the circulation tightly bound to polymeric IgA as a 30 kD molecule designated soluble (s)CD89[27,28,45,46]. The physiological or pathophysiological role of sCD89 is so far unknown.

$Fc_{\alpha/\mu}R$ is abundant in both secondary lymphoid tissues and mesangial cells and is believed to play a regulatory role during inflammation. Furthermore, there is evidence that it plays a role in the primary stages of antimicrobial immune responses. Together with the pIgR, $Fc_{\alpha/\mu}R$ shares an affinity for polymeric immunoglobulins (IgA, IgM)[29,32].

PIgR is expressed on apical surfaces of all mucous membranes as well as in the glandular epithelia of the liver, breast and lacrimal glands. It serves as a shuttle for polymeric IgA and IgM to the luminal side, where they are excreted as secretory immunoglobulin. Furthermore, it binds IgA-immune complexes leading to pathogen- or antigen excretion. Intraepithelial or luminal bacteria and viruses may be bound by pIgR, which thus participates in immune exclusion. PIgR may be translocated to the luminal side without previous binding to a ligand. At the luminal side the extracellular part is detached constituting free secretory components, which carry out several other important immunomodulatory and defense functions on the mucosal surface (reviewed in[30]).

On the IgA molecule there is a site-overlap for Fc-binding to CD89, $Fc_{\alpha/\mu}R$ and pIgR[47].

The asialo-glycoprotein receptor (ASGP-R) expressed on hepatocytes recognizes terminal Gal or N-acetyl glucosamine residues, which both have been detected on IgA[9]. The recognition of Gal may be partially impaired by NeuNAc bound to Gal[48]. ASGP-R-bound IgA is either degraded intracellularly or may escape intact into the hepatic biliary excretion and thus reappear in jejunal fluid. ASGP-R has a much higher affinity for IgA_2 than IgA_1 and is the main known pathway for IgA_2 clearance from the circulation and the reason for the lower serum levels of IgA_2 compared to IgA_1[31].

The transferrin-receptor (CD71) is expressed on bone marrow stromal cells, activated T and B lymphocytes, macrophages, and proliferating cells, in which it is involved in iron transport[33]. It is expressed on human mesangial cells and was shown to bind polymeric IgA_1 with a much higher affinity than monomeric IgA_1[34]. Exposure of human mesangial cells to underglycosylated polymeric IgA1 leads to an up-regulation of the mesangial expression of CD71[32,35].

Recently lectins have attracted interest as sensors for altered glycosylations and thus as possible IgA receptors in the mesangial region. The mannose receptor, a member of the C–type lectins, has been described on dendritic cells, macrophages and mesangial cells[37,49] and has been shown to bind sIgA[38]. C-type lectins are involved in the maintenance of tolerance towards endogenous glycoproteins and may induce inflammatory responses due to structural alterations in glycoproteins.

Among the FcαRs described above CD71, $Fc_{\alpha/\mu}R$, and the mannose-receptor have been shown to be expressed on human mesangial cells. The $Fc_{\alpha/\mu}R$ has an equal affinity for IgM and for polymeric IgA. As mesangial immune deposits in tissue samples from patients with IgAN usually do not contain significant amounts of IgM the $Fc_{\alpha/\mu}R$ is probably not involved in the pathogenesis of the disease[50]. The role of the other receptors as well as sCD89 in the pathogenesis of IgAN is further discussed in the chapter 1.3.6.4.IgA-receptors in IgAN.

1.2. Complement system

The complement system, an important part of the human innate immune system, was discovered more than 100 years ago and characterized by its "complementary" bactericidal activity and role in phagocytosis of cellular debris[51-53]. It is involved in the host-protection against invading pathogens and disposal of immune complexes and apoptotic cells and provides a link between the innate and adaptive immune system. More than 35 proteins collaborate in the complement system to assure efficient, directed activation in specific pathways and their strict control[54].

1.2.1. Activation of the complement system

The complement system can be activated by three specific pathways: the classical pathway, the lectin pathway, and the alternative pathway of activation. The factors which activate each of the three pathways are summarized in Table 3[54-57].

Table 3: Activators of the complement system

Classical pathway	Lectin pathway	Alternative pathway
• Immune complexes • Apoptotic cells • Certain viruses and Gram-negative bacteria • C-reactive protein bound to ligand	• Terminal mannose on microbial surfaces • Polymeric IgA	• Bacteria, viruses, fungi • Tumor cells • Apoptotic cells • IgA

The first step in each of the pathways is the formation of a C3 convertase. The classical and lectin pathways converge via the proteolysis of C4 and C2 to a common C3 convertase (C4b2a) (Figure 3). Within the alternative pathway spontaneous hydrolysis of C3 and the proteolysis of complement factor B (CFB) in the presence of complement factor D (CFD) enables minimal albeit constant activation. The alternative pathway C3 convertase (C3bBb) is formed in response to activation as shown in Figure 3. For durable function it is stabilized by properdin[54,58].

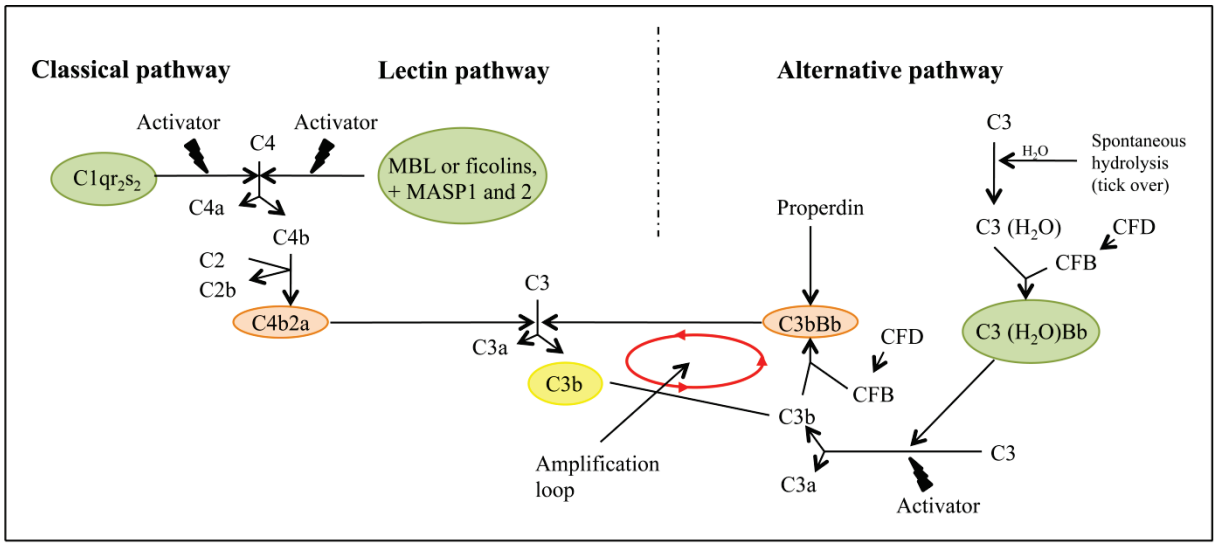

Figure 3: The formation of the C3 convertase. The C3 convertases are indicated in orange. MBL: mannose-binding lectin, MASP: MBL-associated serine proteases, CFB: complement factor B, CFD: complement factor D.

Both C3 convertases cleave C3 to C3b and C3a. A part of the generated C3b will be deposited in proximity to the initiating event and participate in the formation of additional alternative pathway C3-convertases. Thereby any activation will be strongly amplified (amplification loop, Figure 3). The function of the amplification loop is of central importance for effective activation of the complement system[59].

By binding C3b both C3 convertases proceed to form the C5 convertase (Figure 4).

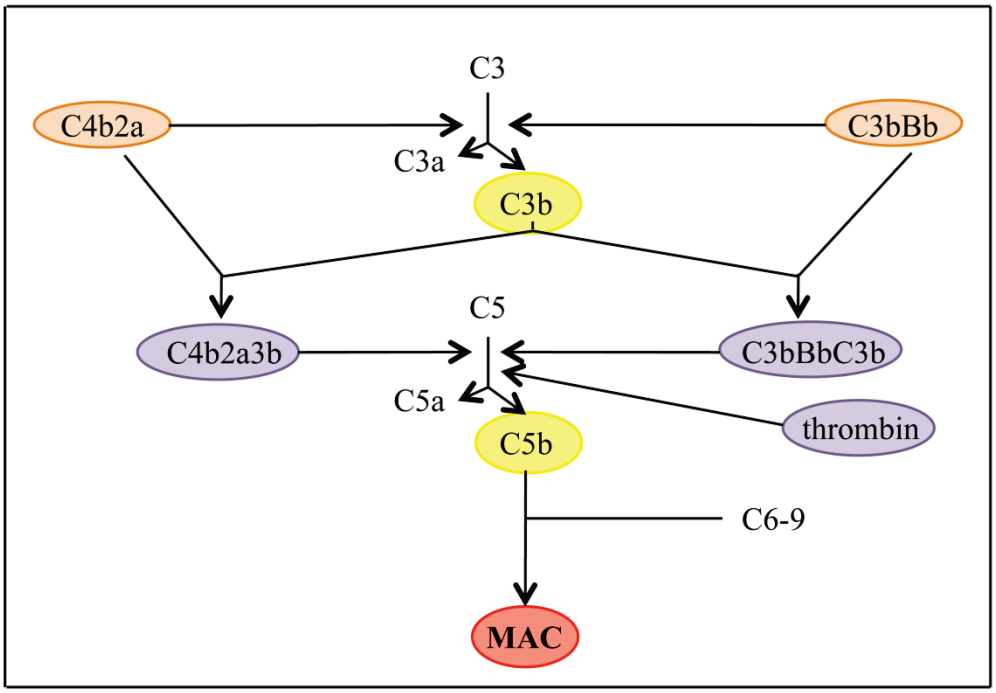

Figure 4: The formation of the C5 convertase and the MAC. The C3 convertases are indicated in orange, the C5 convertases in purple and the MAC in red. MAC: membrane attack complex.

Thus C5 is cleaved to C5b and C5a. C5 convertase bound C5b binds C6 and C7. The C5b67-complex is then released from the C5-convertase to bind to lipid bilayers. Upon further binding of C8 and C9 the membrane attack complex (MAC) is formed. The MAC is a lipophilic membrane insert, which forms pores in the surface membrane of cells or microbes and thus induces lysis[54,58,60-62] (Figure 4).

Thrombin has been described to act as a C5-convertase. This pathway could generate C5a and C5b independent of previous cleavage of C3[63] (Figure 4).

Several split-products generated during complement activation have important immunological functions. Opsonization of antigens is performed by C4b and the C3 cleavage products C3b, iC3b and C3dg, which are retained covalently bound to membranes and recognized by complement-receptors 1-4 on phagocytes[64]. IC3b and C3dg stimulate B-cells and antigen-presenting cells[65]. Through these interactions they stimulate an antibody response to the bound antigens and induce immunological memory, which are an example of a link between innate and adaptive immunity. Binding of C1q, C3b, and C4b to immune complexes and apoptotic cells leads to opsonization followed by clearance. C3a and C5a act as anaphylatoxins thereby attracting and activating leucocytes, enhancing phagocytosis and local vasodilatation. Receptors for C3a (C3aR) and C5a (C5aR) have been described on monocytes, macrophages as well as C3aR in the kidney on podocytes and proximal tubular epithelial cells[54,59,66-68].

Of all complement factors C3 is the most abundant with a serum concentration of about 1.2 mg/ml. It is mainly synthesized in the liver. Extrahepatic sources include endothelial cells, fibroblasts, mesangial cells, monocytes, and polymorphonuclear leukocytes[69-72]. C3 synthesis is up-regulated by pro-inflammatory stimuli such as IL-1, IL-6, and LPS[73,74] and in mesangial cells after exposure to immune complexes[75].

1.2.2. Regulation of the complement system

The activity within the complement system is carefully adapted to the actual needs of the host through a tightly controlled balance between activation and inhibition. Malfunction within this regulation of the complement system results in disease.

Factors which promote activation of the complement system include the supply of complement factors and co-factors such as CFB, CFD, and properdin. Several regulators

inhibit the complement system in order to protect host tissue and prevent depletion of complement factors. The main regulators are summarized in Table 4[59,76-78].

Table 4: Main regulators of the complement system.

Pathway	Localization	Complement regulator	Major functions in the complement system
Alternative	Fluid phase	CFH	Co-factor for CFI in cleavage of C3b Prevents formation and accelerates decay of C3bBb Host cell recognition
		Properdin	Stabilizes the C3bBb convertase
	Membrane	CD46 (MCP)	Co-factor for CFI in cleavage of C3b
Classical/ lectin	Fluid phase	C1q	Activation of the classical pathway
		C1-INH	Blocks serine proteases, suicide substrate for C1r, C1s, and MASP2
		C4-BP	Co-factor for CFI in cleavage of C4b Accelerates decay of C4bC2a
	Membrane	CD35 (CR1)	Clearance of immune complexes Regulation of C3-degradation Accelerates decay of C3 convertase
Common for alternative and classical/ lectin	Fluid phase	CFI	Degrades C3b and C4b in the presence of cofactors
	Membrane	CD55 (DAF)	Accelerates decay of C3 convertases
Terminal	Fluid phase	Clusterin	Inhibits MAC formation
		Vitronectin	Inhibits MAC formation
		CFHR1	Inhibits C5 convertase
	Membrane	CD59 (protectin)	Inhibits MAC formation

CFH: complement factor H, CFI: complement factor I, FHL-1: CFH-like protein-1, C1-INH: C1-inhibitor, C4-BP: C4-binding protein, CFHR1: CFH-related protein 1

Regulation by host recognition

The cleavage of C3 by the C3 convertase generates C3b, which harbors a highly reactive thioester enabling it to bind to carbohydrates and protein-receptors on membranes. Depending on the biochemical properties of the membrane different co-factors are attracted. Polyanionic structures prevalent on host cells attract and bind CFH. CFH is a cofactor for CFI enabling cleavage of C3b into the inactive iC3b. Microbial membranes usually lack polyanions on their surface, which generates an environment that favors the binding of CFB instead of CFH. CFB is susceptible for cleavage by CFD into Bb and Ba and thus new C3-convertases are formed. Thus complement system activation is amplified and focused on surfaces lacking the

protective coating of polyanions, i.e. glycosaminoglycans, phospholipids and sialic acids, such as microbial membranes[54].

The following chapter addresses the structure and function of CFH. A comprehensive description of the precise mechanisms, which underlie the delicate balance of factors others than CFH controlling the complement system, is given in recent reviews[59,76].

1.2.3. Factor H and Factor H-like protein-1

CFH is a glycoprotein present in plasma at a concentration of about 110-615µg/ml. It has a size of 155 kD and is composed of 20 short consensus repeats (SCR, complement control protein modules) each of which consists of 60 amino acids (Figure 5). The *CFH* gene is located within the regulators-of-complement-activation gene cluster on chromosome1q32. Each SCR is encoded in a separate exon in the *CFH* gene[79]. FHL-1 is an alternative splicing product of the *CFH* gene. It is comprised of the N-terminal SCRs 1-7 of CFH with four additional amino acids at its C-terminal and is present in human plasma as a 42 kD protein[80] (Figure 5).

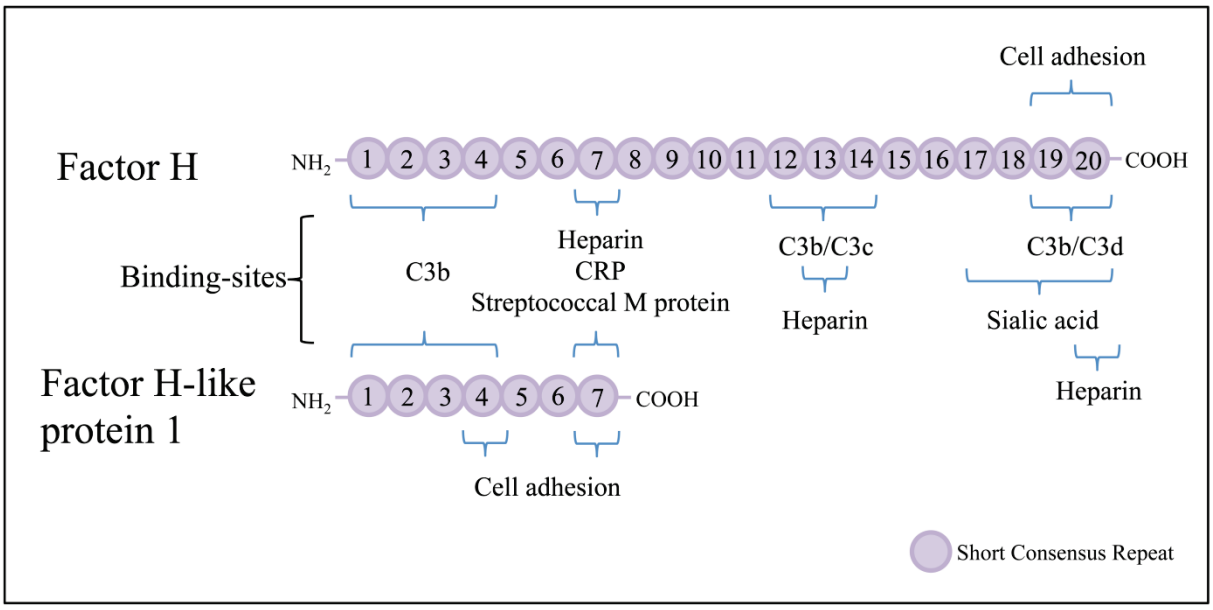

Figure 5: Complement factor H and complement factor H-like protein 1

As mentioned above, CFH has three main regulatory functions in the alterative pathway of complement. It prevents formation and accelerates decay of the C3 convertase, it acts as a co-factor for CFI in the cleavage of C3b and it recognizes host cells based on their membrane composition. Specific binding-sites have been detected on CFH as shown in Figure 5. Surface-binding and host-recognition is located at the C-terminal region at SCR 19 and 20,

where binding sites to sialic acid, heparins and C3b have been detected. The cofactor and decay accelerating activity is located at the N-terminal at SCRs 1-4. M proteins of certain Group A streptococcal serotypes have been shown to bind to CFH in SCR 7[81].

Due to structural homology between FHL-1 and CFH the FHL-1 protein shares the cofactor and decay accelerating activity and a binding site for polyanions and GAS M proteins. Surface-binding of FHL-1 is mediated either by SCR 7 or an Arg-Gly-Asp (RGD) sequence motif in SCR 4, which binds to integrin receptors on cell surfaces (Figure 5)[80].

Despite the functional and structural overlap between CFH and FHL-1 significant differences in their activity have been reported. *In vitro* experiments show an up to 100-fold more effective decay acceleration activity of CFH than FHL-1, which is in accordance with findings of C-terminal mutations in *CFH* with normal FHL-1 in patients with atypical HUS[82,83]. There is thus evidence for a limited importance of FHL-1 in the regulation of the alternative pathway C3 convertase. On the other hand, the binding affinity of FHL-1 to M proteins is stronger than that of CFH, possibly due to the hydrophobic four amino acid tail in FHL-1 not present in CFH[84].

1.2.4. Complement mediated renal disease

Complement-mediated diseases affecting kidneys are summarized in Table 5.

Table 5: Complement-mediated renal diseases

Affected pathway	Disease	Mechanism behind complement activation	Refs
AP	Atypical hemolytic uremic syndrome	• Loss-of-function mutations in CFH (C terminal), risk-associated polymorphisms in CFH, deletions of CFH-related proteins, mutations in CFI, thrombomodulin, CD46 (membrane co-factor protein), clusterin, gain-of-function mutations in C3 or CFB, auto-antibodies to CFH	85
	Dense deposit disease	• Mutations in CFH N-terminal (blocking secretion) or impaired binding to C3b. • Auto-antibodies: C3-nephritic factor or antibodies to CFB/Bb: stabilize alternative pathway C3 convertase, CFB antibodies inhibit the C5 convertase	86 - 88
	C3 glomerulopathy	• Dysregulation of the alternative pathway and MAC-formation – in most cases of unknown origin • Mutations in C3 conferring resistance to CFH, in CFHR5 conferring reduced inhibition of alternative pathway C3 convertase or CFH impairing co-factor and decay accelerating activity and binding to C3b	89
	ANCA-associated vasculitis	• ANCA-activated neutrophilic granulocyte release C3 and CFB, which activate the alternative pathway. Thereby generated C5a attracts neutrophilic granulocytes and primes them for interaction with ANCA.	90
Mainly AP	Acute post-streptococcal GN	• Mainly the alternative pathway is activated. Proposed mechanisms include glomerular deposition of and local activation by streptococcal enzymes (NAPlr, SpeB).	91
CP	Systemic lupus erythematodes (SLE)	• Activation of the classical pathway by immune complexes • Secondary depletion of complement factors: reduced tolerance to auto-antibodies, reduced degradation of apoptotic cells or immune complexes, and impaired synthesis of cytokines • Genetic deficiency of C1q, C4, (C1r/s, C2) confers susceptibility for SLE	92, 93
	Membranous GN	• Activation of the classical pathway most likely due to circulating immune complexes	94
Mainly CP	Ischemia – reperfusion, solid organ transplantation	• Activation of classical pathway • C3aR and C5aR signaling on antigen-presenting cells and T-cells leads to T-cell proliferation and stimulation, and antibody-production (antibody-mediated rejection). • Activation of the alternative pathway (together with classical pathway) involved in host-versus-graft reactions	95

ANCA: anti-neutrophil cytoplasmatic antibodies, GN: glomerulonephritis

1.3. IgA nephropathy

1.3.1. Clinical features

IgA nephropathy (IgAN), the most common primary form of kidney inflammation, is most commonly diagnosed at age 10-40 years with a peak incidence between 20-30 years of age. It may, however, affect patients at any age[96]. During the early phase of the disease the majority of cases do not display clinical signs. Thus these patients would be unaware of the disease unless they are diagnosed due to screening or coincidental sampling. Indeed, in one study 60% of cases diagnosed with idiopathic IgAN were found by chance [97]. Isolated microscopic hematuria is found in approximately 62 % of cases at the time of debut. Macroscopic hematuria and proteinuria in conjunction with infections, usually affecting the upper respiratory tract, appear in 26 % of cases. This clinical picture is more common in patients aged 10-30 years and hardly seen in patients older than 40 years. Acute nephritic or nephrotic syndrome is seen in about 12 % of cases[50,98]. A serious complication is the development of malignant hypertension[99].

1.3.2. Pathology

IgAN was formally defined by Berger and Hinglais as a primary inflammation of the kidney characterized by mesangial cell proliferation, matrix expansion and immune deposits containing predominantly IgA[100]. The light microscopy features include proliferation of mesangial cells and matrix. During advanced stages of disease other lesions are found including diffuse endocapillary proliferation, crescent formation, segmental sclerosis or necrosis, glomerulosclerosis, interstitial fibrosis, tubular atrophy, and vascular affection. Strong immunofluorescence staining for IgA is the defining feature, staining is found in the mesangial region and occasionally also in glomerular capillary loops. Besides IgA immunofluorescence almost always demonstrates C3 and sometimes even minor amounts of IgG, IgM, lambda and kappa light chains within the same regions. Immune electron microscopy demonstrates that the IgA and C3 are localized to the mesangial matrix, glomerular basement membrane, and in glomerular capillary loops[50,96,101-106].

The Working Group of the International IgA Nephropathy Network and the Renal Pathology Society proposed a new classification for pathological findings in kidney biopsies in 2009. Four pathologic variables (mesangial hypercellularity, endocapillary hypercellularity,

segmental glomerulosclerosis, and tubular atrophy or interstitial fibrosis) were evaluated and the resulting score (MEST score) was shown to have prognostic significance independent of clinical features in both children and adults[107-109].

1.3.3. Diagnostic work-up

The diagnosis IgAN may be clinically assumed based on symptoms and the presence of hematuria in a urine sample, but is more definitively determined by kidney biopsy showing the pathological features described above. In a patient with mesangioproliferative glomerulonephritis the detection of marked IgA staining in mesangial immune deposits is considered indicative of IgAN[96].

1.3.4. Epidemiology

IgAN is the most common form of primary glomerulonephritis worldwide. The world-wide incidence of IgAN was estimated to be about 2.5/100 000/year[110]. Incidence may, however, vary in different populations. In previous studies from Europe and North America the incidence was reported to be between 1 - 4 cases/ 100 000/ year[50,97,111-113] whereas it was found to be as high as 4.5/100 000/year in Japanese children[114]. In another Japanese investigation 1.6 % of graft kidneys biopsied before transplantation exhibited pathological features compatible with IgAN[115].

Variations in reported rates of incidence may partly be due to differences in diagnostic routines. Urine-screening programs[116] may detect many cases that lack any clinical symptoms and would not have been diagnosed under other circumstances. Furthermore, kidney biopsy practices vary significantly between countries. Kidney biopsy is performed in cases with uncomplicated microscopic hematuria in some Asian countries, detecting many clinically asymptomatic cases with excellent long-term prognosis, which would increase the reported incidence in these countries. Conversely, it is not customary to perform a kidney biopsy in many Western countries unless the patient develops increasing or persistent proteinuria or affected renal function. Differences in incidence may, furthermore, depend on the genetic background, as suggested by the finding that IgAN is uncommon in afro-Americans or blacks in South Africa when compared to whites or Asians living in the same surroundings[117,118]. An extremely high prevalence of IgAN was found in Native Americans from New Mexico[119] and Australian aborigines[120]. Another factor that obviously influences reported incidence rates is

the fact that lower socioeconomic status is related to limited health care access and therefore underestimation of the diagnosis in these groups[121].

IgAN affects males twice (Japan) to six times (Northern Europe and USA) more often than females[50]. Most cases are sporadic cases, but in about 10-15 % of the cases IgAN is familial[122].

1.3.5. Outcome and predictors of outcome

In about 20 % (15 – 40 %) of cases IgAN will proceed to end-stage renal disease (ESRD) within 20 years[50,123]. Disease progression and thus prognosis of IgAN depend on the clinical features and pathological findings in the kidney biopsy. Hypertension, impaired renal function at time of diagnosis, persistent proteinuria, especially if exceeding 1 g/d, and a high histopathological MEST score have been shown to be predictors of poor renal survival in both children and adults with IgAN[124-134]. In adults obesity was shown to be a further independent risk factor in IgAN[135,136]. Obesity in children may cause renal damage, known as obesity-related nephropathy[137], but its influence on the course of IgA nephropathy has so far not been addressed in studies. These indicators of risk for progression of IgA nephropathy, also helpful in the detection of high-risk cases with need for intensive medical treatment, may not be valid on a case-to-case basis, as even cases without risk-factors may proceed to ESRD[138].

1.3.6. Pathogenesis

Despite being the most common form of primary glomerulonephritis world-wide the pathogenesis of IgAN is largely unknown. The following section will describe what is known thus far.

1.3.6.1. *Genetic factors*

The fact that ethnicity may influence disease incidence and that about 10 - 15 % of cases are familial indicates that genetic factors may be of importance in the pathogenesis of IgAN[139]. Much effort has therefore been devoted to genetic investigations and genome-wide association studies (GWAS) of clusters of patients or families with IgAN.

A Japanese case-control association study suggested that susceptibility to IgAN in the Japanese population was related to polymorphisms or a missense mutation within the gene for the polymeric immune globulin receptor (pIgR) causing defective IgA transcytosis and thus increased serum levels of mucosal polymeric IgA_1[140,141].

GWAS carried out on a European cohort of patients with IgAN reported an association risk-alleles for IgAN with the major histocompatibility complex (MHC)[142]. Recently GWAS on two independent large cohorts of Chinese and European ancestry in the USA identified five common loci, of which three were polymorphisms located in the MHC. One locus could be defined as a polymorphism in the intron 12 of the gene for CFH, which was correlated with a deletion spanning over the gene encoding factor H-related protein 3 and 1. The fifth locus detected in the GWAS was related to an intronic polymorphism on chromosome 22q, which includes genes for cytokines involved in mucosal immunity and inflammation. All polymorphisms in the detected loci were, however, associated with a decreased risk for the development of IgAN. The cumulative effect of the protective gene variants was estimated to explain about 4-7% of disease variance and a ten-fold variation of the inter-individual risk for IgAN[143].

Despite these findings the obscurity of the genetic background associated with IgAN supports the theory of a combination of a complex genetic background and environmental factors necessary for disease development[139,144-146], which is further emphasized by the fact that the clinical course of IgAN may differ in genetically identical monozygotic twins[147].

1.3.6.2. *Infectious agents*

As the outbreak or relapse of IgAN is regularly preceded by infections, commonly affecting the upper respiratory tract, infectious agents have been suspected to be involved in the pathogenesis of IgAN. There is evidence that circulating IgA-binding antigens may play a critical role as mediators of glomerular injury during the development of IgAN[148]. Furthermore, infections could lead to the release of proinflammatory cytokines, which either systemically or locally may provide the necessary stimulus to precipitate latent inflammation[149].

There is circumstantial evidence for involvement of group A streptococci (GAS, *Streptococcus pyogenes*) in the pathogenesis or initiation of IgAN[150-152], but even other infectious agents have been implicated as tonsillar infections with *Haemophilus parainfluenzae*[153-156], respiratory infections with methicillin-resistant *Staphylococcus aureus*[157-159] or infections with enteroviruses[160].

1.3.6.3. *IgA_1 in IgAN*

The hallmark of IgAN is the deposition of IgA in the mesangial area. IgA is prevalent as two isotypes in humans (IgA_1 and IgA_2) and may be monomeric or polymeric. IgA_1 contains

several O-linked sugars in its hinge-region, some of which may differ in their composition (see section on IgA). Several studies have shown that the serum levels of underglycosylated polymeric IgA_1 are elevated in patients with IgAN compared to controls[161,162]. Circulating immune complexes in IgAN contain mainly IgA_1[163] and IgA in renal immune deposits consist predominantly of underglycosylated polymeric IgA_1[164,165].

An increased production of underglycosylated polymeric IgA_1 is dependent on the reduced expression or function of C1GalT1 or its chaperone Cosmc within IgA-producing plasma cells in the bone marrow or peripheral lymphoid tissue (see section on IgA)[166-168]. As the level of underglycosylated polymeric IgA_1 is elevated in some of the unaffected first-degree relatives to patients with IgAN the reduced expression of C1GalT1or Cosmc may be genetic determined[169]. However, no single mutation or other genetic determinant that could explain the alteration has been found so far (see section on Genetic causes). Furthermore, the ortho-glycosylation of other serum proteins such as IgD and C1 inhibitor was found to be normal in patients with IgAN, which were able to produce other possible variations of IgA_1 ortho-glycosylation during an immunological response to neoantigens.

The alteration of IgA_1 ortho-glycosylation may thus not be based on a genetically determined glycosylation defect, but more likely on an altered control of $pIgA_1$ production and galactosylation[170]. Along these lines there is evidence supporting the hypothesis that the reduced glycosylation of IgA_1 could be reactive, possibly due to Th2 cytokines released during the course of infections. In particular IL-4 has been shown to down-regulate the expression of C1GalT1 and Cosmc and the activity of the C1GalT1 in human B-cells, which results in a reduced glycosylation of the secreted IgA_1 (see Figure 2)[171-173].

Findings from immunization experiments in patients with IgAN and investigations addressing the regulation of IgA synthesis suggest an aberrant mucosal type of immune response to certain antigens, in which the mucosal type of IgA_1 is produced at systemic sites[174-180]. However, the actual pathophysiological background to this altered regulation of IgA synthesis in patients has so far not been completely elucidated.

IgA-containing immune complexes (IC) are regularly found in sera of patients with IgAN. Serum levels of IC increase during exacerbations[181] and they are considered to be a source for mesangial IgA-deposits[25]. Several attempts have been made to clarify the nature of the antigen involved in IC formation. The conclusions drawn from these studies are summarized

here. Altered glycosylation leading to underglycosylation of IgA_1 may lead to the presentation of antigenic structures within the hinge region, which could promote an antibody response to these neo-epitopes. The generated specific anti-hinge region IgG or IgA would react with the underglycosylated IgA_1, thus forming a complex[163,182]. A second theoretical possibility would be that antimicrobial IgG could cross-react with structures in the underglycosylated hinge-region and thus form an immune complex with IgA[183]. In either case the immune complex containing IgG could then be deposited in the mesangium, possibly by binding to the FcγR prevalent on human mesangial cells[184]. However, as most of the kidney samples from patients with IgAN actually lack significant amounts of IgG the actual importance of this mechanism remains unclear. A third plausible antigen contributing to the formation of immune complex with IgA could be the $Fc_\alpha R1$ (CD89). Elevated serum levels of underglycosylated polymeric IgA_1 cause an increased shedding of the extracellular domains of CD89 from myeloid cells, thus forming soluble CD89, which would react with IgA[27,46]. Finally, underglycosylated polymeric IgA1 could react with IgA-binding streptococcal M proteins and thus be deposited in the mesangial space (see PAPERs 1 and 3).

Binding of IgA or ICs containing IgA to mesangial cells could occur directly as polymeric IgA_1 lacking terminal sialic acid or galactose has been shown to exhibit an increased affinity to mesangial extracellular matrix proteins such as fibronectin and type IV collagen[185,186]. Alternatively binding could occur through IgA-receptors on mesangial cells (see next chapter).

In vitro experiments have shown that mesangial deposition of underglycosylated polymeric IgA_1 exerts an inflammatory response in renal cells. Upon exposure human mesangial cells react with an up-regulation of interleukin (IL)-6[187,188], IL-8[188], tumor necrosis factor (TNF)-α [189,190], monocyte chemotactic peptide (MCP-1)[191,192], platelet activating factor (PAF)[193] and transforming growth factor (TGF)-β[187,194,195] and down-regulation of vascular endothelial growth factor (VEGF)[196]. Exposure of human mesangial cells to large IgA-immune-complexes derived from pediatric or adult patients with active IgAN leads to mesangial cell proliferation[197,198].

The amount of sialic acids in underglycosylated polymeric IgA_1 has been investigated and found to be increased implying a possible role of highly anionic sialic acids in the surface binding of IgA_1[199]. However, contradictory results were found using mass-spectrometry. A

decreased sialation could lead to an increased presentation of and facilitated immunological reactions to GalNAc[200].

In some patients elevated serum levels of secretory IgA_1 ($sIgA_1$) as well as mesangial deposits of these antibodies have been detected. Detected $sIgA_1$ correlated with the amount of hematuria in these patients[201].

1.3.6.4. *IgA-receptors in IgAN*

Of the human IgA-receptors implicated in the pathogenesis of IgAN CD71, mannose receptor have been described to be expressed on human mesangial cells. CD89 is prevalent as soluble receptor (sCD89) in the circulation[28,34,35,49].

CD71 expression is up-regulated on mesangial cells in the presence of underglycosylated polymeric IgA_1. Binding of polymeric IgA to CD71 results in a mesangial cell proliferation and the release of the pro-inflammatory cytokine interleukin (IL)-6 and the profibrotic transforming growth factor (TGF)-β *in vitro*[36,187]. Like CD71, CD89 shows specific interactions with underglycosylated polymeric IgA_1. Shedding of sCD89 from myeloid cells is increased by underglycosylated polymeric IgA_1 *in vitro* and sCD89 circulates in the blood bound to polymeric IgA[27,28,46]. Increased serum levels of sCD89 have been found to be associated with disease progression of IgAN[202]. *In vitro* experiments show that IgA-bound sCD89 significantly increases the effect of IgA binding to CD71 on mesangial cells[35,36].

The potential importance of the mannose-receptor in autoimmune glomerulonephritis is indicated by results of studies of the role of mannose-deficiency in autoimmune disease. Mannosidase II-deficient mice develop spontaneously glomerulonephritis with glomerular depositions of C3, IgA, IgG, and IgM. *In vitro* exposure of mesangial cells to serum from mannosidase II-deficient mice caused mesangial cell activation and production of pro-inflammatory cytokines. The activation was mediated by a mannose-dependent binding mechanism[203]. The N-glycans of IgA in IgAN have, however, been reported not to differ from those in controls[20].

Galactin-8, a lectin expressed on and secreted by various human tissues including renal cells, is the only human galectin with high affinity for IgA_1[204,205] due to a special preference for α2,3-linked NeuNAc and further binding of β1,3-bound galactose[206]. Both these linkages are prevalent in normoglycosylated forms of IgA_1. The function of galectin 8 includes the regulation of cell growth, transformation, apoptosis, adhesion, and interactions, immune

responses and inflammation[207-209]. A reduced binding to galactin 8 has been detected for IgA from sera of patients with IgAN compared to controls[210]. The importance of these findings for the pathogenesis of IgAN remains to be shown.

1.3.6.5. *Cytokines*

Several cytokines have been shown to be of importance for the development of the histopathological picture and clinical features characterizing IgAN.

Interleukin-6

Urinary IL-6 was found to be elevated in IgAN during disease progression and may thus be useful to monitor disease activity in patients[211,212]. Kidney samples of patients with IgAN show positive staining for IL-6 in the mesangial region and using in situ techniques IL-6 was found to be up-regulated in mesangial cells from patients with IgAN[213,214]. In cases with advanced tubulointerstitial damage IL-6 was even detectable in tubular regions by immune histochemistry[213].

In vitro experiments have shown that IL-6 induces mesangial cell proliferation and extra-cellular matrix expansion in renal glomeruli[215]. The nephritogenic effect of IL-6 may be further amplified as it induces the secretion of MCP-1 from human mesangial cells *in vitro*, which in turn leads to the recruitment and activation of lymphocytes, granulocytes and monocytes. This induction is dependent on the co-delivery of the soluble receptor for IL-6 (see below)[216]. MCP-1 collaborates with IL-6 in inducing collagen synthesis by HMC and could be involved in the expansion of the mesangial matrix seen in IgAN[217]. IL-6 enhances IgA secretion from plasma cells, which could contribute to the elevated IgA synthesis seen in a part of patients with IgAN[218,219].

The synthesis of IL-6 is induced in various tissues by viral and bacterial infections, and proinflammatory cytokines such as TNF-α, IL-1, platelet-derived growth factor (PDGF), and interferon (INF)-γ[220]. Underglycosylated polymeric IgA_1 stimulates the synthesis and secretion of IL-6 from human mesangial cells *in vitro*[187,198,221,222].

IL-6 executes its effects by binding to its receptor, which consists of two subunits. The α-subunit (IL-6R), a 80kD transmembrane glycoprotein, guarantees ligand specificity, whereas gp130, shared by other cytokines of the IL-6 family, is the signal transducing subunit. IL-6 binding to the α-subunit leads to generation of a complex with gp130 and further down-stream signaling resulting in activation of nuclear transcription. The extracellular part of IL-6R,

either detached from a cell or secreted as an alternative splicing product, is prevalent in the circulation as soluble (s)IL-6R. sIL-6R is able to bind IL-6 and associate with gp130, implying the possibility to induce IL-6 signaling in cells that only express gp130, which may be the case on HMC[216,223].

TGF-β and other cytokines

TGF-β is of major importance in the development of peritubular fibrosis and glomerular sclerosis in renal inflammatory processes in general (reviewed in[224]). The level of expression of TGF-β in kidney samples of patients with IgAN was found to be related to the development and progression of glomerulosclerosis, renal tubular injury and peritubular fibrosis[214,225,226].

VEGF synthesized and secreted from podocytes is involved in the repair of glomerular damage and a deficient VEGF supply has been implicated in the development of proteinuria (reviewed in[227,228]). Reduced expression of VEGF due to podocyte injury, as seen in advanced stages of IgAN, may lead to endothelial cell loss and consecutive development of glomerulosclerosis[228].

PDGF have been shown to exert a strong proliferative effect on human mesangial cells thereby causing mesangial cell proliferation and matrix expansion. PDGF-B and –C and PDGF-α and -β receptors have been shown to be up-regulated in the mesangial region of kidney samples from IgAN patients (reviewed in[229]).

Other proinflammatory cytokines of importance in the pathogenesis of IgAN include TNF-α[189,195], which may be involved in the development of proteinuria, IL-8[230,231], and MCP-1[232]. The latter two attract neutrophilic granulocytes and monocytes to the mesangial and peritubular region and may thus be involved in tissue damage. As the experiments described herein are restricted to the analyses of IL-6 the role of other cytokines will not be further discussed.

1.3.6.6. *Toll-like receptors*

Toll-like receptors (TLR) are type 1 transmembrane proteins crucial for the detection of exogenous pathogen-associated or endogenous danger-associated molecular patterns (PAMPs or DAMPs, respectively). So far 10 different TLRs have been described in humans. Of these TLR1, -2, -4, -5, -6, and -10 are expressed on the cellular surfaces, where they are mainly responsible for recognition of microbial membrane components and endogenous DAMPs. Beside their indisputable importance in the defense against microbial attacks, inappropriate

TLR-signaling has been found to be involved in acute and chronic inflammation and systemic autoimmune disease[233,234].

In patients with IgAN TLR4 was found to be up-regulated on circulating mononuclear cells[235] and in kidney samples, where its up-regulation correlated with that of TGF-β, IL-6, and MCP-1[236]. The up-regulation of these cytokines in HK-2 cell lines was previously found to be dependent on TLR4-signaling[237].

TLR-9 is an intracellular TLR, which in human is mainly expressed in endosomes of various immune cells and function as a detector for bacterial or viral DNA[238]. In two Japanese cohorts of patients with IgAN a single nucleotide polymorphism (SNP) in the gene for TLR-9 (TT genotype, rs352140) was significantly correlated with the risk of disease progression as estimated by clinical and pathological findings[239]. Further support for a role of TLR-9 in the pathogenesis of IgAN comes from the ddY mouse, a strain of mice with increased IgA serum level and spontaneous development of an IgAN-like kidney disease, which therefore has been employed as an animal model for IgAN[240]. Five weeks after nasal challenge of these mice with CpG-oligodeoxynucleotides, which are agonistic ligands for TLR-9, higher renal injury scores and more mesangial IgA-deposits and proteinuria were observed when compared to non-CpG-oligodeoxynucleotides challenged ddY mice or CpG-oligodeoxynucleotides challenged BALB/c mice[239].

Taken together, there is evidence for a possible involvement of signaling via TLR4 and/or TLR9 as well as a SNP in TLR 9 in the pathogenesis of IgAN.

1.3.6.7. *The complement system in IgAN*

The complement system is activated in IgAN, which is confirmed by the common finding of mesangial depositions of C3 as well as other complement components in kidney samples from patients[241-243]. Thus the question arises, which pathway of complement activation actually is responsible for this activation.

In vitro evidence suggests that polymeric IgA_1 may be a strong activator of both the alternative and the lectin pathway[55,57] but not of the classical pathway[244].

Of all complement factors C3 and properdin are most often detected in the proximity of mesangial immune deposits in IgAN[241-243] and in a majority of cases increased serum levels of soluble split products of C3 such as C3a, iC3b or C3d have been reported[245-247]. Furthermore, in the urine of patients with IgAN CFH levels are elevated and correlate to disease activity[248].

These findings indicate that activation of the alternative pathway C3 convertase is involved in the pathogenesis of IgAN in the majority of cases.

C3 synthesis has been detected in human mesangial cells using in situ techniques on renal samples from patients with IgAN but not in controls[70,249]. The mesangial synthesis of C3 is up-regulated by exposure to pro-inflammatory cytokines or immune complexes[75,250]. Further *in vitro* experiments revealed that human mesangial cells stimulated with pro-inflammatory cytokines express CFB[251]. CFD as well as properdin show relatively high expression in the glomeruli of normal kidneys[252]. Thus all the components required for activation of the alternative pathway C3 convertase are available in human mesangial cells. C3 split products and MAC in mesangial immune deposits in kidney samples from patients with IgAN co-localize with the regions with in-situ detected C3 synthesis[70]. These findings indicate that the activation of the alternative pathway in the mesangial region involve locally produced complement factors. *In vitro* experiments further show that human mesangial cells change their phenotypic appearance due to exposure to C3 and switch to a phenotype with increased cell proliferation and synthesis of mesangial matrix[253].

MBL was detected co-localizing with IgA, L-ficolin, MASPs, and C4d in about 25% of kidney biopsies from patients with IgAN. Cases with MBL deposits had more severe histological damage in kidney samples and a higher grade of proteinuria than those without MBL deposits[254]. Others found deposits of C4 in the absence of C1q as well as increased levels of circulating C4 activation products in a subpopulation of IgAN[245,255]. These findings are in accordance with the theory that the lectin pathway may be activated in a subgroup of patients with IgAN with worse prognosis.

Thus the complement system could be activated via the alternative pathway as well as the lectin-binding pathway in IgAN.

In a recent study in a cohort of 46 patients with IgAN the CFH gene was sequenced and investigated for C-terminal single nucleotide polymorphisms (SNPs) and frequencies compared to healthy controls. The investigators concentrated on C-terminal SNPs as all included patients had normal serum levels of CFH. N-terminal structural alterations of CFH could interfere with the excretion of the protein and thus result in reduced serum levels. Three different SNPs, known to confer risk for development of hemolytic uremic syndrome, were investigated, but no significant correlation with the development of IgAN was detected[256].

1.3.7. Malignant hypertension and thrombotic microangiopathy in IgAN

Malignant hypertension is defined as a significant elevation of blood pressure and evidence for acute arteriolar injury diagnosed by the funduscopic finding of hypertensive retinopathy[257]. The incidence of malignant hypertension in IgAN varies greatly between different studies. In Chinese cohorts of patients with IgAN it varied between 0.5 -1.2 % whereas it was as high as 5 - 15% in Spanish and French cohorts [99,258-261]. The variation of reported incidence of malignant hypertension could reflect the different routines for diagnosis of IgAN in Asia versus the Western world (see Epidemiology). It could, however, be due to genetic differences or differences in the definition of malignant hypertension as well.

Thrombotic microangiopathy (TMA) is another serious condition characterized by occlusive, intravascular formation of thrombi. The condition is usually associated with consumptive thrombocytopenia, microangiopathic hemolytic anemia, renal manifestations (hematuria, renal failure) and other signs of organ ischemia[262]. Pathology shows thickening and swelling of vessel walls and detachment of the endothelial cell from the basement membrane with subendothelial accumulation of amorphous material. The intraluminal space is partially or complete obstructed with platelet thrombi[263]. TMA may be caused by hemolytic uremic syndrome or thrombotic thrombocytopenic purpura, but has been associated with other conditions as well, such as malignant hypertension, systemic lupus erythematosus, malignancy, disseminating intravascular coagulopathy, and pre-eclampsia[264]. A recent study reported TMA lesions in diagnostic kidney biopsies in 53% of 128 patients presenting with IgAN. Among the patients with TMA 96 % had hypertension, which only in about a fourth of cases was controlled and in about 18.5 % fulfilled the criteria for malignant hypertension[99].

1.4. Henoch-Schönlein purpura

1.4.1. Clinical features

Henoch Schönlein purpura (HSP) is a systemic vasculitis affecting small vessels and capillaries. Cutaneous symptoms are essential for the diagnosis and thus found in 100% of cases. Initially skin lesions may appear as vesicular or macular rashes before transforming into painless, palpable purpura. Rarely bullous lesions may occur. Petechiae, ecchymoses, or urticarial lesions may complete the picture, which in children is often polymorphic, whereas it is more likely to be monomorphic in adults. Cutaneous necroses are rarely seen in children, but are found in 60 % of adults with HSP. Purpura are most often found on the lower legs, arms and buttocks, but may spread to the trunk and face as well.

Joint affection, mainly as self-resolving oligoarthritis of the knees or ankles, is seen in up to 82 % of patients. Arthralgia or arthritis may precede cutaneous symptoms in about 25 % of cases.

Abdominal symptoms affect 50-75 % of cases. These may consist of mild to severe colicky abdominal pain as well as intestinal bleeding, which may become massive and life-threatening. Rare features include intussusception, pancreatitis, hydrops of the gallbladder, and protein-loosing enteropathy.

Renal affection (Henoch-Schönlein nephropathy, HSN) occurs in up to 60 % of cases. Most commonly patients develop microscopic or macroscopic hematuria with or without low-grade proteinuria (about 80 % of cases with abnormal urine analysis). Nephritic or nephrotic syndrome or a combined nephritic-nephrotic syndrome may develop in about 7 % of cases. Patients may develop acute renal failure and hypertension. In patients with HSN symptoms develop in 91 % within 6 weeks and in 97 % within 6 months after debut of HSP. In adults renal affection tends to develop later than in children.

Urogenital symptoms of HSP include orchitis, which may be found in up to 27 % of boys, and ureteral stenosis. Headache is commonly reported in patients with HSP, but severe complications caused by vasculitis in vessels of the central nervous system (CNS) or bleeding rarely occur. Likewise, pulmonary bleeding is rarely seen in children with HSP but may cause severe complications (reviewed in[265-267]).

1.4.2. Pathology

HSP displays features of systemic vasculitis, with inflammation affecting mainly capillaries, arterioles and venoles. The pathological lesion, termed leukocytoclastic inflammation, includes endothelial swelling, fibrinoid necrosis of blood vessel walls, infiltration of neutrophilic granulocytes with nuclear fragmentation, and immune deposits containing predominantly IgA_1. The perivascular region is infiltrated with neutrophilic granulocytes and mononuclear cells. Leukocytoclastic vasculitis has been detected in affected organs, but even in clinically unaffected skin samples. In case of kidney affection the renal pathology resembles that of IgAN as described above and is graded according to the classification by the International Study for Kidney Disease in Children[266,268,269].

1.4.3. Definition

HSP is diagnosed by clinical and pathological criteria defined in classifications. Recently the classification of the American College of Rheumatology[270] was updated. The new criteria include as a mandatory criterion non-thrombocytopenic, palpable purpura with lower limb predominance or, if purpura is visualized elsewhere, a skin biopsy showing IgA deposits. Besides the mandatory purpura the detection of at least one of the following clinical or histopathological signs is required to diagnose HSP: (1) diffuse abdominal colicky pain, (2) histopathology showing a leukocytoclastic vasculitis with immune deposits containing predominantly IgA or a mesangioproliferative glomerulonephritis with predominant IgA deposits, (3) arthritis or arthralgia, and (4) renal manifestations such as hematuria or proteinuria[271].

1.4.4. Epidemiology

HSP is the most common form of vasculitis in childhood, with an incidence rate varying in different countries between 10.5-20.4/100.000 children per year. Differences in incidence rates could reflect the involvement of genetic and/or environmental factors in the pathogenesis of the disease. They may also depend on an underestimation of the real number of cases if investigations are based on patients treated at secondary or tertiary centers, whereas many uncomplicated cases may be not recognized as HSP, dealt with within primary care or not seeking medical attention at all[265,272]. HSP is present worldwide and typically occurs during the autumn and winter period[266]. Even if HSP may develop at any age most cases occur between 2 and 6 years of age[273,274] and 90 % of cases before the age of 10 years[275].

Boys are slightly more often affected than girls (1.2/1) and white or Asian children more often than black children[274].

1.4.5. Outcome and predictors of outcome

In most cases, especially in children, HSP is an acute, self-limited and benign condition lasting for approximately two to three weeks. The cutaneous lesions usually resolve within five to seven days, but new ones may develop within eight weeks. Recurrence of symptoms usually restricted to the skin and gastrointestinal tract are experienced by 15 – 40 % of children with HSP within three months after debut[266].

With the exception of rare, severe, acute complications such as CNS, pulmonary, or massive intestinal bleedings the prognosis of HSP is dependent on the development of HSN and the concomitant risk of developing end-stage renal disease (ESRD). The relative risk for poor renal outcome was found to be related to the clinical picture at onset[276]. The risk for development of long-term impaired renal function dependent on the initial urinary findings in children and adults are summarized in table 6,based on two recent studies.

Table 6: Risk for long-term renal impairment in patients with HSP[267,277]

Number of cases (%)	Initial urinary analysis	Risk for long-term renal impairment* in percent
1133 **children**		1.8
746 (65.8)	Normal	0
387 (34.2)	Abnormal	5.4
305 (27)	Isolated hematuria and/or proteinuria (<40mg/m^2/h)	1.6
82 (7.2)	Nephritic or nephrotic syndrome	19.5
250 **adults**		38
18 (7.2)	Absence of hematuria	17
208 (83.2)	Microscopic hematuria	37
24 (9.6)	Macroscopic hematuria	67
81 (32.4)	Proteinuria (<1g/d)	26
169 (67.6)	Proteinuria (>1g/d)	41

*Renal impairment in children was defined as serum creatinine level above the upper limit of normal, in adults as creatinine clearance [CrCl] <50 ml/min.

Using multivariate Cox regression analysis a study on a group of patients with biopsy-proven HSN including 83 children and 163 adults detected older age at debut, female gender, and higher grade of proteinuria at follow-up (mean 3 g/d vs 1 g/d) as poor prognostic factors.

1.4.6. Etiology, pathogenesis

The etiology of HSP as well as its pathogenesis are largely unknown.

1.4.6.1. Genetic factors

Familial clustering of HSP has been described and suggests a genetic background to the disease[278,279]. In several countries and ethnic groups different HLA types have been associated with susceptibility for HSP[268,280]. The different HLA type-associations may explain differences in manifestations between different ethnic groups, but, so far, no conclusions could be drawn from these studies to explain the pathogenesis of HSP. Positivity for HLA-B35 was found to increase the risk for the development of HSN[281].

A polymorphism in the angiotensinogen gene (M235T) may confer risk for the development of HSN[282,283].

Polymorphisms in the gene for angiotensin 1-converting enzyme (*ACE*) may be involved in the pathogenesis of HSP or HSN, although data are conflicting. The insertion (I)/deletion (D) genotype of a polymorphism in *ACE* may confer susceptibility to HSP[282,283]. The DD polymorphism was related to persistent proteinuria in patients with HSN in one study[284], whereas in another study no correlation was found between the prognosis of HSN and the *ACE* genotype[285].

Variations in the complement C4 protein gene may confer susceptibility to the development of HSP. C4 null isotypes have been described to be prevalent in a significantly higher proportion of patients with HSP and HSN than controls[286-288]. A partial or complete deficiency of C4 could be related to impaired clearance of immune complexes and thus play a role in the pathogenesis of HSP[289]. Complement deficiency is, however, uncommon and transient in patients with HSP[290].

Investigations addressing polymorphisms in genes encoding for proinflammatory cytokines (TNF-α, IL-1b, IL-8, TGF-β and VEGF) have so far not revealed any predisposing factors for HSP[291,292].

Familial mediterranean fever (FMF) is an autoinflammatory disease caused by a mutation in the MEFV gene, which in 7 % of cases is associated with HSP[293]. There is a high prevalence of children with *MEFV* mutations among HSP patients in countries with relative abundance of FMF[294,295]. The implication this association has on the general pathogenesis of HSP is, if at all, unclear.

1.4.6.2. *Infectious and non-infectious agents*

HSP is usually preceded by infections, in up to 95 % of cases localized in the upper respiratory tract, and appears in clusters in families[296-298]. The incidence of HSP is highest during early childhood and shows distinct seasonal variations with a peak during autumn and winter[274]. Both early childhood and the autumn-winter season are periods with frequent infections. Thus, clinical observations suggest an important role of infections in the etiology and pathogenesis of HSP.

Several studies have shown a circumstantial relation of infections with group A streptococci and the development of HSP[299-302]. Others found serological evidence for an association with infections with other bacteria such as *Bartonella henselae*[303] or viruses such as parvovirus B19[304] and hepatitis C virus[305].

Non-infectious agents have been found to be associated to the development of HSP especially in adults. These include certain drugs such as angiotensin-converting enzyme inhibitors, angiotensin II-receptor antagonists, antibiotics, and non-steroidal anti-inflammatory drugs as well as insect bites, vaccinations or food allergies[266].

1.4.6.3. IgA_1 in HSP

IgA deposits in HSP are composed of immune-complexes mainly consisting of IgA_1[165]. Serum samples from HSN patients were found to have elevated levels of underglycosylated polymeric IgA_1 compared to controls[306]. However, in children with HSP without renal involvement the levels were not higher than those of controls[307]. Underglycosylated polymeric IgA_1 has been found to exhibit an inflammatory and proliferative effect on mesangial cells (see IgA_1 in IgAN). Taken together, underglycosylated polymeric IgA_1 seems to be involved in the development of HSN, but its role in the pathogenesis of HSP per se remains unclear.

1.4.6.4. *Mediators of inflammation*

The acute phase of systemic vasculitis is generally characterized by vascular leukocytic infiltration and activation of innate immunity. An elevated level of inflammatory cytokines are usually detectable in the serum and affected tissues in these diseases.
IL-6, TNF-α, tumor necrosis factor-like weak inducer of apoptosis (TWEAK), IL-8, TGF-β, and VEGF have been found to be up-regulated during the acute stage of HSP[308-313].

Tissue samples of affected skin areas from patients with HSP show epidermal staining with IL-6[309]. Serum levels of IL-6 were significantly higher in patients with HSP during the acute phase of disease than in controls and also higher in patients with HSN than HSP without renal affection[308]. IL-6 displays a wide variety of pro-inflammatory properties and promotes the secretion of IgA[219]. For the possible role in the development of HSN see the chapter "Cytokines in IgAN". IL-6 displays, besides its various pro-inflammatory effects, even anti-inflammatory effects by inhibiting TNF-α and IL-1 and activating IL-1ra as well as IL-10[314].

TNF-α is produced by macrophages and T cells in affected skin areas during HSP. Serum levels of TNF-α were higher in patients with HSN than HSP without renal engagement[308]. It stimulates the presentation of adhesion molecules and receptors on leukocytes and endothelial cells thereby directing inflammatory events. Furthermore, endothelial cells stimulated with TNF-α were shown to bind IgA with higher affinity[309]. These findings suggest, that TNF-α could be involved in the accumulation of granulocytes and endothelial sequestration of IgA as seen in affected tissues in HSP[308].

TWEAK, a member of the TNF superfamily, which binds to specific receptors on endothelial cells, is involved in the regulation of cell growth, angiogenesis, apoptosis, and inflammation. *In vitro* evidence suggests that TWEAK may induce cytokine production by human microvascular endothelial cells via up-regulation of the production of IL-8 and CCL-5 leading to a leucocyte migration into affected vessels[310,315] which are common aspects of the HSP lesion.

Sera and IgA from patients with HSP induce the secretion of IL-8 from endothelial cells *in-vitro*[311,316]. IL-8 is a potent chemoattractant for polymorphonuclear neutrophilic granulocytes (PMNs). Levels of leukotriene B4, also a potent chemo-attractant and activator of PMNs, are elevated both in serum and urine in patients with HSN compared to those with HSP. Furthermore, the levels of leukotriene A4, which counter-balance the effects of leukotriene

B4 and inhibit the synthesis of proinflammatory cytokines (e.g. IL-6, IL-8, TNF- α), are decreased in patients with HSN[317].

The role of VEGF in HSP is not clear-cut. Serum levels of VEGF were significantly higher during the acute phase of HSP than during remission. However tissue staining for VEGF showed more intense staining for VEGF in the epidermis and vascular bed during the resolution phase than during the acute phase of HSP[313]. High serum levels of VEGF could influence endothelial permeability, which may enhance capillary leakage and facilitate the extravasation and perivascular deposition of immune complexes. The increased tissue staining during the resolution phase, on the other hand, suggests a possible function of VEGF in the resolution of vascular damage.

T helper cells (Th) are a sub-population of lymphocytes, which have an important role in adaptive immune responses. Dependent on the surrounding cytokine environment naïve Th differentiate into subtypes with different functions[318]. In patients with HSP an elevated number of Th2 and Th17 with increased synthesis of IL-5 and IL-13 have been found together with increased serum levels of IL-4, IL-6, and IL-17A[319]. The differentiation towards Th2 is stimulated by exposure to IL-4 and towards Th17 by TGF-β combined with IL-6. By secreting IL-4 Th2 exhibit a stimulatory effect on B cells and promote the generation of plasma cells. Further secretion of IL-5 or IL-13 from Th2 leads to an antibody switch in plasma cells towards the generation of IgA or IgE, respectively. Th17 secrete IL17, which in turn stimulates the expression of pro-inflammatory cytokines such as IL-1, IL-6, and cell-adhesion factors and promotes leukocyte migration to the sites of inflammation. Th17 has been implicated in the pathogenesis of autoimmune diseases[318]. An imbalance of Th with Th2 and TH17 predominance, as seen in HSP, could explain elevated serum levels of IgA and IgE, the expression of pro-inflammatory cytokines and leukocyte infiltrations into affected tissues seen in HSP[265,320].

If the pieces of this jigsaw puzzle are put together potential origins of cardinal symptoms of HSP emerge. Neutrophilic infiltration of the perivascular region may be mediated by TNF-α, TWEAK, IL-8, chemo-attractant leukotrienes, VEGF and/or Th17 and the extravasation and deposition of IgA by IL-6, TNF-α, VEGF, and Th2. The development of HSN could be related to the prevalence of underglycosylated polymeric IgA_1 (see previous chapter), the effect of IL-6, TNF-α, and a disturbed balance between chemo-attractant and counteracting leukotrienes.

The contact system, which induces liberation of bradykinin or other vasoactive kinins from high-molecular kininogen, has been found to be activated in HSP, which could contribute to the development of clinical features such as inflammation, vasodilatation, edema and pain[321].

Increased reactive oxygen species, lipid and protein oxidation, and nitric oxide level detectable during the acute phase of HSP are believed to reflect secondary events and vascular damage[322-324].

1.5. Evidence for a common pathogenetic background of IgAN and HSP

A common background for the development of IgAN and HSP has been suggested as both conditions share several common clinical and pathological features. The debut or exacerbation of both is commonly preceded by infections, often situated in the upper respiratory tract and caused by Group A streptococci (GAS)[150,296,300]. The reason for the increased susceptibility of patients with IgAN and HSP towards infections with GAS has so far not been resolved. A possible explanation could be reduced synthesis and secretion of specific sIgA following neoantigens as observed in patients with IgAN[177]. An impaired secretion of sIgA could lead to insufficient mucosal eradication and increased colonization with GAS.

Another common finding in patients with IgAN and HSN is a relative elevation of serum levels of underglycosylated polymeric IgA_1[162,307]. Tissue immune deposits contain predominantly IgA_1[165] and the renal lesion is similar for both HSN and IgAN[102].

1.6. Group A streptococci

1.6.1. Streptococci

Streptococci are spherical, Gram-positive bacteria growing in chains or pairs. According to their effect on erythrocytes during growth on blood-agar they can be divided into α-hemolytic streptococci (incomplete hemolysis), β-hemolytic streptococci (complete hemolysis), or γ-hemolytic streptococci (no hemolysis)[325]. Rebecca Lancefield discovered that the different species of streptococci carry specific carbohydrates on their cell wall and accordingly classified them into groups (Group A-N, R&S, and non-Lancefield streptococci)[326]. Among the β-hemolytic streptococci, the most important pathogen is group A streptococcus (GAS),

which also is known as *Streptococcus pyogenes*, reflecting the fact that it often is detected in cultures from purulent infections.

Strains of GAS can be further subclassified. The most important of these classifications is based on the structure of the surface-bound M protein, which allows division into M types (see below)[327]. On the basis of structures within the C-terminal conserved part of the M protein, GAS strains can also be classified into two major classes, designated class I and class II[328]. This division into class II and class I strains largely corresponds to a division of GAS isolates into SOF^+ and SOF^- strains, reflecting ability (or lack thereof) to produce the apoproteinase 'serum opacity factor' (SOF)[329]. An additional typing system is based on the trypsin resistant T antigens, which allow for a division of GAS into approximately 25 T types[330]. The T antigens were recently demonstrated to be components of surface-localized pili and they are most likely important virulence factors[331].

1.6.2. Group A streptococcal disease -epidemiology

GAS is an important human pathogen with an estimated prevalence of 111 million cases of GAS pyoderma (impetigo contagiosum) and more than 616 million cases of GAS pharyngitis per year[332]. Approximately 26 % of all upper respiratory tract infections in children are caused by GAS[333]. The estimated prevalence of severe disease caused by GAS is at least 18 million cases causing at least 517 000 deaths per year. Rheumatic heart disease, a non-suppurative complication of GAS disease, accounts for the major part of severe cases with a prevalence of at least 15.6 million cases causing 233 000 deaths each year, whereas invasive disease was estimated to affect 663 000 new cases causing death in 163 000 patients per year. Acute post-streptococcal glomerulonephritis, another non-suppurative complication of GAS infection, occurs in approximately 470 000 cases per year, world-wide, causing death in about 5000 cases[332]. Other features of GAS infections include the pharyngitis-associated, toxin-mediated scarlet fever, erysipelas, cellulitis and the potentially life-threatening toxic shock syndrome and necrotizing fasciitis.

1.6.3. M proteins

GAS expresses on its surface a dimeric, coiled-coil (i.e. two α-helical coiled proteins/dimers coiled together), fibrillar protein termed M protein due to the "matt" appearance of colonies formed by M protein-expressing streptococci as opposed to the "glossy" appearance of those without M proteins[327]. M proteins are important virulence factors of GAS and their most

studied property is their ability to confer resistance to phagocytosis, a property that most likely depends on the ability of the M proteins to bind human plasma proteins[334-339].

The structure of the M protein varies between strains, but all M proteins share a common basic structure including a relatively conserved C-terminal region, which is required for anchoring of the M protein to the peptidoglycan layer of the bacterial cell wall, a semi-variable domain and a hypervariable N-terminal region. The different regions of M proteins include homologous sequence repeat blocks, designated A-D repeats, which contain seven-residue repeats of non-polar aminoacids. D repeats are localized C-terminal within the M protein and are almost identical between strains, whereas A–repeats within the hypervariable N-terminal region of M proteins in some GAS strains differ between strains. The further N-terminal the repeats are expressed the more they vary in number and structure between strains[329,340].

M proteins are encoded by *emm* genes in the bacterial genome and are part of the Mga regulon, indicating that the expression of M proteins can be instantly regulated by Mga in response to environmental changes[341,342]. Sequencing of the 5' end of *emm* genes allows for subdivision of GAS into more than 200 M types[343]. Many GAS strains also express M-like proteins, for which the genes (*mrp* or *enn*) are closely linked upstream and downstream to the *emm* gene encoding the M protein. In particular, most strains expressing a class II (SOF^+) M protein have three adjacent genes encoding Mrp, M and Enn proteins, consecutively[344]. Based on the presence of these additional genes and their structure, GAS strains can be assigned to five *emm*-patterns denoted A-E. The M proteins associated with these different patterns show differences in structure and binding properties (Figure 6). Strains with A-C *emm*-pattern are believed to predominantly cause throat infections, whereas D strains preferentially cause cutaneous infections. E-pattern streptococci are found in specimens from both pharyngeal and cutaneous infections and are therefore called generalists[345]. Most class II/SOF^+ GAS serotypes express E-pattern M proteins and class I/SOF^- serotypes express A-D-pattern M proteins[346].

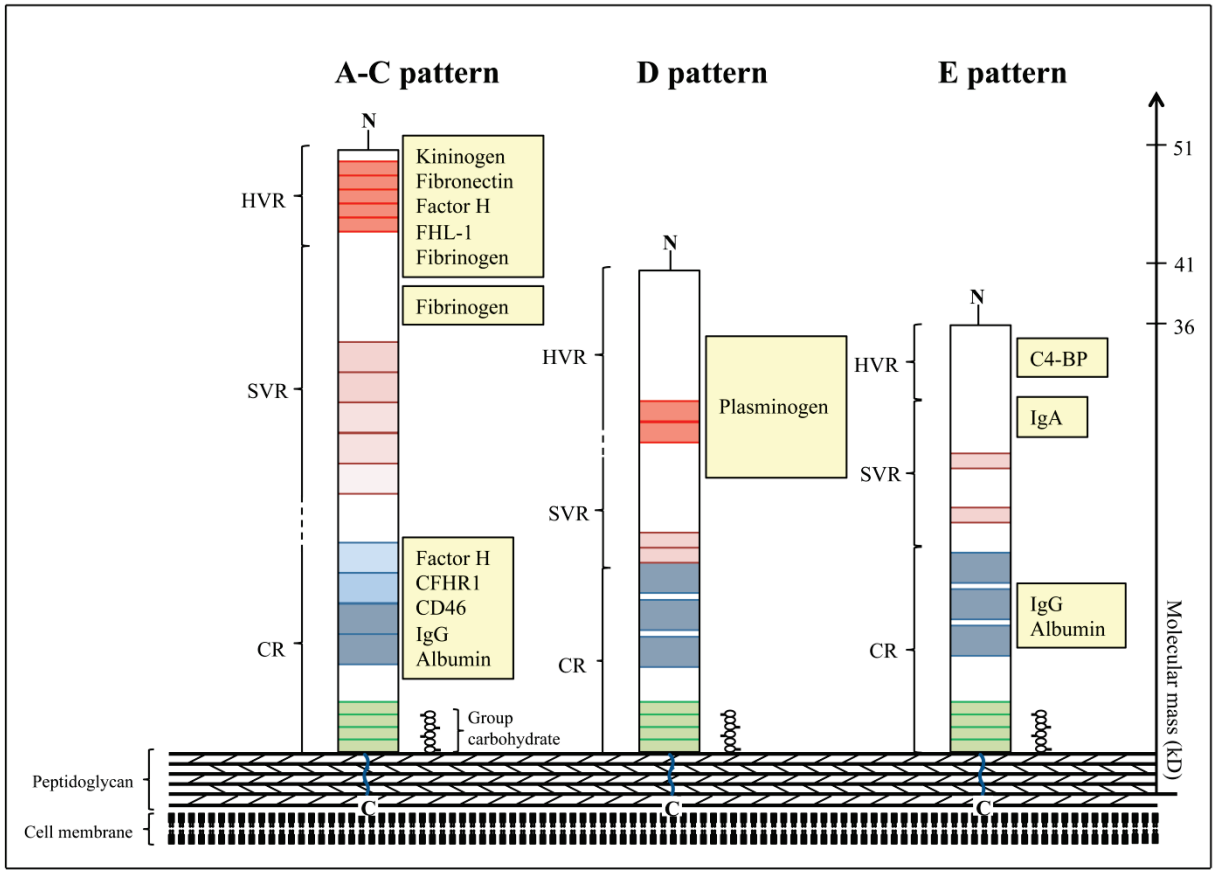

Figure 6: Principal structure of streptococcal M proteins and their protein binding sites
The principal structure and size of streptococcal M proteins[345,346] as well as specific binding-sites for proteins within different regions of M-proteins dependent on the classification of M proteins into emm-types are depicted[167,347-365]. A-repeats are shown in red, B-repeats in purple, C-repeats in blue, and D-repeats in green. A-C-repeats may vary in number between strains. HVR: hypervariable region, SVR: semivariable region, CR: conserved region, FHL-1=: factor H-like protein-1, CFHR1: complement factor H related protein 1, C4-BP: C4-binding protein. Modified from[346].

Approximately 20% of the different GAS M types belong to the A-C pattern. The remaining GAS M types can be equally divided into those with D and E pattern[366]. In clinical GAS isolates, however, strains of the A-C pattern represent much more than 50%. The size of an M protein may vary between isolates of the same serotype without interfering with their function[367]. In general, M proteins with an A-C-pattern are larger than those with a D or E pattern (average molecular mass of approximately 51, 41, and 36 kD respectively)[346] (Figure 6).

M proteins bind to a variety of human plasma proteins. Whether a certain plasma protein can bind to an M protein seems to follow the structure of the M protein reflecting the *emm*-pattern (A-E) of the strain. Protein binding to M proteins has, however, only been investigated in a limited number of the known GAS M serotypes (reviewed in[346]). The N-terminal part of M proteins with the *emm* A-C pattern have been reported to harbor binding sites for

kininogen[347], fibrinogen[348,349], fibronectin[350-353], complement factor H (FH), FH-like protein-1[354,355], FH-related protein-1[356] and CD46[167,357]. The N terminal part of the M protein belonging to the D-pattern were shown to bind to plasminogen[358,360] and those of the E-pattern express binding sites for IgA[359,361,363] and complement factor 4-binding protein[361,362,364,365] (Figure 6).

Furthermore, many M proteins of the A-C and E-pattern share the expression of binding sites for IgG[361,368-372] and albumin[368-371,373,374] in their conserved, C-terminal regions (Figure 6).

In vitro experiments suggest that M proteins may be detached from the bacteria during the course of infection either due to the action of proteases from neutrophilic granulocytes or streptococcal cysteine proteinase[336,375].

1.6.4. IgA-binding regions of group A streptococcal M proteins

M proteins expressed by GAS strains belonging to pattern E have a binding site for IgA, which is located in the semi-variable part of these M proteins, i.e. the region located between the N-terminal hypervariable region and the conserved region (Figure 6). The IgA-BRs show species-specificity only for IgA of human origin[376]. The molecular properties of this IgA-binding region (IgA-BR) have been studied for several M serotypes of the E pattern (M4, M22, M28, and M60)[359,361,363,377-380]. The amino acid sequence of the IgA-BRs from M4, M22, and M60 exhibit considerable sequence homology (42% for M4, M22 and M60, 76% for M4 and M60)[379,381,382]. An IgA-binding M protein binds at to IgA at the $C_{\alpha 2}$-$C_{\alpha 3}$ inter-domain region, at a site that overlaps with binding to the human IgA-receptor $Fc_{\alpha}R1$ (CD89)[380] (Figure 1A).

1.6.5. GAS and renal disease

1.6.5.1. *Acute post-streptococcal glomerulonephritis*

Acute post-streptococcal glomerulonephritis (APSGN) is a non-suppurative complication following infections with certain "nephritogenic" strains of GAS. APSGN may be associated with pyoderma (e.g. M49, M2, M60, M42, M56, and M57 strains –in order of prevalence among clinical isolates) or pharyngitis (e.g. M1, M4, M25, and some M12 strains). M49, M2, M60, M4, and M25 belong to the SOF^{+}/E emm-pattern[329]. APSGN exhibits a distinct pathological renal lesion with endothelial and mesangial hypercellularity, polymorphonuclear cell infiltration[383], and granular deposition of C3 and often IgG in capillary loops and the

mesangial region[384]. Electron microscopy displays typical hump-like, most often sub-epithelial localized deposits of immune complexes[385].

M proteins and other streptococcal proteins have been proposed to be involved in the pathogenesis of APSGN. The complement system is in most cases activated via the alternative pathway, which may be caused by streptococcal exotoxins (SpeB, NAPl1). However, the exact pathogenetic background of the disease remains, to be elucidated (reviewed in[91]).

1.6.5.2. *Evidence for streptococcal involvement in other forms of glomerulonephritis*

Post-infectious glomerulonephritis (PGN) may develop following infections with various other bacteria and viruses[386]. In some patients with PGN immunofluorescence reveals predominant deposition of IgA besides other typical pathological features resembling those described for APSGN. IgA-dominant PGN is associated with streptococcal infections in about 20% of cases[387].

There is circumstantial evidence for a relation between infection with GAS and the later development of IgA nephropathy and Henoch-Schönlein purpura. Epidemiological observations also suggest a possible relation between the occurrence of membranoproliferative glomerulonephritis and GAS infections[97,388].

2. The present investigation

2.1. Aims

The overall aim of the studies included in this thesis was to investigate the role of GAS serotypes expressing IgA-binding M proteins and the complement system in the pathogenesis of IgAN and HSP.

Specific aims

Paper 1

1. To investigate whether GAS IgA-binding M proteins are detectable in tissue samples from children with IgAN and HSP
2. To investigate whether IgA-binding M proteins co-localize with IgA in the mesangial region of kidney samples from patients with IgAN

Paper 2

3. To investigate, whether children with IgAN have elevated serum levels of IgG-antibodies to the IgA-binding region of GAS M proteins compared to age-matched controls

Paper 3

4. To investigate the binding-affinity of the IgA-binding M protein from GAS serotype 4 (M4) to IgA_1 dependent on the level of O-glycosylation and the size of IgA_1
5. To investigate binding of M4 to human mesangial cells
6. To investigate if M4 and IgA_1 stimulation of human mesangial cells induces IL-6 and C3 synthesis and secretion

Paper 4

7. To investigate the genome of a patient with IgA nephropathy and thrombotic microangiopathy for mutations in complement factor H

2.2. Patients and Materials

2.2.1. Streptococcal proteins, peptides and antibodies

Group A streptococcal proteins and peptides derived from streptococcal proteins used in the experiments in the present investigation are shown in Figure 7.

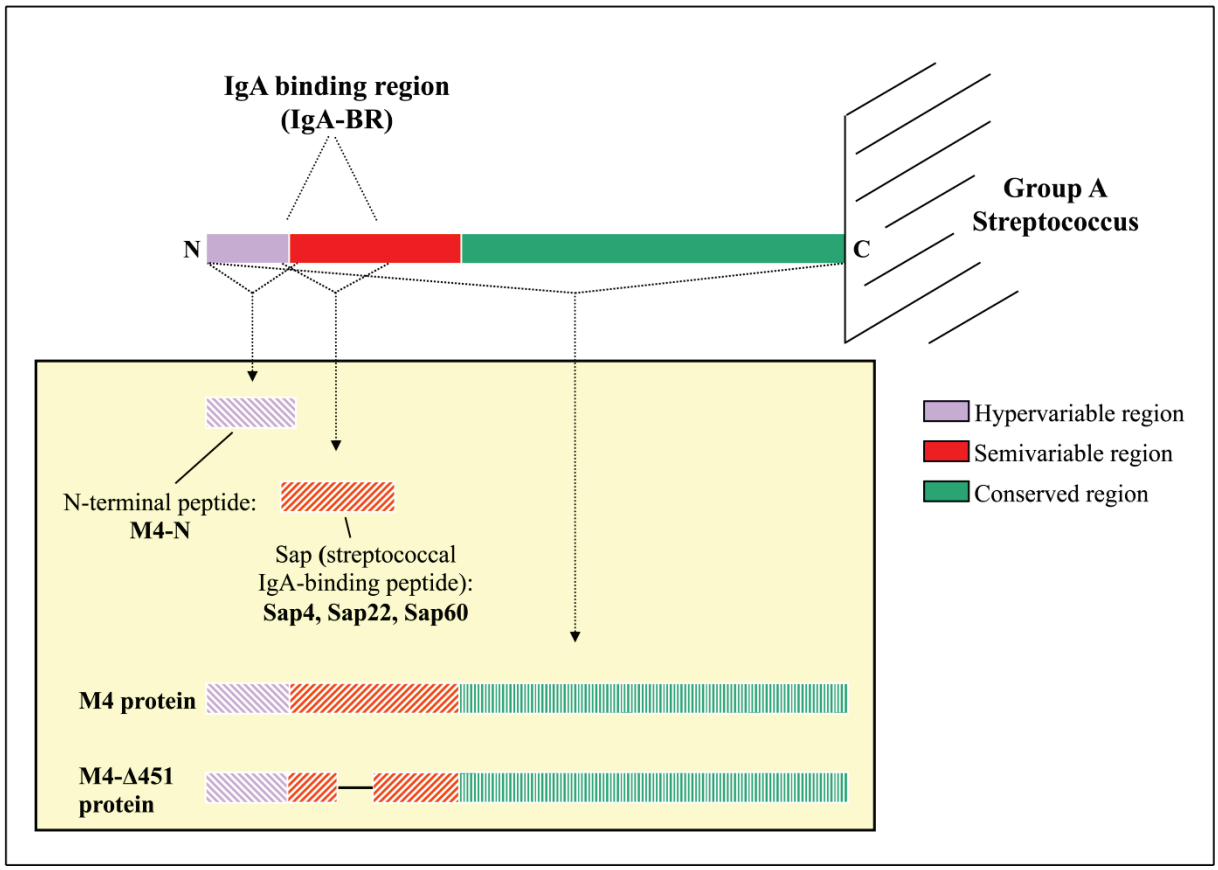

Figure 7: Streptococcal proteins and peptides used in experiments

The principal structure of the M protein, mutated M protein and peptides used in experiments are depicted. The IgA-binding region of M proteins of emm-pattern type E (M4, M22, M60) is shown. M4-N corresponds to the N-terminal 45 amino acids from the M4 protein with a C-terminal tyrosine-cysteine[364,378]. Sap4 and Sap22 (streptococcal IgA-binding peptide derived from M4 or from M22, respectively) consist of amino acids 35-83 in the M4 or M22 proteins, and Sap60 (derived from M60 protein) of amino acids 44-92 in the M60 protein. All Saps and N-terminal peptides contained C-terminal elongations with cysteine to allow for dimerization[378]. The M4-Δ451 protein constitutes the complete M4 protein with a 10 amino acid deletion within the IgA-binding region, thus precluding IgA-binding[359].

Streptococcal IgA-binding peptides (Sap)

We had access to synthetic peptides analogous to the IgA-binding region (IgA-BR) of the M4, M22, and M60 proteins termed streptococcal IgA-binding peptide (Sap)4, Sap22, and Sap60, respectively[378] (Figures 6 and 7). As a control we used a non-IgA binding peptide termed M5-N, corresponding to the N-terminal 50 amino acids from the M5 protein with a C-terminal addition of tyrosine-tyrosine-cysteine[364].

IgG-fractions of polyclonal antibodies to the Saps[364] were used to detect IgA-BR in tissue samples (Paper I). The three Saps were used in an ELISA to detect antibodies to IgA-BR (Paper II).

Using Saps instead of the entire M proteins precluded confounding effects related to the IgG-binding region in the conserved part of M proteins (Figure 6). IgA-BR may both share structural similarities and differences between serotypes. This is the reason for a varying degree of cross-reactivity of antibodies against these regions[379,381,382]. Thus we used polyclonal rabbit antibodies directed against the Saps (corresponding to IgA-BRs) of three different E-pattern M proteins (M4, M22 and M60) in order to increase the chance to detect deposits of IgA-BRs in patient tissues. Similarly, using the Saps from the three serotypes increased the possibility to detect serum antibodies to IgA-BRs in the patients. Detection of IgA-BRs in tissues and antibodies to these antigens allowed for an investigation of a role of IgA-BRs in the pathogenesis of IgAN.

M proteins

The complete M protein of Group A streptococcus serotype 4 (M4) and a mutant protein with a deletion within the IgA-BR (M4-Δ451)[359] (Figure 7) were used for investigation of the binding affinity of M protein to IgA and stimulation of human mesangial cells (Paper III). IgG fractions of polyclonal rabbit antisera to M4[389] were used to investigate binding of M4 to human mesangial cells by flow cytometry (Paper III). The mutant variant lacked the ability to bind IgA enabling us to study the specific effects of IgA and IgA-binding M proteins on mesangial cells, separately and in complex.

M4 originates from one of the most common GAS serotypes in clinical isolates[390], thus making it a suitable representative of the group of IgA-binding M proteins. However there are structural differences between IgA-binding M proteins and the results obtained regarding stimulation of mesangial cells with M4 may not be valid for all IgA-binding M proteins. The use of a mutant non-IgA-binding M protein enabled us to investigate the role of the IgA-BR within M proteins for IL-6 and C3 secretion by human mesangial cells.

N-terminal regions from M proteins of Group A streptococci

We had access to peptides representing the N-terminal region of the IgA-binding M protein of Group A streptococcus serotype 4 (M4-N) and the non IgA-binding M protein from GAS serotype 5 (M5-N). These peptides have been previously described[359,364,377,378] (see Figure 7 for M4-N). M4-N and a polyclonal antibody to this peptide[364] were used in immunohistochemistry experiments to further characterize the M proteins in immune deposits. Furthermore, the anti-M4-N antibodies were used as a positive control for Sap4 staining, indicating that more than one anti-M4 antibody detected deposits (Paper I), and in the ELISA as a control for detection of antibodies to the IgA-BR of M4 (Paper II). M5-N served as a negative control for the ELISA to detect antibody levels to a common non-IgA-binding M protein (Paper II).

2.2.2. Tissues and blood samples from patients and controls,

Patients with IgAN (n=21, 6 girls and 15 boys, median age 12.5 years, range: 3-19) and HSP (n=17, 7 girls, 10 boys, median age 13 years, range 7 to 18) treated at the Department of Pediatrics, Lund University Hospital, between 1994 and 2006 were included in these studies (Paper I, II). IgAN was defined as the presence of hematuria and/or glomerulonephritis with or without proteinuria in patients in whom renal biopsy showed mesangioproliferative glomerulonephritis with immune deposits of IgA in glomerular mesangial cells and matrix. HSP was defined as per the criteria of the American College of Rheumatology[270]. Thirteen of the 17 HSP patients had nephropathy (HSN) manifesting as the occurrence of hematuria and proteinuria (Paper I).

The current diagnostic work-up for IgAN at Lund University hospital recommends kidney biopsy only in cases with clinical signs of a progressive, complicated disease. As all our patients were diagnosed by kidney biopsy the study group may represent more severe cases of disease. However, as kidney biopsy is needed to confirm the diagnosis IgAN no cases without biopsy were included. Likewise, the high proportion of HSN in patients treated for HSP suggests a selection bias as mainly complicated cases of HSP were referred for treatment at a tertiary center.

Patient tissue samples included kidney biopsies from 16 patients with IgAN (from five patients with IgAN no biopsy was available) and 13 patients with HSN as well as skin biopsies from five patients with HSP. From one HSP patient both a kidney and skin biopsy was available. Furthermore, we had access to control kidney samples from 10 patients with

diseases others than IgAN and HSP whose biopsies showed mesangial IgA deposits (SLE nephritis, membranous glomerulonephritis, Goodpasture's disease and post-streptococcal glomerulonephritis). Normal kidney samples were available from three patients (one sample taken from a 10-year old girl investigated because of hematuria but deemed histologically normal, and two histologically normal sections from renal nephrectomy samples taken from two adult renal cancer patients) and histologically normal skin resection tissues from two unidentified breast cancer patients (Paper I).

Blood samples were available from all IgAN patients (n=21). The samples were taken within a median of 3 months (range 0 –14 months) after the onset of clinical disease (as defined by the presence of symptoms). Sera were used in ELISA for detection of antibodies against IgA-binding regions of group A streptococcal M proteins serotypes 4, 22 and 60 (Paper II) and to detect circulating IgA-binding regions of group A streptococcal M proteins (Paper I).
Control sera were available from age-matched pediatric outpatients (n=83) suffering from diseases other than vasculitis or nephropathy and five healthy adults (Paper II) and from five healthy adults (Paper III).

Serum and whole blood sample was taken from one patient with IgAN and her father and used for measurement of factor H levels as well as the sequencing of the factor H gene (Paper IV).

The study was approved by the ethics committee of the Medical Faculty, Lund University. All samples were obtained with the informed written consent of healthy adults and all patients, or their parents when patients were younger than 15 years. The 83 control sera used in paper II were stored for use in scientific investigations according to bio-bank regulations.

Primary human mesangial cells

Commercially available primary human mesangial cells were used in cell stimulation experiments. The cells were defined as mesangial by their stellate-like morphology and positive staining for smooth muscle actin. The mesangial cells were cultured in serum-free media to avoid stimulation by fetal calf serum proteins containing bovine IgA. The proliferation of mesangial cells was reduced before the start of stimulation by using maintenance medium to mimic physiological conditions (Paper III).

2.3. Methods and results

2.3.1. PAPER I

The overall aim of the first study was to investigate tissue samples of patients with IgAN and HSP for deposits of IgA-binding streptococcal M proteins and define their localization.

The main finding in this study was the demonstration of IgA-binding streptococcal M proteins co-localizing with IgA in the mesangial region of kidney biopsies taken from patients with IgAN and HSN as well as in skin samples from patients with HSP.

2.3.1.1. *Characterization of the IgA-binding region in different group A streptococcal M proteins*

IgA-BR of M proteins from three different GAS serotypes (4, 22, and 60) were characterized using synthetic peptide analogues termed Sap4, Sap22, and Sap60, respectively. Overall they showed 42% amino acid identity. The peptides retained the IgA-binding properties of the original M proteins and were thus considered suitable for the study. Using polyclonal rabbit IgG to the three Sap peptides we confirmed differences in antigenicity as well as a certain degree of cross-reactivity between the three Sap peptides.

2.3.1.2. *Detection of IgA-binding M proteins in tissue samples*

IgA-binding M protein residues were detected in the mesangial region and around glomerular capillary walls in 17 of 29 kidney biopsies (10 of 16 from patients with IgAN and 7 of 13 from patients with HSN) using immunohistochemistry. Considering the limited number of GAS serotypes investigated these findings were surprisingly and suggested cross-reactivity with other GAS serotypes not investigated in this study. Furthermore, these results indicated that IgA-binding M proteins may be deposited in most tissue samples from patients with IgAN and HSN.

Five of the kidney samples that stained for the IgA-BR of M4 were further tested for the presence of the hypervariable N-terminal (by antibodies directed to the peptide M4-N). Three of these five kidney samples exhibited positive staining for the M4 N terminal region suggesting that the deposited M protein residue may include larger regions of the M protein encompassing more than the IgA-BR. The remaining two cases, which did not stain positively for the N terminal region of M4, may actually not have had M4 in their mesangial deposits, but instead deposits of M proteins from other GAS serotypes cross-reacting with the IgA-BR of M4, but not with the N terminal of M4. Alternatively, but less likely, only a minor part of

the M protein including the IgA-BR, but not its N-terminal region, may be deposited in the mesangial region in these cases.

Two of the patients with IgAN showing positive staining for the IgA-binding M4 protein in their kidney biopsies had throat swabs taken 19 or 31 days before the renal biopsy was obtained. Both cultures showed growth of GAS serotype M4 detected by DNA sequencing. Thus throat infections with GAS, which are common findings in patients with IgAN[150], could be associated with deposits of M proteins in the mesangial region. However, the development of IgAN will take a longer time than 19-31 days corresponding to the time interval between throat culture and kidney biopsy in these cases. This finding therefore suggests an exacerbating effect on the mesangial deposition of M proteins or that the patients may have had previous GAS infections with M4.

Nine out of ten control kidney samples with other renal diseases stained negatively for IgA-binding M proteins, which suggests that the deposition of IgA-binding M proteins is not a common event even in cases with renal IgA deposits. The one disease control sample staining for the IgA-BR of GAS serotype 4 was from a patient with acute post-streptococcal glomerulonephritis. M4 is known to be a "nephritogenic" GAS serotype and deposits of M proteins have previously been detected in renal samples from patients with post-streptococcal glomerulonephritis[91].

Four of five skin biopsies from patients with HSP showed positive perivascular staining for IgA-binding M proteins. None of the control skin samples exhibited staining. In one patient with HSN both a skin and kidney sample was available and both showed positivity for serotype M60. Although the results were found using a low number of investigated cases they indicate that IgA-binding streptococcal M proteins are involved in the pathogenesis of HSP.

2.3.1.3. *Ultrastructural localization of IgA-binding M proteins and co-localization with IgA*

Three renal tissues (from one patient with IgAN with positive staining in immunohistochemistry for the IgA-BR of M60, one patient with HSN with positive staining for the IgA-BR of M4 and one control) were investigated using immune electron microscopy. In patients IgA-binding M protein residues could be detected co-localizing with deposited IgA in electron-dense deposits in the mesangial matrix and in minor amounts in the glomerular basement membrane (GBM), the subendothelial and subepithelial region of the GBM and the urinary space.

The findings were conclusive in the two samples investigated and we assume that they are representative for the rest of the patients.

2.3.1.4. *Mass spectrometry for detection of IgA-binding M proteins in a skin sample*

To verify our findings we used an antibody-independent method, i.e. selected reaction monitoring mass spectrometry on one ample skin biopsy from a patient with HSP, in which immunohistochemistry had shown positive staining for the IgA-BR of M22. The investigation revealed that an IgA-BR of a group A streptococcal M protein, possibly M22, was present in the biopsy, thus confirming the immunohistochemistry results.

2.3.2. PAPER II

The overall aim of this study was to investigate whether children with IgAN develop an antibody response to GAS expressing IgA-binding M proteins.

The results evidence for infections with GAS serotypes expressing IgA-BR in their M proteins in patients with IgAN.

2.3.2.1. *Detection of antibodies to IgA-binding regions of streptococcal M proteins*

To detect levels of antibodies to IgA-BR of streptococcal M proteins we developed an ELISA, which specifically measures IgG to the IgA-BR of M proteins from GAS serotype 4, 22 and 60, the N-terminal of M4 (M4-N), and as a negative control the N-terminal of the non-IgA-binding M5 (M5-N). Blood samples from patients with IgAN (n=21) were taken at around the time of the clinical onset of IgAN (median deviation from clinical onset was 3 months). Patient samples were compared to sera from age-matched controls (n=83). Ten of the 21 patients with IgAN had evidence for a recent infection with GAS as detected by throat cultures or serological assays for streptococcal infections (anti-streptolysin and/or anti-DNase B).

Combined antibody levels to the three IgA-BRs (from M4, 22 and 60) in IgAN patients were significantly higher than in controls ($p=0.016$). The difference was even more pronounced in the subgroup of patients with evidence for a recent streptococcal infection ($p=0.008$), which had higher levels of antibodies to IgA-BRs than the subgroup of patients without evidence for a recent streptococcal infection ($p=0.03$).

In patients the antibody levels to the IgA-BR of the M4 protein correlated significantly to those to the N-terminal of the M4 protein ($r=0.683$, $p=0.001$), which suggests an antibody response to a larger part of the M protein than the IgA-BR alone.

The results suggest that infections with GAS expressing IgA-binding M proteins are more common in children with IgAN than in controls.

The main drawback of this study is that we had access to IgA-BRs from only three different GAS serotypes expressing IgA-BRs in their M proteins. Despite antigenic cross-reactivity between certain IgA-BRs the three GAS serotypes available would most probably not cover all IgA-binding M serotypes.

2.3.3. PAPER III

The overall aim of the third study was to investigate the binding affinity of M4 to different types of IgA_1, the binding of M4 to mesangial cells, and the effect of stimulation with M4 and underglycosylated polymeric IgA1 on IL-6 synthesis and secretion as well as C3 secretion from mesangial cells.

The results of this study showed that M4 had a significantly higher affinity for underglycosylated polymeric IgA_1 compared to other forms of IgA_1. M4 exerted a significant stimulatory effect on IL-6 synthesis and secretion from mesangial cells. Co-stimulation of M4 with underglycosylated polymeric IgA_1 resulted in a synergistic up-regulation of both the synthesis and secretion of IL-6 from mesangial cells, which was related to M4 binding to IgA. Furthermore, underglycosylated polymeric IgA_1 induced C3 secretion from mesangial cells. The release of IL-6 and C3 from mesangial cells may contribute to the renal lesion seen in IgAN.

2.3.3.1. *Binding affinity of IgA-binding M proteins to IgA_1*

IgA1 was purified from sera from patients with IgAN and controls and separated into four fractions: normoglycosylated monomeric IgA_1, underglycosylated monomeric IgA_1, normoglycosylated polymeric IgA_1, and underglycosylated polymeric IgA_1. The binding affinity of M4 to IgA_1, dependent on size and ortho-glycosylation status in the hinge region, was investigated by BIAcore and ELISA.

Both methods showed a significantly higher binding affinity of M4 towards polymeric than monomeric IgA_1. Furthermore, M4 bound preferentially underglycosylated polymeric IgA_1 more than normoglycosylated polymeric IgA_1.

2.3.3.2. *Binding of M4 to primary human mesangial cells*

Using flow cytometry we showed that M4 binds to primary human mesangial cells. Future experiments will investigate the regions within the M protein responsible for this binding as well as which mesangial cell receptors are involved.

2.3.3.3. *IL-6 synthesis and secretion and C3 secretion from human mesangial cells stimulated with M4 and IgA_1*

Human mesangial cells were stimulated with M4, non-IgA binding M4-Δ451, underglycosylated polymeric IgA_1 and each of the two streptococcal proteins co-stimulated with underglycosylated polymeric IgA_1, for different time intervals. IL-6 synthesis by the mesangial cells was detected by real-time PCR from cell lysates. IL-6 and C3 secretion in cell supernatants was detected by ELISA.

A significant up-regulation of IL-6 synthesis and secretion from mesangial cells was induced by M4, M4-Δ451, underglycosylated polymeric IgA_1, and either of the two streptococcal proteins co-stimulated with IgA_1, compared to unstimulated mesangial cells. M4 as well as M4-Δ451 and IgA_1 exerted a synergistic effect on IL-6 synthesis and secretion by mesangial cells.

Underglycosylated polymeric IgA_1 induced C3 secretion from mesangial cells. Although the M4 proteins alone did not induce C3 secretion, co-stimulation with underglycosylated polymeric IgA_1 and either M4 or M4Δ451 enhanced the stimulatory effect of underglycosylated polymeric IgA_1 on mesangial C3 secretion.

The results suggest that IgA-binding M proteins, such as M4, detached from the bacterium during an infection, preferentially bind underglycosylated polymeric IgA_1 in the circulation. After deposition in the mesangial region the M4 and underglycosylated polymeric IgA_1 induce an inflammatory response in mesangial cells corresponding to that seen in IgAN. The IgA binding property of the M protein does not seem to be involved in this stimulatory effect but we speculate that IgA binding is necessary for the reaction to occur as it is by this means the M protein will reach the mesangial cell. The common finding of C3 in mesangial deposits during IgAN may be partly due to stimulated production by mesangial cells as shown in this study.

2.3.4. PAPER IV

The overall aim of the fourth study was to investigate whether genetic alterations in *CFH* could be involved in the clinical picture seen in a patient with IgAN complicated with TMA.

2.3.4.1. *Detection of a novel mutation and polymorphisms in a patient with IgAN*

We describe a 14-year-old patient with IgAN, malignant hypertension and TMA. Sequencing of the patient's *CFH* gene revealed a novel heterozygous mutation in exon 2, A48S (nucleotide position 142 32 G>T, alanine>serine). As the patient's blood sample exhibited reduced levels of CFH we suspect that the mutation may interfere with cellular secretion of CFH. Furthermore, sequencing showed the presence of three heterozygous polymorphisms (-257 c/t, 2089 a/g and 2881 g/t) shown to be associated with an increased risk of development of atypical hemolytic uremic syndrome[391].

The mutation in exon 2 could potentially lead to a decreased serum level of CFH and thus increased activation of the complement system. The polymorphisms could confer an increased risk for the development of TMA in this patient with IgAN.

2.4. Discussion

This study detected IgA-binding streptococcal M proteins co-localizing with IgA in tissue samples from patients with IgAN and HSP (Paper I). Furthermore we showed that children with IgAN have an antibody response to IgA-Br of M proteins from three different GAS serotypes suggesting that they have a higher prevalence of infections with GAS serotypes that express IgA-binding regions on their M proteins than age-matched controls (Paper II). The IgA-binding M protein from GAS serotype 4 had a higher binding affinity for underglycosylated polymeric IgA_1 than for other forms of IgA_1. M4 bound to mesangial cells and exerted, together with underglycosylated polymeric IgA_1, a stimulatory effect on IL-6 synthesis and secretion of mesangial cells. Stimulation of mesangial cells with underglycosylated polymeric IgA_1 induced a significant secretion of C3 (Paper III). In the last study we described an IgAN patient with a novel mutation in *CFH* leading to reduced serum levels of CFH and three HUS-risk polymorphisms in *CFH* (Paper IV).

Based on these findings I envisage the following sequence of events leading to the pathogenesis of IgAN and HSP (Figure 8). Children with IgAN commonly suffer from infections caused by IgA-binding M protein-expressing GAS. M proteins may bind IgA from secretions in the upper respiratory tract, which confers protection from phagocytosis of the bacterium[334]. IgA in these secretions is predominantly polymeric IgA_1[8]. During infections M proteins may be detached from the bacteria due to the effect of streptococcal or neutrophilic granulocyte-derived proteases[336,375]. M proteins bind to IgA on the $Fc_\alpha R$ binding site and the formed complex may thus escape phagocytosis[380]. The detached M proteins, in part bound to underglycosylated polymeric IgA_1, gain access to the circulation. IgA-binding M proteins, such as M4, exhibit a significantly higher affinity towards underglycosylated polymeric IgA_1 than to other forms of IgA_1 prevalent in serum. The relative amount of underglycosylated polymeric IgA_1 is increased in patients with IgAN and HSN[161,307]. Thus most of the M proteins circulating and deposited in affected tissues will be bound to this type of IgA. A certain portion of the M proteins may, after gaining access to the circulation, be bound by anti-M protein IgG. However, these anti-M protein IgG may as well bind to M proteins, which are already bound via Fcα-binding to underglycosylated polymeric IgA_1, and thus form immune complexes (Figure 8A).

M protein and underglycosylated polymeric IgA_1 pass through the fenestrated glomerular endothelium to the mesangial space and bind to mesangial cells. Both antigens collaborate in

inducing an inflammatory response in mesangial cells such as synthesis and secretion of IL-6. Increased local production of IL-6 may lead to mesangial cell proliferation and matrix expansion[215], which are typical light microscopic features in renal lesions associated with IgAN and HSN[50,265]. In addition, underglycosylated polymeric IgA_1 induces the secretion of C3 from mesangial cells. C3 in mesangial deposits may therefore originate, in part, from local synthesis by mesangial cells stimulated with underglycosylated polymeric IgA_1. This stimulatory effect on C3 secretion by mesangial cells would be slightly enhanced by the presence of M4. Thus underglycosylated polymeric IgA_1 and M protein provide a basic substrate for complement system activation.

The presence of C3b on the cell surface could allow complement activation, via the alternative pathway, to occur on the cell surface in the presence of dysfunctional or low levels of CFH. Impaired secretion of CFH from cells due to an N terminal mutation affecting its tertiary structure would result in reduced regulation of the alternative pathway C3-convertase. The resulting increased activity of the complement system would cause more severe inflammatory damage and would impact the clinical picture of IgAN (Figure 8B). Known HUS risk-associated polymorphisms in *CFH* could confer the risk for development of TMA in patients with IgAN. Many patients with newly diagnosed IgAN have evidence of renal TMA which may complicate the renal prognosis[99].

The difference in mesangial response to stimulation with M4 and underglycosylated polymeric IgA_1 regarding secretion of IL-6 or C3 support the hypothesis that the effect of the two antigens is mediated by different pathways. This hypothesis is further supported by the finding that the co-stimulatory effect of underglycosylated polymeric IgA_1 and M4 or M4Δ451 did not differ significantly, which suggests that the IgA-binding property of the M protein was not involved in this effect and both agonists exerted their effects separately (Figure 8B). Thus M proteins may play an important role in the pathogenesis of IgAN and HSP.

As streptococcal infections are rather common other factors are likely to contribute to the pathogenesis of IgAN and HSP as well. The scenario described above is probably dependent on a, thus far, undefined predisposition in individuals prone to the disease. A common finding in patients with IgAN and HSN is the prevalence of underglycosylated polymeric IgA1 in serum, which was even detected in healthy relatives to patients with IgAN[122,161,307]. The

alteration of IgA_1 could be the predisposing factor, which could make individuals susceptible to the development of IgAN.

The main conclusion of the present investigation is therefore, that infections with GAS expressing IgA-BR on their M proteins are involved in the etiology and pathogenesis of IgAN and HSP. Alterations in CFH may contribute to an increased activation of the alternative pathway of complement on the mesangium and predispose towards the development of TMA.

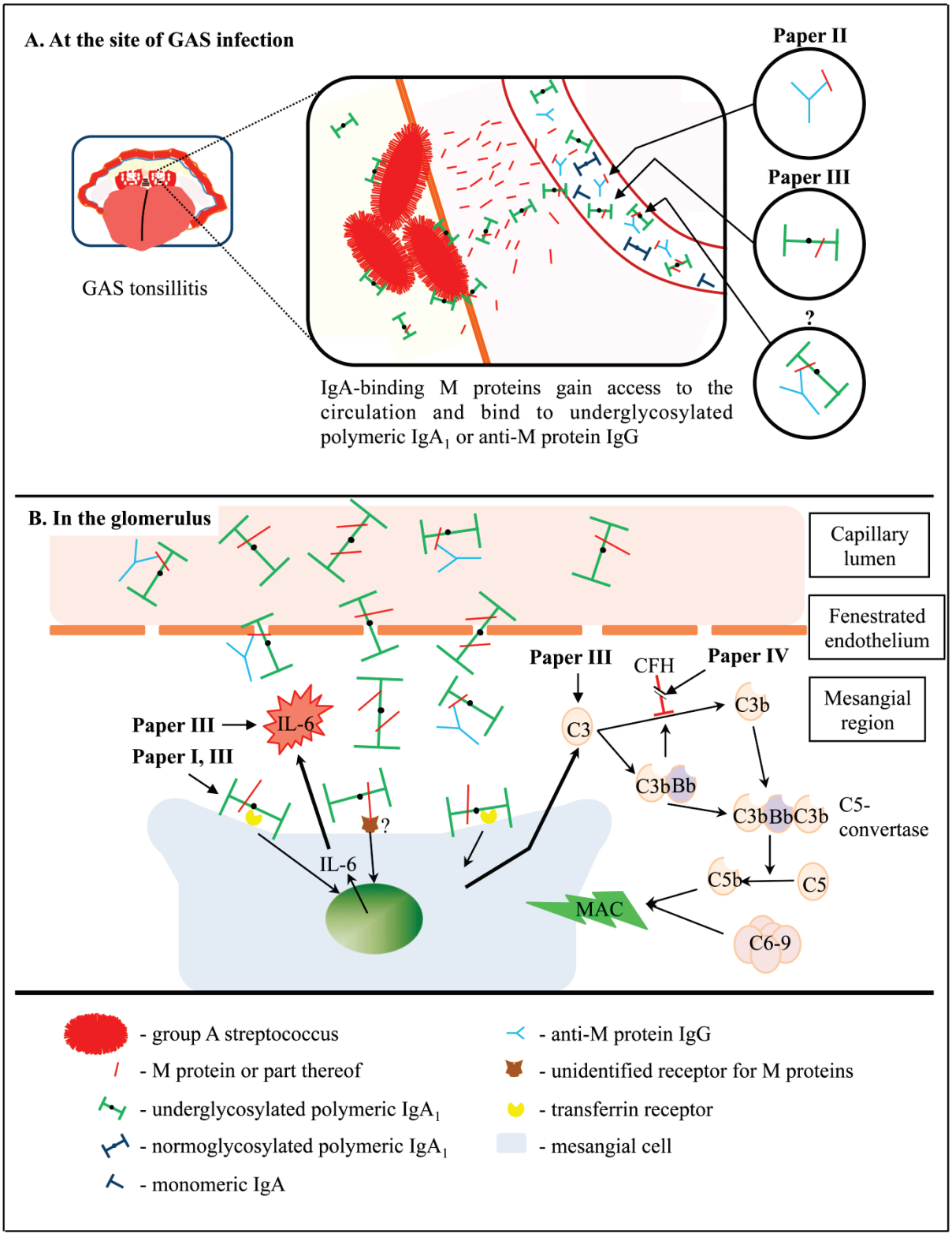

Figure 8: The role of streptococcal IgA-binding M proteins and N-terminal mutations in CFH in the pathogenesis of IgAN

Populärvetenskaplig sammanfattning (svenska)

IgA-nefropati (IgAN) är den vanligaste formen av primär inflammation av njurarna och Henoch-Schönlein purpura (HSP) den vanligaste formen av kärlinflammation i barndomen. HSP kan också drabba njurarna och kallas då Henoch-Schönlein nefropati (HSN). Orsaken till båda sjukdomar är inte helt klarlagd ännu.

Båda kännetecknas av vävnadsinlagringar med immunoglobulin A (IgA), ett slags äggvita eller antikropp, som kroppen producera för att försvara sig på slemhinnor och i blodet. Det finns två olika slags IgA: IgA_1 och IgA_2. IgA_1innehåller ett område (hinge-region) som binder sockerkedjor av varierande längd. IgA finns vidare som enskilda IgA (monomert IgA) eller i en annan form där två eller flera IgA har satt ihop sig (polymert IgA). Vävnadsinlagringar i IgAN eller HSP utgörs av underglykosylerat (korta sockerkedjor), polymert (flera IgA ihop) IgA_1.

I njurvävnadsprover från patienter med IgAN hittar man ofta bredvid det nämnda IgA även komplement faktor 3 (C3) och andra faktorer som antyder att komplement-systemet är aktiverat. Komplement-systemet är en viktig del av det medfödda försvaret (immunsystemet) och sammansätts av ett tretiotal äggvitor, som samspelar i den kaskadartade aktiveringen av komplement-systemet eller kontrollen över den. Systemet är mycket effektivt och ställa till med stora skador ifall kontrollen över den rubbas. Den viktigaste äggvitan som kontrollera komplement-systemet är komplement faktor H.

En annan gemensam nämnare till IgAN och HSP är att utbrotten av båda vanligtvis föregås av luftvägsinfektioner, som ofta orsakas av grupp A streptokocker. Dessa är bakterier som orsakar bland annat halsfluss, lunginflammation eller svinkoppar. De har på sin utsida en äggvita, som kallas M protein och som skiljs åt i strukturen mellan streptokockstammarna (serotyperna). Vissa av stammarna har M proteiner som binder IgA genom en IgA-bindande region. M-proteiner kan lösas ifrån bakterierna i samband med infektioner.

I de första tre studierna undersökte vi huruvida det finns ett samband mellan IgA-bindande M proteiner från streptokocker och utvecklingen av IgAN eller HSP.

I första studien undersökte vi vävnadsprover av patienter med IgAN och HSP avseende spår av IgA-bindande M proteiner och påvisade IgA-bindande M proteiner i många av dessa. Vi såg att de IgA-bindande M proteiner fanns på samma ställen som IgA i njurarna av patienter med IgAN och HSN.

I andra studien jämförde vi antikroppshalten (immunoglobulin G, IgG) mot IgA-bindande regioner av M-proteiner i blodprover av en grupp barn med IgAN med blodprover från lika gamla kontrollbarn. Mängden av antikropparna mot de undersökta IgA-bindande regioner var signifikant högre i patientgruppen, vilket tyder på att dessa i högre utsträckning drabbats av infektioner med streptokocker, som har IgA-bindande M proteiner.

I tredje studien undersökte vi bindningen av det IgA-bindande M proteinet från en streptokock-serotyp (M4) till IgA_1 och kunde visa att den har en mycket större dragningskraft

(bindningsaffinitet) mot underglykosylerat polymert IgA_1 än mot andra typer av IgA_1. Sedan visade vi att M-proteinet binder till njurceller och att dessa som ett svar börja tillverka och utsöndra ett inflammatoriskt budämne (interleukin-6, IL-6). Tillverkningen och utsöndringen tilltog betydligt när vi stimulerade njurcellerna med M-proteinet tillsammans med underglykosylerat polymert IgA_1. Även utsöndringen av komplement faktor 3, en viktig äggvita i aktiveringen av komplement-systemet, från njurcellerna ökade klart genom att låta underglykosylerat polymert IgA_1 inverka på dessa.

I fjärde studien beskriver vi en flicka med IgAN, som utvecklat högt blodtryck och en komplikation som kännetecknas av proppbildningar och sönderfall av röda blodproppar i de små kärlen i filterenheterna (glomeruli) av njurarna (trombotisk mikroangiopati, TMA). Vi undersökte patientens arvsmassa ifrån ett blodprov avseende förändringar i arvsanläggningen (genen) för komplement faktor H. Vi fann att en hittills okänd mutation stört utsöndringen av faktor H ifrån de cellerna som producerar den. Vidare hittade vi tre stycken avvikelser, som tidigare har beskrivits kunna öka risken att utveckla en annan orsak till TMA (hemolytisk uremiskt syndrom, HUS) och som kan ha inverkat på utvecklingen av TMA i patienten.

Sammanfattningsvis så visade vi i våra studier att IgA-bindande M-proteiner från grupp A streptokocker sannolikt är delaktiga i sjukdomsutvecklingen (patogenesen) av IgAN. Genetiska förändringarna av faktor H skulle kunna leda till ökad aktivering av komplementsystemet och därmed ha inflytelse i det kliniska förloppet hos patienten med IgAN.

Populärwissenschaftliche Zusammenfassung (deutsch)

Die IgA-Nephropathie (IgAN) ist die gewöhnlichste Form von primärer Nierenentzündung und Schönlein-Henoch Purpura (HSP) die gewöhnlichste Form von Gefäßentzündung im Kindesalter. HSP kann die Nieren betreffen –eine Komplikation die Schönlein-Henoch Nephropathie (HSN) genannt wird. Die Ursache beider Erkrankungen ist noch nicht gänzlich klargelegt.

Beide Krankheiten weisen charakteristische Gewebeeinlagerungen von Immunoglobulin A (IgA) auf. IgA ist eine Art von Eiweiß oder Antikörper, den der Körper produziert um sich an Schleimhäuten oder im Blut zu verteidigen, und liegt in zwei Formen vor: IgA_1 und IgA_2. IgA_1 beinhaltet einen Teil, die so genannte Hinge-Region, in welcher Zuckerketten von verschiedener Länge gebunden werden können. Weiter kann IgA als einzelne IgA (monomeres IgA) oder als eine Verbindung von zwei oder mehreren IgA (polymeres IgA) vorliegen. IgA_1 in Gewebeeinlagerungen bei IgAN oder HSP ist in erster Linie unterglykosyliert (kurze Zuckerketten) und polymer (mehrere IgA zusammen).

In Gewebeproben von Patienten mit IgAN findet man häufig neben IgA auch Komplementfaktor 3 (C3) und andere Eiweiße, die andeuten, dass das Komplementsystem aktiviert ist. Das Komplementsystem ist ein wichtiger Teil des angeborenen Verteidigungssystems (Immunsystem) und setzt sich aus ca 35 Eiweißen zusammen, welche in der kaskadenartigen Aktivierung oder der Kontrolle darüber zusammenwirken. Das System

ist extrem effektiv und kann bei gestörter Kontrolle zu weitreichenden Schäden führen. Das wichtigste Eiweiß in der Kontrolle über das Komplementsystem ist der Komplementfaktor H.

Ein weiterer gemeinsammer Nenner von IgAN und HSP ist, dass dem Ausbruch von beiden für gewöhnlich Luftwegs-Infektionen vorrausgehen, und dass diese häufig von Streptokocken verursacht sind. Streptokokken sind Bakterien die Mandelentzündungen (Angina), Lungenentzündungen oder Hautentzündungen (Impetigo contagiosum, Grindflechte) verursachen können. Auf ihrer Außenseite sitzen Eiweiße, die so genannten M-Proteine, die sich in ihrer Struktur zwischen den verschiedenen Streptokockenstämmen (Serotypen) unterscheiden. Ein Teil der Serotypen hat M-Proteine, die IgA binden können. M-Proteine können im Zusammenhang mit Infektionen von den Bakterien losgelöst werden.

In den ersten drei Studien untersuchten wir den Zusammenhang zwischen IgA-bindenden M-Proteinen von Streptokokken und der Entwicklung von IgAN oder HSP.

In der ersten Studie untersuchten wir Gewebeproben von Patienten mit IgAN und HSP auf Spuren von IgA-bindenden M-Proteinen. Wir wiesen IgA-bindende M-Proteine in den meisten dieser Gewebeproben nach. Weiter konnten wir zeigen, dass die IgA-bindenden M-Proteine sich an den gleichen Stellen in den Nieren von Patienten mit IgAN und HSN befanden wie IgA.

In der zweiten Studie verglichen wir die Menge von Antikörper (Immunoglobulin G, IgG) gegen die IgA-bindenden Regionen von M-Proteinen in Blutproben von Kindern mit IgAN mit der in Blutproben von gleichaltrigen Kontrollkindern. Die Menge der Antikörper gegen die IgA-bindenden Regionen war signifikant höher in der Patientengruppe, welches andeutet, dass diese in höherer Ausstreckung an Infektionen mit Streptokokken mit IgA-bindenden M-Proteinen erkrankt waren.

In der dritten Studie konnten wir zeigen, dass die Anziehungskraft (Bindungsaffinität) eines IgA-bindenden M-Proteins gegenüber unterglykosyliertem polymerem IgA_1 deutlich grösser ist als gegenüber alla anderen Typen von IgA_1. Das M-Protein bindet sich an Nierenzellen (Mesangiumzellen), welche als Reaktion damit anfangen Entzündung-Botenstoffe (Interleukin-6, IL-6) zu produzieren und auszuscheiden. Die Produktion und Ausscheidung von IL-6 nahm deutlich zu als wir mit M-Protein und underglykosyliertem polymeren IgA_1 zusammen stimulierten. Nach Inkubation mit IgA_1 stieg auch die Ausscheidung von Komplementfaktor 3, einem wichtigen Protein des Komplementsystemes, von den Nierenzellen.

In der vierten Studie beschreiben wir ein Mädchen mit IgAN, welches einen Bluthochdruck und eine Komplikation in Form von Gefäßverschlüssen in den Filtereinheiten der Nieren (Glomerulus) samt Zerfall der roten Blutkörperchen in den Blutgefäßen erlitt (thrombotische Mikroangiopathie, TMA). Wir untersuchten die Erbmasse des Patienten, gewonnen aus einer Blutprobe, angesichts der Erbanlage (Gen) für den Komplementfaktor H. Wir fanden, dass eine Mutation die Ausscheidung von Faktor H aus der ihn produzierenden Zelle störte und damit eine unkontrollierte Aktivierung des Komplementsystemes verursachte. Außerdem

fanden wir drei Abweichungen, die dafür bekannt sind das Risiko zur Entwicklung einer anderen Ursache für TMA (Hämolytisches, urämisches Syndrom, HUS) zu erhöhen.

Zusammenfassend haben wir in unseren Studien gezeigt, dass IgA-bindende M-Proteine von Streptokokken wahrscheinlich an der Krankheitsentwicklungen (Pathogenese) von IgAN und HSP beteiligt sind. Veränderungen von Faktor H könnten zu unkontrollierter Aktivierung des Komplementsystemes führen und deshalb von Bedeutung für den klinischen Verlauf von IgAN sein.

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Tissue Deposits of IgA-Binding Streptococcal M Proteins in IgA Nephropathy and Henoch-Schönlein Purpura

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IgA nephropathy (IgAN) and Henoch-Schönlein purpura (HSP) are diseases characterized by IgA deposits in the kidney and/or skin. Both may arise after upper respiratory tract infections, but the pathogenic mechanisms governing these diseases remain unclear. Patients with IgAN (n = 16) and HSP (n = 17) were included in this study aimed at examining whether IgA-binding M proteins of group A streptococci could be involved. As M proteins vary in sequence, the study focused on the IgA-binding-region (IgA-BR) of three different M proteins: M4, M22, and M60. Renal tissue from IgAN and HSP patients and skin from HSP patients were examined for deposits of streptococcal IgA-BR by immunohistochemistry and electron microscopy using specific antibodies, and a skin sample from a HSP patient was examined by mass spectrometry. IgA-BR deposits were detected in 10/16 IgAN kidneys and 7/13 HSP kidneys. Electron microscopy demonstrated deposits of IgA-BRs in the mesangial matrix and glomerular basement membrane, which colocalized with IgA. Skin samples exhibited IgA-BR deposits in 4/5 biopsies, a result confirmed by mass spectrometry in one patient. IgA-BR deposits were not detected in normal kidney and skin samples. Taken together, these results demonstrate IgA-BR from streptococcal M proteins in patient tissues. IgA-BR, would on gaining access to the circulation, encounter circulatory IgA and form a complex with IgA-Fc that could deposit in tissues and contribute to the pathogenesis of IgAN and HSP. ***(Am J Pathol 2010, 176:608–618; DOI: 10.2353/ajpath.2010.090428)***

Tissue deposits containing IgA characterize IgA nephropathy (IgAN) and Henoch-Schönlein purpura (HSP), two conditions affecting kidney function. IgAN is the most common primary glomerulonephritis worldwide. Its predominant clinical feature is episodic macroscopic hematuria usually coinciding with upper respiratory tract infections. Symptoms may, however, vary from microscopic hematuria to a severe nephritic-nephrotic syndrome. End-stage kidney disease occurs in 30% to 40% of patients within 20 years. Histopathologically IgAN is characterized by mesangial cell proliferation and in progressive cases crescent formation as well as glomerular sclerosis, interstitial fibrosis, and tubular atrophy. Ultramorphologic findings show mesangial deposits of immune complexes containing predominantly IgA.[1,2]

HSP is the most common form of vasculitis in childhood. It may affect many organs, but usually presents as skin lesions, varying from purpura to bullous intradermal bleedings, arthritis, gastrointestinal involvement with pain and/or bleeding. Renal involvement occurs in up to 50%

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of cases[3] and is known as Henoch-Schönlein nephropathy (HSN). HSN may manifest as microscopic or macroscopic hematuria as well as glomerulonephritis or nephrotic syndrome. Approximately 20% of HSN cases will develop renal failure.[4] The histopathological lesion termed leukocytoclastic vasculitis is characterized by inflammation of small vessels with perivascular polymorphonuclear leukocyte and mononuclear cell infiltrates. Immune deposits in affected organs contain IgA, and renal pathology resembles that seen in IgAN.[1,3]

The IgA mesangial deposits in kidneys of patients with IgAN and HSP are primarily composed of galactose-deficient IgA1.[5–7] The mechanism by which under-glycosylated IgA1 deposits in the mesangium, possibly in complex with IgG,[8,9] has not been determined. Environmental antigens have been proposed to contribute to the disease but have not been consistently associated with mesangial deposits.[9] Although the etiology of IgAN and HSP is unclear, these diseases are often preceded by infections, primarily of the upper respiratory tract, and an infectious agent has therefore been suspected. There is circumstantial evidence for involvement of group A streptococcus (GAS, *Streptococcus pyogenes*),[10–15] but infections with other bacteria[16,17] as well as viruses[18] have been implicated as well.

In this study we hypothesized that GAS infection could trigger IgAN and/or HSN, because GAS is a very common cause of upper respiratory tract infection, and because many GAS strains bind IgA-Fc.[19–21] The ability of a GAS strain to bind human IgA results from the presence of an IgA-binding region (IgA-BR) in the surface-localized M protein.[22,23] The fibrillar M protein, which is a major virulence factor of GAS, varies in sequence between strains[24] allowing classification of GAS isolates into more than 120 M serotypes.[25] The exact function of the IgA-BR in an M protein is not known, but there is evidence that it contributes to bacterial phagocytosis resistance.[26] The IgA-BR of an M protein represents a distinct domain that can be studied in isolated form, as a peptide that binds IgA.[27,28] Such IgA-binding peptides, designated Sap (streptococcal IgA-binding peptide), were used in the experiments described herein.

To analyze whether IgA-binding streptococcal M proteins are present in affected tissues of patients with IgAN and/or HSP, and colocalize with IgA, we used antibodies to the IgA-BR of three different M proteins M4, M22, and M60. Of note, M4 and M22 are among the most common serotypes of clinical GAS isolates.[29] As the IgA-BRs of different M proteins vary extensively in sequence,[22,23] the use of antibodies to three different serotypes enhanced our chances to detect tissue deposition of an IgA-BR.

Materials and Methods

Synthetic Peptides and Rabbit Antisera

The streptococcal IgA-binding peptides (Sap), designated Sap4, Sap22, and Sap60, are synthetic peptides derived from the M4, M22 and M60 proteins, respectively.[22,27] These M proteins were earlier designated Arp4, Sir22, and Arp60, respectively. By focusing the analysis on Sap peptides, rather than on intact M proteins, we could avoid the problem that many IgA-binding M proteins have a separate binding site for human IgG-Fc, a property that could complicate experimental conditions.[30,31]

Each Sap peptide has a length of 49 amino acid residues. The Sap4 and Sap22 peptides were derived from amino acids 35 to 83 of M4 and M22, whereas Sap60 was derived from amino acids 44 to 92 of M60. Native M-proteins form dimers but Sap peptides may not do so spontaneously.[27] A C-terminal cysteine residue, not present in the M protein, was therefore included in each Sap peptide to promote dimerization via a disulfide-bond and assure the IgA-binding ability of the peptides.[27,28] The 47-aa residue M4-N peptide corresponds to residues 1 to 45 of the N-terminal of the mature M4 protein, with the C-terminal addition of a tyrosine-cysteine sequence, not present in the M4 protein.[32] The M5-N peptide was derived from the N-terminal amino acid residues 1 to 50 in the non–IgA-binding M5 protein, with the C-terminal addition of a tyrosine-tyrosine-cysteine sequence.[27] M5-N was used as a negative control. All peptides were synthesized at the Department of Clinical Chemistry, Lund University, Malmö General Hospital, Sweden.

The specific IgA-binding properties of the Sap peptides immobilized in columns were tested using human serum applied to HiTrap columns (Amersham Bioscience, Uppsala, Sweden) containing the immobilized Sap peptides. Bound protein was eluted with acetate buffer pH 4.0. Normal human serum and the eluates were analyzed by SDS-PAGE. IgA heavy chains (H) and light chains (L) were identified by immunoblotting using specific antibodies as previously described.[27]

Rabbit antisera against dimerized Sap peptides were raised as described.[32] IgG fractions of the rabbit anti-Sap sera were further purified on a Protein A-Sepharose column (Amersham Biosciences) for tissue studies. Antigenic properties and cross-reactivity between different Sap peptides was analyzed using rabbit antisera and Sap peptides immobilized in microtiter wells as described.[32] The anti-Sap4 antiserum had a lower titer than the anti-Sap22 and anti-Sap60 antisera. The anti-Sap4 antibodies were therefore concentrated and purified by applying the serum to a Sap4 column.[26]

The anti-Sap antisera generated in rabbits would be expected to react with Sap but not with human IgA. Cross reactivity of the IgG-fractions of the anti-Sap sera with IgA was ruled out by ELISA using microtiter wells (Maxisorp, Nunc, Roskilde, Denmark) coated with normal human IgA (5 μg/ml, Sigma Aldrich, St. Louis, MO), probed with rabbit-anti–Sap-IgG (anti-Sap4 0.45 μg/ml, anti-Sap22 2.8 μg/ml, anti-Sap60 2.35 μg/ml, final concentrations). Bound antibodies were detected with goat anti–rabbit-HRP (0.3 μg/ml, Dako Cytomation, Carpinteria, CA) at OD 490 nm. Furthermore, the interaction was tested by Western blot under nonreducing conditions using normal human sera (n = 8, diluted 1/100 [v/v]) in sample buffer (3.2% [w/v] sodium dodecyl sulfate [Bio Rad, Hercules, CA], 8% glycerol [Sigma-Aldrich], 1% bromophenol blue [LKB Products AB, Bromma, Sweden] in 0.01 mol/L Tris buffer, pH 6.8 [ICN Biomedicals, Aurora, OH]) probed

with rabbit anti-Sap-IgG (anti-Sap4 0.9 μg/ml, anti-Sap22 3.7 μg/ml, anti-Sap60 9.4 μg/ml). Sap peptides (0.1 mg/ml) in control-serum or PBS were used as positive controls. Bound antibodies were detected with goat anti-rabbit-HRP (0.15 μg/ml). The anti–Sap-IgG did not bind to IgA using both of the methods, and in Western blot the anti-Sap did not react with any serum protein.

Subjects

Patients with IgAN (n = 16, 3 girls and 13 boys, median age 12.5 years, range: 3 to 19 years) and HSP (n = 17, 7 girls, 10 boys, median age 13 years, range 7 to 18 years) treated at the Department of Pediatrics, Lund University, between 1994 and 2008 were included in the study. IgAN was defined as the presence of hematuria and/or glomerulonephritis with or without proteinuria in patients in whom renal biopsy showed mesangioproliferative glomerulonephritis with immune deposits of IgA in glomerular mesangial cells and matrix. HSP was defined as per the criteria of the American College of Rheumatology.[33] Thirteen of the 17 HSP patients had nephropathy (HSN) manifesting as the occurrence of hematuria and proteinuria. The high proportion of HSN in patients treated for HSP reflects the clinical spectrum of patients referred to a tertiary center.

Clinically relevant data at the time of biopsy were obtained from the patients' hospital charts. Data regarding proteinuria, serum creatinine levels, as well as biopsy findings such as the presence of crescents, mesangial IgG deposits and tubulo-interstitial affection, were recorded. Proteinuria was measured by one of the following methods: albumin/creatinine clearance assayed using Hitachi Modular-P (Roche Diagnostics Scandinavia AB, Bromma, Sweden), urine albumin/creatinine index, quantified using Hitachi Modular-P, or by total quantitation of 24-hour urine albumin secretion. All analyses were performed by accredited hospital laboratories.

Microbiological Investigation of Patients

Evidence for GAS infection was investigated by microbiological (throat culture at time of onset) or serological methods. Anti-streptolysin O and/or anti-DNase B were assayed at routine hospital laboratories using kits from BioSystem S.A., Barcelona, Spain (anti-streptolysin O) and Dade Behring, Deerfield, IL, USA (anti-DNase B) in serum samples obtained within 0 to 17 months, median 1.5 months, after clinical debut of IgAN or HSP.

Three patients with IgAN had positive serology, and three had a positive throat culture for GAS. Two of these GAS isolates were saved. Thus 6/16 IgAN patients (37.5%) had evidence of recent streptococcal infection. Likewise, two patients with HSP had positive serology and one had a positive GAS throat culture; altogether 3/17 patients with HSP (17.6%) had evidence for recent streptococcal infection. The two GAS isolates taken from patients with newly diagnosed IgAN were obtained during an episode of acute pharyngitis with onset of macroscopic hematuria. These strains were of serotype M4 as determined by DNA sequencing.[34]

Tissue and Blood Samples

Renal biopsies were available from all patients with IgAN (n = 16). Biopsies that had been paraffin-embedded and saved at the Department of Pathology were used. Glomeruli were detected in all biopsies after routine diagnostics was completed. Crescent formation was found in nine biopsies and tubulo-interstitial affection (tubular atrophy, interstitial inflammation, and fibrosis) in eight biopsies. Biopsies were obtained at a median of eight months after the onset of disease (range, 2 to 72 months). The 16 biopsies used for immunohistological investigation had a median of 5 glomeruli (range, 1 to 24), a total of 76 glomeruli were detected. Renal biopsy was performed in 13/13 HSN cases within a median of 4 months after clinical debut of HSP (range, 0 to 60 months). Glomeruli were detected in all of the biopsies and showed mesangioproliferative glomerulonephritis with immune deposits of IgA in the glomerular pericapillary and mesangial regions. Crescents were noted in 8/13 biopsies and tubulo-interstitial affection in 5/13. The 13 biopsies used for immunohistological investigation had a median of 4 glomeruli (range, 1 to 13), a total of 80 glomeruli were detected. Skin biopsies were performed in five HSP cases during the acute stage of the disease and showed leukocytoclastic vasculitis, with pericapillary deposits containing IgA. Two of these five patients with HSP developed nephropathy. For one patient both a skin and a kidney sample were available.

Renal tissue from disease controls was chosen from individuals diagnosed with conditions other than IgAN and HSN whose renal biopsies showed glomerular IgA deposits. Disease control renal biopsies were available from 10 patients with: SLE-nephritis (n = 4, median age 12 years, range 8 to 16 years), membranous glomerulonephritis (n = 4, median age 49 years, range 10 to 65 years), Goodpasture's disease (n = 1, age 65 years), and poststreptococcal glomerulonephritis (n = 1, age 52 years). Normal renal tissue was available from three patients. One kidney biopsy, obtained from a 10-year-old girl as part of investigation of hematuria, was deemed normal by the hospital pathologist. Two kidney samples were obtained from nephrectomized kidneys of two adults (one female and one male) with renal cancer who had not received any chemo- or radiotherapy before surgery. Tissue was taken from an area unaffected by cancer and evaluated as normal by the hospital pathologist. Two histologically normal skin resection biopsies taken from unidentified adults with breast cancer were available.

Blood (serum or citrated plasma) samples, taken within a median of three months after onset of clinical disease (as defined by the presence of symptoms, range 1 to 13 months) and a median of 0 months before biopsy (range 0 to 2 months), were available from 12 patients (IgAN: n = 7, HSP: n = 5). Control blood samples were obtained from five healthy adults. Venous blood was collected in

4-ml vacutainer Hemogard SST tubes for serum or in 5-ml vacutainer tubes, containing 0.5 ml 0.129 mol/L sodium citrate for plasma (tubes from Becton Dickinson, Plymouth, UK). Samples were centrifuged at 2000*g* for 10 minutes and frozen at −20°C until assayed.

The study (patients and controls) was approved by the ethics committee of the Medical faculty of Lund University, and samples were obtained with written informed consent of all patients and controls, or the parents when patients were younger than 15 years.

Immunohistochemistry of the Kidney and Skin Biopsies

Immunohistochemsitry was performed on deparaffinized tissue sections as previously described[35] with certain modifications. Briefly, endogenous peroxidase activity was blocked by incubation in 10% methanol and 3% H_2O_2 (v/v) for 25 minutes at room temperature, and unspecific binding sites were blocked by incubation with normal goat serum (Dako, Glostrup, Denmark, 1/70 [v/v]). Detection of IgA-BR was performed using rabbit anti-Sap IgG (on renal tissue: anti-Sap4 0.38 μg/ml, affinity-purified anti-Sap4 0.06 μg/ml, anti-Sap22 0.7 μg/ml, anti-Sap60 0.39 μg/ml; on skin tissue: anti-Sap4 0.25 μg/ml, anti-Sap22 1.4 μg/ml, anti-Sap60 0.39 μg/ml) in 2.5% bovine serum albumin (BSA, MP Biomedicals Irvine, CA), 5 mmol/L Tris (Sigma-Aldrich), 0.9% NaCl (Scharlau Chemie SA), pH 7.6. Similarly, tissues were labeled with rabbit anti-M4-N (N-terminal of the M4 protein) at 0.14 μg/ml. As the secondary antibody peroxidase-conjugated goat anti-rabbit antibody (Envision$^+$-system, DakoCytomation) was used. Positive staining labeled brown.

Antibody specificity was tested by using either preimmune rabbit sera IgG in equimolar amounts to the anti-Sap, or by anti-Sap preincubated with a molar excess (20:1) of the specific Sap antigen for 1 hour at 37°C. Specificity of the secondary antibody system was tested by omitting the primary antibody. Slides were examined by light microscopy (Axiostar Zeiss, mounted with AxioCam MRc5 camera, Carl Zeiss AB, Stockholm, Sweden). AxioVision AC software version 4.4 (Carl Zeiss AB) was used for image processing.

Electron Microscopy

Kidney samples were sectioned and prepared for transmission electron microscopy as previously described.[36] Detection of IgA-BR was performed using rabbit anti-Sap60 IgG at a final concentration of 4.7 μg/ml or anti-Sap4 at 4.5 μg/ml in incubation buffer: 0.5% BSA, 0.1% gelatin (BioRad, Richmond, CA), 20 mmol/L NaN3 (Sigma Aldrich) in PBS. For detection of IgA monoclonal mouse anti-human IgA (2 μg/ml in incubation buffer, Dako) was used. Tissue sections were incubated with the primary antibody at 4°C overnight. The secondary antibody was goat anti-rabbit IgG:gold 10 nm (for the Sap antibody) or goat anti-mouse IgG:gold 6 nm (for the IgA antibody, Aurion, Washington, PA) diluted 1/20 (v/v) in incubation buffer. Grids were examined as described.[37] Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) was used for image processing. Gold particles (6 and 10 nm) were considered to be associated if their proximity was 30 nm or less as each conjugated IgG molecule is 15 nm.[38,39]

Specificity of the anti-Sap antibodies was tested by preincubating with the specific Sap antigen (molar excess of Sap 28:1). For the anti–IgA-antibody normal mouse-IgG_1 (Dako) was used as the negative control. Specificity of the secondary antibody was tested by omission of the primary antibody.

ELISA for Detection of Complexes between IgA-BR and IgA in Blood Samples

ELISA microtiter wells (Maxisorp) were coated with the IgG-fraction of polyclonal rabbit anti-Sap4 (0.09 μg/ml), anti-Sap22 (0.18 μg/ml), or anti-Sap60 (0.10 μg/ml) followed by patient or control sera (1/100 [v/v]) in phosphate-buffered saline-Tween 0.05% (Medicago, Uppsala, Sweden) containing 0.1% BSA. As a positive control normal serum, to which IgA-BR (from M4, M22 or M60) 50 μg/ml was added, was used. To detect IgA bound to IgA-BR in serum, rabbit anti-human IgA-HRP (0.32 μg/ml, DakoCytomation) was added. Detection was performed at OD 490 nm.

Selected Reaction Monitoring Mass Spectrometry

Selected reaction monitoring mass spectrometry[40] was used to detect the presence of IgA-BR in skin. An ample skin sample from one patient with HSP was assayed for the presence of IgA-BR from M22 and compared with skin samples from the two controls.

Deparaffinized skin samples were suspended in lysis buffer consisting of 8 mol/L urea (Sigma-Aldrich, Steinheim, Germany), 30 mmol/L Tris (Fluka Chemie GmbH, Buchs, Switzerland), 5 mmol/L MgAc (Sigma-Aldrich), pH 8.5, ground with a grinding-pestle, sonicated, vortexed, and centrifuged at 14,000 rpm for 10 minutes. The supernatant was diluted fourfold with sterile H_2O to reduce the urea concentration. Sap22 (15 pmol/μl) in 0.1% formic acid (JT Baker, Tamro, Mölndal, Sweden) was used as the positive control. Human plasma samples with or without added Sap22, at concentrations of 1.5 pmol/μl to 0.25 fmol/μl, were also used as detection controls.

All samples were adjusted to equivalent protein amounts using the Bicinchoninic Acid Assay Kit (Pierce, Rockford, IL) and a pH of 8.5. Samples were reduced with 45 mmol/L dithiothreitol (Sigma-Aldrich) for 15 minutes at 50°C and after cooling to room temperature underwent alkylation using 100 mmol/L iodoacetic acid (Sigma-Aldrich) for 15 minutes in the dark at room temperature. The proteins were then digested by adding 1/25 (w/w) bovine trypsin (Sigma-Aldrich) overnight at 37°C. Digestion was terminated by addition of 88% formic acid. Samples were stored at −20°C until analyzed.

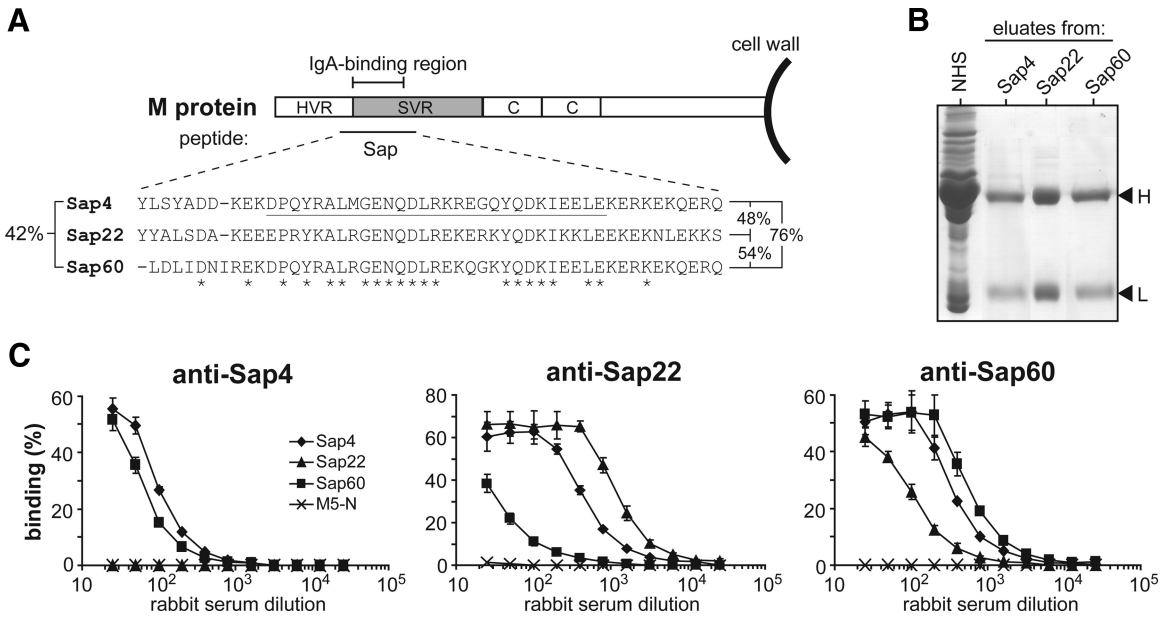

Figure 1. Characterization of the IgA-binding region of three streptococcal M proteins. **A:** Location and sequence of IgA-binding regions. The M protein is covalently attached to the bacterial cell wall and includes an N-terminal hypervariable region, a semivariable region, and a relatively conserved C-repeat region. Many M proteins have an IgA-binding region, as indicated. Synthetic peptides that include this region are designated Sap. The alignment shows the sequences of Sap4, Sap22, and Sap60 (derived from the M4, M22, and M60 proteins, respectively). The 29-residue sequence underlined in Sap4 represents the shortest region known to confer IgA-binding ability in an intact M protein.[22] **Asterisks** indicate residues identical in all three Sap peptides. Residue identity (%) for all three peptides is indicated to the **left** and pair-wise identities (%) are shown to the **right**. **B:** Sap peptides specifically bind IgA. Normal human serum (NHS) was applied to columns containing different immobilized Sap peptides. Proteins bound were eluted and analyzed by SDS-PAGE under reducing conditions, using NHS as a control. **Arrowheads** indicate the position of IgA heavy chains (**H**) and light chains (**L**). **C:** Antigenic cross-reactivity among Sap peptides. The three Sap peptides, and the control non-IgA binding peptide M5-N, were immobilized in microtiter wells and analyzed for capacity to bind rabbit antibodies directed against Sap peptides, as indicated. Data from three experiments with duplicate samples are presented as means ± SD.

Each sample (1.5 pmol) was loaded onto a trap column (Zorbax 300SB-C18, 5 μm, 5 × 0.3 mm) and separated on a Zorbax 300SB-C18 3.5 μm, 150 mm × 75 μm using an Agilent 1100 series capillary and nanopump 2D-separation system (Agilent Technologies, Santa Clara, CA). The peptides were eluted using a binary solvent system. Analysis was performed using a triple quadrupole mass spectrometer ThermoFinnigan TSQ Quantum (Thermo Finnigan, San Jose, CA) equipped with a nanospray source and a metallic emitter (Proxeon Biosystems A/S, Odense, Denmark).

Based on the known preference of trypsin for lysine or arginine residues and the sequence of the IgA-BR of the M22 protein (Figure 1A) six plausible cleavage products were predicted. Analysis of trypsin-digested Sap22 and Sap22-spiked plasma showed that only one predicted cleavage product, the peptide ALRGENQDLR (mass 1171 Da, mass/charge [*m/z*] ratio 586 [M2H+]), displayed a distinct peak (defined as more than threefold the background signal). This peak was eluted at 32.97 minutes. In normal human plasma it was not detected. A Blast Search with the UniProtKB release 13.2 database using NCBI BLASTP 2.2.17 showed that the analyzed peptide sequence does not occur in any known human protein, but exclusively in M-proteins of certain IgA binding GAS serotypes. Further experiments analyzed the predicted breakage of the filtered parent peptide specifically after Asp (D) and Asn (N) during passage through the collision cell and therefore transition of the *m/z* ratio from 586 to 884 and 641 in the daughter fragments.

Statistics

Statistical evaluation was performed using SPSS version 14.0 (Chicago, IL). The correlation between clinical/pathological features and IgA-BR-deposits was analyzed using the Fisher exact test. P values <0.05 were considered significant.

Results

Characterization of the IgA-Binding Region in Different M Proteins

The studies reported here used the M4, M22, and M60 proteins, which bind human IgA-Fc.[20,30,31] The IgA-BRs of the three M proteins were characterized in isolated form, as synthetic peptides designated Sap4, Sap22, and Sap60, respectively (Figure 1A). Overall, these three peptides show 42% amino acid residue identity. Affinity chromatography of whole human serum on immobilized Sap peptides showed that each peptide specifically binds IgA among all proteins in serum (Figure 1B). Thus, all three Sap peptides retained the properties of the IgA-BR of the corresponding intact M protein, implying that these peptides could be used for immunological comparisons of the IgA-BRs.

To compare the antigenic properties of the three Sap peptides, we used rabbit antisera and peptides immobilized in microtiter wells (Figure 1C). As expected, this analysis showed that the Sap peptides have different antigenic

Table 1. IgA-BR in Tissue Samples from Patients with IgAN and HSP

	IgA-BR of M4		IgA-BR of M22		IgA-BR of M60		Total	
	No. of positive samples/total	No. of labeled glomeruli/total in positive samples (%)	No. of positive samples/total	No. of labeled glomeruli/total in positive samples (%)	No. of positive samples/total	No. of labeled glomeruli/total in positive samples (%)	No. of positive samples/total	No. of labeled glomeruli/total in positive samples (%)
IgAN (kidney)	7/16*	17/44 (39%)	0/16	—	6/16	50/68 (74%)	10/16†	67/112 (60%)
HSN (kidney)	4/13*	12/26 (46%)	2/13	12/12 (100%)	6/12	23/43 (53%)	7/13†	47/81 (58%)
Total	11/29‡	29/70 (41%)	2/29	12/12 (100%)	12/28‡	73/111 (66%)	17/29†	114/193 (59%)
HSP (skin)	0/5	—	2/5	—	2/5	—	4/5	—

*Immunohistochemistry with polyclonal rabbit anti-Sap4 IgG showed positive glomerular staining in one patient with IgAN and three with HSN. Using affinity-purified anti-Sap4 IgG antibodies, positive staining was identified in an additional six patients with IgAN and one with HSN.

†Labeling with more than one antibody in the same sample occurred in some patients: with all three antibodies in two patients with HSN, and with two antibodies (anti-Sap4 and anti-Sap60) in one patient with HSN and in three patients with IgAN.

‡The number of kidneys investigated varied because of a lack of sufficient tissue for analysis with all antibodies.

properties although a certain degree of antigenic cross-reactivity was found. For example, anti-Sap4 reacted well with Sap4 and Sap60 but did not react with Sap22. These data indicate that it is essential to use antisera to different Sap peptides to detect tissue deposition of IgA-BRs.

Detection of the IgA-Binding Region of M Proteins in Kidney Samples from IgAN Patients and Kidney and Skin Samples from HSP Patients

Immunohistochemical investigation was performed to determine whether IgA-BRs of the M4, M22, or M60 proteins were deposited in the kidneys of patients with IgAN and HSN, and the skin of patients with HSP, using rabbit antibodies against the three Sap peptides.

In renal biopsies reactivity was seen in the mesangial region and around capillary walls in 17/29 biopsies (10/16 IgAN patients and 7/13 HSN patients, Table 1, Figure 2, A–C). Samples in which staining for an IgA-BR was detected were subject to quantification showing that 59% of glomeruli exhibited staining for IgA-BRs (Table 1). Labeling with more than one antibody in the same sample occurred in some patients: with all three antibodies in two HSN patients, and with two antibodies (anti-Sap4 and anti-Sap60) in one HSN patient and three IgAN patients.

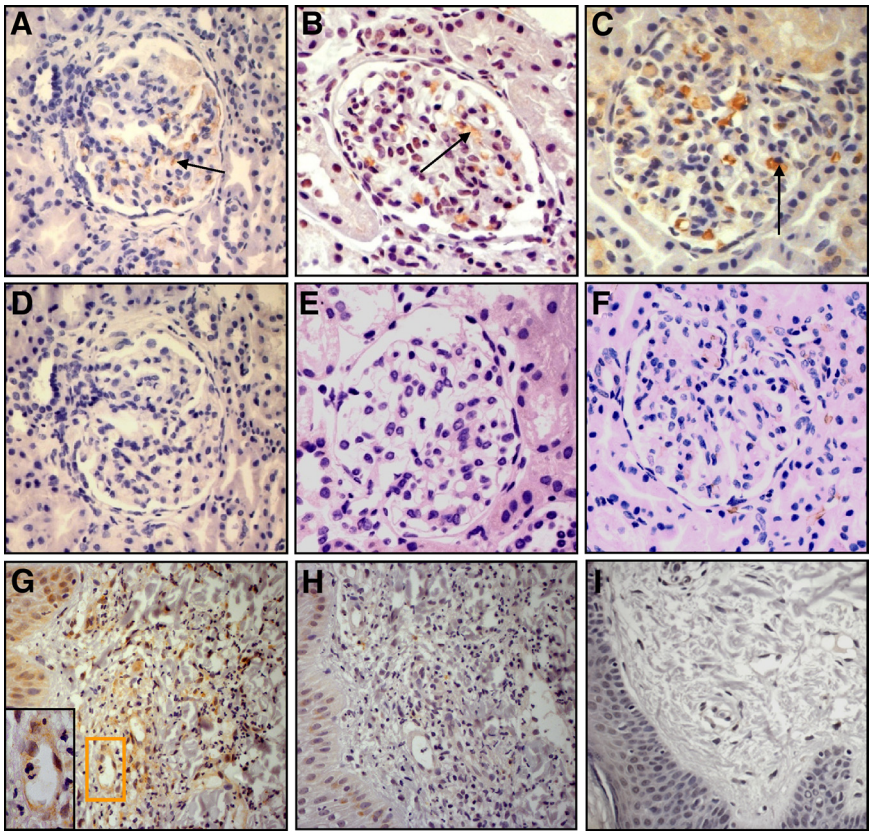

Figure 2. IgA-binding regions of streptococcal M proteins in kidney samples from IgAN patients and kidney and skin samples from HSP patients. **A:** Renal cortex from a patient with IgAN labeled with anti-Sap60 (ie, antibodies to the IgA-BR of GAS serotype 60). Positive labeling is brown and noted in the mesangial area (see **arrow**). **B:** Renal cortex from a HSP patient labeled with anti-Sap4. **C:** Renal cortex from a HSP patient labeled with anti-Sap60. **D:** Renal cortex from the same patient as in **A**. Lack of labeling when the biopsy was incubated with preimmune rabbit serum. **E:** Normal renal cortex stained with anti-Sap60. **F:** Renal cortex from the same patient as in **C**. Specificity demonstrated by lack of labeling when the antibody was preincubated with its specific antigen (Sap60). **G–H:** Skin sample from a patient with HSP showing typical leukocytoclastic vasculitis with perivascular polymorphonuclear leukocyte and mononuclear cell infiltrates. **G:** Labeling with anti-Sap22 with pericapillary staining, magnified in the **inset**. **H:** Lack of reactivity when the primary antibody was preincubated with its specific antigen, Sap22, demonstrating specificity. **I:** Skin sample from a control strained with anti-Sap22. All panels counterstained with hematoxylin and shown at magnification × 400.

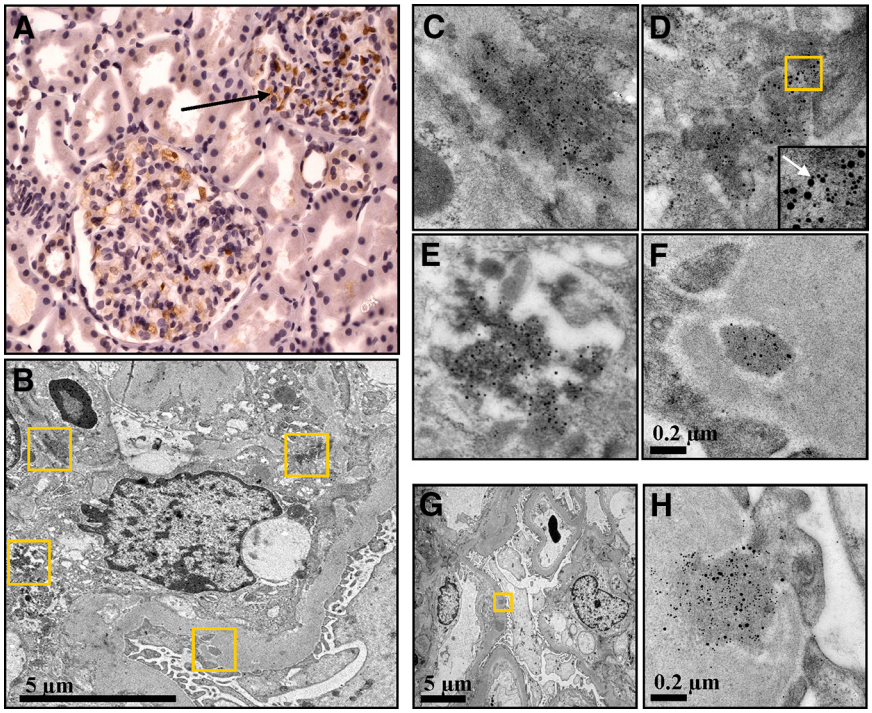

Figure 3. Ultrastructural colocalization of the IgA-binding region of M60 and IgA in the mesangial matrix of a patient with IgAN. **A:** Immunohistochemistry showing positive labeling for anti-Sap60 in the mesangial area from a patient with IgAN (see **arrow** pointing to brown labeling, counterstained with hematoxylin, magnification × 400). **B–H:** Immuno-electron microscopy of a renal biopsy from a patient with IgAN. Anti-Sap60 was conjugated to 10-nm gold particles and anti-IgA to 6-nm gold particles. The boxes in **panel B** are magnified in panels **C–F** (**upper left** in **C**, **upper right** in **D**, **lower left** in **E**, **lower right** in **F**). **C–E:** Electron-dense deposits of IgA-BR and IgA in the mesangial matrix. The box in panel **D** is magnified in the **inset** in which the **arrow** points to IgA-BR and IgA in close proximity. **F:** Electron-dense deposits of IgA-BR and IgA in the GBM. **C–F:** all at the same magnification. **G:** Electron-dense deposits in the GBM. **H:** Magnification of the boxed area in panel **G**. Colocalization of IgA-BR and IgA is indicated by the proximity (<30 nm) of the two different gold conjugates.

Control samples of normal renal tissue were negative (Figure 2E).

A GAS strain of serotype M4 was isolated from throat cultures of two patients with IgAN with pharyngitis and macroscopic hematuria. These throat cultures were taken 19 and 31 days before the respective renal biopsies were obtained. The biopsies, taken within two months after the onset of symptoms, exhibited deposits in the mesangial matrix that stained positively with antibodies to the IgA-BR of M4 (data not shown).

To study whether the deposited protein included a larger part of the IgA-binding M protein, we studied renal biopsies from patients that were positive for the IgA-BR of M4 (Table 1). These samples were analyzed for reactivity with antibodies to M4-N, a synthetic peptide derived from the N-terminal hypervariable region of the M4 protein (Figure 1A). Among five kidney biopsies analyzed, three stained positively for M4-N, suggesting that the streptococcal protein deposited in renal tissue may indeed be larger than the IgA-BR (data not shown).

In skin biopsies from patients with HSP perivascular deposits of IgA-BR were found in 4/5 samples (Figure 2G, Table 1). Two patients with HSP (without nephropathy) were positive for IgA-BR from M22, whereas two patients with HSN were positive for IgA-BR from M60. For one of the HSN patients both a skin and a kidney sample were available and positive labeling for the IgA-BR of M60 was demonstrated in each sample. Control samples of normal skin exhibited negative staining (Figure 2I).

Labeling for IgA-BR was found to be specific as it could be abolished by blocking the antibody with the specific antigen (Figure 2, F and H). Moreover, tissue that labeled positively with anti-Sap gave negative results when incubated with preimmune rabbit serum (Figure 2D). Kidneys from disease controls did not label for IgA-BRs (data not shown) with the exception of the renal sample taken from the patient with poststreptococcal glomerulonephritis, which showed positive labeling for IgA-BR of M4 in the mesangial region (data not shown).

Ultrastructural Localization of IgA-Binding Regions of M Proteins and Colocalization with IgA in Kidney Samples

The results described above indicated that IgA-BRs were present in many renal samples from patients with IgAN (Figure 3A) and HSN. Ultrastructural investigation of the localization of IgA-BRs was performed on three renal samples. One of these samples was from a patient with IgAN, whose kidney labeled for the IgA-BR of the M60 protein, one sample was from a patient with HSN, whose kidney labeled for the IgA-BR of the M4 protein, and one sample was from the pediatric control whose renal tissue was normal.

Immunogold labeling for the IgA-BR of the M4 or M60 proteins was found in the respective renal tissues from the two patients. Electron-dense deposits were primarily demonstrated in the mesangial matrix but also in the glomerular basement membrane (GBM, Figure 3, B–H for IgA-BR of M60, data not shown for IgA-BR of M4), the subendothelial and subepithelial regions of the GBM, the urinary space (between the GBM and podocyte foot processes), as well as around the capillary walls. No labeling was noted in other renal cells and regions or in the non-tissue background. The IgA-BR clustered in the same areas as IgA and colocalization was indicated by the proximity (≤ 30 nm) of the anti-Sap-antibodies and IgA (Figure 3D). All labeling of IgA-BR colocalized with labeled IgA.

Table 2. Correlation between Clinical or Pathological Findings and Presence of IgA-BR from M4, M22, and M60 in Renal Biopsies from Patients with IgAN and HSN

	IgA-BR positive (n = 17)	IgA-BR: negative (n = 12)	Correlation
Significant proteinuria*	12	7	P = 0.24; ns
Affected renal function†	1	2	P = 0.31; ns
Presence of crescents‡	11	6	P = 0.22; ns
Mesangial IgG deposits‡	5	3	P = 0.32; ns
Tubulo-interstitial involvement‡	7	6	P = 0.26; ns

ns indicates not significant.

*Significant proteinuria was arbitrarily defined using one of several clinical laboratory methods as follows: albumin/creatinine-clearance ratio of >0.7 (reference value <0.01); or a urine albumin/creatinine index of >60 g/mol (reference value <3.8 g/mol); or a urine albumin >1g/L (reference value <25 mg/L); or an annotation in the patient chart describing heavy proteinuria.

†Affected renal function was defined as a ≥30% elevation of serum creatinine above the upper normal limit related to age.

‡Biopsy findings determined by light microscopy (crescents, tubulo-interstitial involvement) or immunofluorescence (IgG deposits).

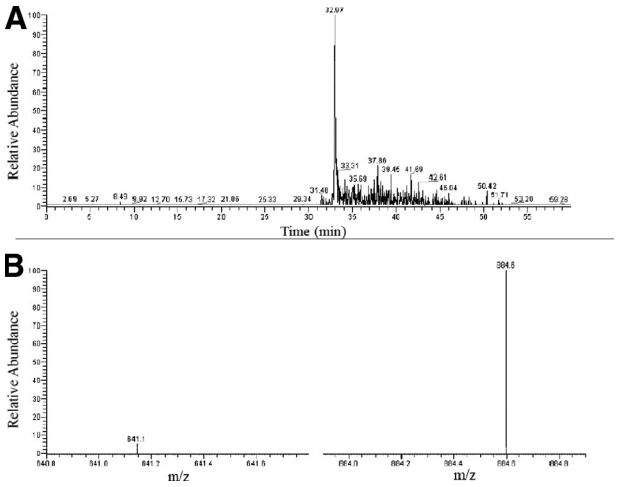

Figure 4. Detection of the IgA-BR from M22 in a skin sample from a patient with HSP. Mass spectrometry analysis of a trypsin-digested skin sample from a patient with HSP (same sample as in Figure 2, G and H). **A:** In the patient's sample a distinct peak was eluted at 32.97 minutes, the expected retention time for a peptide with the amino acid sequence ALRGENQDLR with a mass/charge (m/z) ratio of 586.313 (M2H+), which is present in the IgA-BR of M22. **B:** The molecule eluted at 32.97 minutes comprises two daughter ions with a m/z ratio of 641 and 884 in accordance with breakage in the parent peptide after aspargine and aspartic acid.

Quantification of the immunogold labeling in six different mesangial regions in renal tissue from the IgAN patient showed 163 IgA-BR-related 10-nm gold particles and 468 IgA-related 6-nm gold particles/μm^2. Immunogold labeling in five different mesangial regions in renal tissue from the HSN patient showed 164 IgA-BR–related 10-nm gold particles and 401 IgA-related 6-nm gold particles/μm^2. Reactivity with all three antibodies (anti-Sap4 and anti-Sap60 as well as anti-IgA) was not detected in the control renal tissue (data not shown). Furthermore, labeling for IgA-BR was abolished when the corresponding antibody was preincubated with the antigen (Sap4 or Sap60), and labeling for IgA was abolished when anti-IgA was replaced with an irrelevant mouse IgG_1 antibody. No labeling was detected when the primary antibodies were omitted (data not shown). These data provide additional evidence for the deposition of IgA-BRs of M proteins in kidneys of patients with IgAN and HSN and show that the IgA-BRs colocalize with IgA.

Lack of Correlation between Clinical or Renal Pathological Features and Detection of IgA-BR in Kidney Samples

The clinical and renal pathological features found in patients are summarized in Table 2 and further divided according to the detection of IgA-BR in renal tissue. The clinical/pathological manifestations did not show any significant correlation with the presence or absence of IgA-BR in kidney samples. The analysis was, however, limited to studies of the presence of three M proteins. Thus it cannot be ruled out that IgA-BRs from other M proteins may have been present in the tissues. The lack of correlation between the presence or absence of IgA-BRs and clinical/pathological features should therefore be interpreted with caution.

Investigation of Blood Samples for Circulating IgA-BR-IgA Containing Complexes

Blood samples from patients with positive labeling for IgA-BR in tissue samples were investigated for the presence of complexes containing IgA-BR and IgA-Fc by ELISA. No circulatory complexes between IgA-BR and IgA were detected. In addition, Western blot analysis of patient or normal sera did not detect any protein that reacted with the anti–IgA-BR antibodies. Thus circulating IgA-BR could not be identified by these methods.

Direct Detection of the IgA-BR of M22 in a Skin Sample

In an attempt to directly demonstrate the presence of an IgA-BR in the tissue of a patient, we analyzed a skin sample from a patient with HSP, found to be positive for the IgA-BR of M22, for whom sufficient material was available for this type of analysis. A trypsin digest of the tissue was analyzed by mass spectrometry. Control extracts were prepared from two samples of normal skin. A distinct peak with an estimated retention time of 32.97 minutes and comprising two ions was present in the patient's sample but not in the controls (Figure 4, A and B). This peak corresponds to a unique sequence present only in the IgA-BR of certain M proteins, including M22. These data indicate that an IgA-BR was present in the patient's tissue.

Discussion

Deposition of IgA in glomeruli is the pathological hallmark of IgAN and HSN. Electron microscopy of IgAN typically shows electron dense deposits consisting of IgA in the mesangial matrix, GBM, and glomerular capillary loops.[41,42] The means by which IgA is trapped in the mesangium is unclear as the human mesangial cell is devoid of the IgA receptor FcαRI (CD89)[43] but may express the transferrin receptor CD71 and the Fc α/μ receptor, both capable of binding IgA.[44,45] In the present study we demonstrate the presence of IgA-binding M proteins from GAS in proximity of IgA in the kidneys of many IgAN and HSN patients. The antibodies used to detect IgA-BRs in patient samples were highly specific and the direct detection of the IgA-BR of the M22 protein in the skin of one HSP patient further supported these findings. Thus, the data suggest that IgA-BRs of M proteins deposit in the kidney during IgAN and HSN and in the skin in HSP, and that these bacterial antigens could contribute to IgA deposition in patient tissues.

IgAN and HSP are frequently preceded by a respiratory infection. The results presented here suggest that a respiratory infection caused by GAS expressing an IgA-binding M protein may have occurred before symptoms of IgAN or HSP developed and support the view that infections with GAS play an important role in the pathogenesis of IgAN and HSP.[10–15] Because GAS infections seldom cause bacteremia or septicemia, the presence of M proteins in tissue is most probably not associated with bacterial invasion. Under certain conditions M proteins are presumably detached from the bacterial wall during bacterial penetration of the mucosal layer in the respiratory tract[46,47] and enter the systemic circulation. Alternatively, M proteins—or fragments thereof—may be released from phagocytes or antigen-presenting cells that have engulfed whole bacteria. The finding that some kidneys, in which IgA-BR of M4 were deposited, also stained for the N-terminal fragment of M4 (M4-N) suggests that the deposited fragment of M protein is larger than the IgA-BR.

IgA-BRs of M proteins bind IgA-Fc with high affinity,[27,48] and immobilized IgA-BR can be used to deplete human serum of IgA.[27] Thus IgA-BRs detached from bacteria should rapidly bind IgA present in the circulation and form circulating complexes, in which the bacterial protein is bound to the Fc part of IgA. Mesangial entrapment of an IgA-BR would then represent deposition of IgA in complex with an M protein or an M protein fragment. Alternatively, IgA-binding M proteins could first deposit in the kidney, followed by complex formation with IgA from the circulation, but this scenario seems less likely. Although IgA-BRs in complex with IgA were not detected in sera from patients whose kidneys exhibited deposits of IgA-BR, this result does not rule out the possibility of such circulatory complexes occurring earlier on in the course of disease, or at levels not detected by our techniques. A discrepancy between the occurrence of tissue deposits colocalizing with IgA and the lack of circulating complexes may be temporal as the disease may start long before symptoms occur and thus complexes present in tissue may no longer be detectable in serum. It would be intriguing to study the role of GAS in the development of IgAN *in vivo*, but, because the IgA-BRs of streptococcal M proteins show species specificity for human IgA[48] animal models cannot be used for exploring the deposition of IgA-BR containing complexes.

The ultrastructural localization of IgA-BR and IgA follows the pattern described in IgAN.[41,42,49] IgA-containing immune complexes have been detected in mesangium, capillary walls, GBM, and in the urinary space, but it is as yet unclear how immune complexes are deposited. Current models suggest that complexes may be passively trapped in the mesangium, because of the relatively high intraglomerular pressure within the capillaries, which have a large and highly permeable surface. Alternative models hypothesize *de novo* formation of immune complexes in the mesangium as IgA binds to either a mesangial autoantigen or to a planted antigen.[50] IgA-BR and IgA were detected not only in the mesangial matrix but also in the GBM and between the GBM and podocytes. The immune complexes are positively charged and the GBM is negatively charged, thus explaining affinity.[51]

Galactose-deficient IgA1 has been implicated in the pathogenesis of IgAN and HSN.[6,52–54] The mechanism by which the glycosylation deficit occurs is still unknown, but there is evidence that increased production of Th2 cytokines promotes IgA production and causes a defective glycosylation of IgA.[55] Interestingly, tonsillar lymphocytes derived from patients with IgAN produce underglycosylated IgA1,[56] suggesting that tonsillitis caused by GAS may elicit the release of underglycosylated IgA1 that deposits in kidneys in complex with an IgA-BR. As *in vitro* studies have indicated that streptococcal M proteins may increase production of Th2 cytokines in lymphocytes from patients with IgAN,[57] a link can be envisaged between streptococcal infection and defective glycosylation. Thus, there is no contradiction between our data and the hypothesis that underglycosylated IgA plays a key role in IgAN and HSN.[7]

The current investigation concentrated on three IgA-binding M proteins of different serotypes, but IgA-binding M proteins of several other serotypes also exist.[19,23,30] Among the three serotypes studied here, M4 and M22 are commonly identified among clinical GAS isolates,[29] providing an explanation for the presence of the corresponding IgA-BR in some patient samples. It was more surprising that the IgA-BR of the unusual serotype M60 was common among the samples. Possibly, IgAN and HSP are associated with infections caused by GAS strains of unusual serotypes. However, our data show that the IgA-BRs of different M proteins show some antigenic cross-reactivity, suggesting that samples identified as positive for a certain IgA-BR may actually contain the IgA-BR of another M protein. Thus, a biopsy shown to be positive for a certain IgA-BR does not necessarily reflect an infection with GAS of the corresponding serotype. In agreement with this, several biopsies reacted with antiserum to more than one Sap peptide. It follows that biopsies that were negative in our tests may have contained an IgA-BR that was not detected with the antisera

used. Our data are therefore compatible with the hypothesis that most cases of IgAN and HSP may be associated with tissue deposition of the IgA-BR of a streptococcal M protein. Based on this assumption it is not surprising that the patients' clinical and pathological features did not correlate significantly with the presence or absence of IgA-BR, as even those patients who did not exhibit tissue deposits of IgA-BR may have deposits of IgA-BR from an M serotype not tested for.

Microbiological investigation showed evidence of recent streptococcal infection in some patients. In particular, we isolated two GAS strains of serotype M4 in the throat cultures obtained from newly diagnosed IgAN patients during episodes of IgAN associated with acute pharyngitis, suggesting a causal relationship, as these patients also were positive for the IgA-BR of M4 in their renal biopsies. These results should, however, be interpreted with caution as the precipitating event causing IgAN could have occurred before the throat culture and renal biopsy was obtained. Pharyngitis caused by GAS M4 may thus have precipitated or exacerbated an episode of hematuria in these patients but was not necessarily the initiating event.

All but one control renal tissue stained negatively for the IgA-BR of GAS serotypes M4, 22, or 60. The one disease control sample that stained positively for the IgA-BR of GAS serotype M4 was taken from a patient with poststreptococcal glomerulonephritis. M4 is one of the known nephritogenic GAS serotypes causing poststreptococcal glomerulonephritis after upper respiratory tract infections,[58] and the presence of M proteins in renal biopsies from patients with poststreptococcal glomerulonephritis, primarily in the glomerular basement membrane and in the mesangial region, has previously been suggested.[59,60] Thus these results are in line with previous observations.

In summary, this study used two independent methods to demonstrate the presence of IgA-BRs of streptococcal M proteins in the kidney and skin of many patients with IgAN and HSP. These data, and the colocalization of IgA-BR and IgA, suggest a pathogenetic role of these M proteins in the etiology of IgAN and HSP, and focus interest on the role of GAS infection in the pathogenesis of these diseases.

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Short Communication

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Antibody response to IgA-binding streptococcal M proteins in children with IgA nephropathy

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Abstract

Background. IgA nephropathy (IgAN), the most common glomerulonephritis worldwide, is characterized by mesangial deposits containing predominantly IgA. IgAN commonly occurs or exacerbates after upper respiratory tract infections such as streptococcal pharyngitis. Certain group A streptococci express M proteins with IgA-binding regions (IgA-BRs). We have previously shown that these IgA-BRs co-localize with mesangial IgA in IgAN.

Methods. Blood samples from patients with IgAN (n=21) and age-matched controls (n=83) were assayed by ELISA to detect an IgG antibody response to the IgA-BRs of the M4, M22 and M60 proteins. Antibodies were assayed for each IgA-BR separately and the results were combined.

Results. Antibody levels to the IgA-BRs were significantly higher in IgAN patients than controls (P=0.016), particularly in patients with recent streptococcal infection (P=0.008).

Conclusions. The results suggest that children with IgAN had a previous infection with a streptococcal strain expressing an IgA-binding M protein.

Keywords: children; group A streptococci; IgA nephropathy; M protein

Introduction

IgA nephropathy (IgAN) is a common form of glomerulonephritis characterized by recurrent episodes of macroscopic haematuria, variably progressing to renal failure in which renal biopsies demonstrate predominantly galactose-deficient IgA1 deposits in the mesangium [1–3]. IgAN is often preceded by infections [4–10], primarily of the upper respiratory tract. Many cases are preceded by streptococcal pharyngitis [10] and clinical observations indicate that tonsillectomy may improve the outcome of IgAN [11].

Group A streptococci (GAS) express surface-localized M proteins, which have an N-terminal hypervariable region that determines the M type of the strain. Certain M proteins bind human IgA-Fc via a semi-variable region [IgA-binding region (IgA-BR)] [12–15]. In a previous study, we showed that most renal biopsies from paediatric IgAN patients contained mesangial deposits of IgA-BRs, which co-localized with IgA, suggesting a pathogenic role for IgA-BRs in IgAN [6]. The aim of the current study was to analyse the antibody response to IgA-BRs in children with IgAN.

Materials and methods

Subjects and blood samples

Patients with IgAN (n=21) and age-matched controls (n=83) were included in the study. Blood (serum or citrated plasma) samples were taken within a median of 3 months (range 0–14 months) after the onset of clinical disease (as defined by the presence of symptoms) in all patients. Detailed information regarding the patients and controls and blood samples taken are available as Supplementary data. The study was approved by the ethics committee of the Medical Faculty, Lund University and blood samples were obtained with informed written consent of all patients or their parents when patients were younger than 15 years.

Evidence for streptococcal infection in patients

Throat cultures and serological assays for streptococcal infection were available for 18 of 21 patients. Evidence for recent streptococcal infection was found in 10 of 18 patients at the initial presentation of IgAN. For the methodology, see the Supplementary data.

Synthetic peptides and rabbit antisera

The IgA-BRs of the M4, M22 and M60 proteins were available as synthetic peptides designated Sap4, Sap22 and Sap60, respectively [6]. The N-terminal hypervariable regions of M4 (M4-N) and of the non-IgA-binding M5 protein (M5-N) were also available as synthetic peptides [16]. Rabbit antisera to the peptides were raised as described [6,16].

Detection of IgG antibodies to the IgA-BRs of M4, M22 and M60

Serum IgG antibody levels to the Sap4, Sap22 and Sap60 peptides and also to the non-IgA-binding M4-N and M5-N peptides were measured by ELISA. Immunoblotting was used to test the specificity of the secondary antibody. For detailed descriptions, see the Supplementary data.

Statistics

Statistical evaluation was performed using SPSS version 17.0 (Chicago, IL). Differences in antibody levels were evaluated by the Mann–Whitney

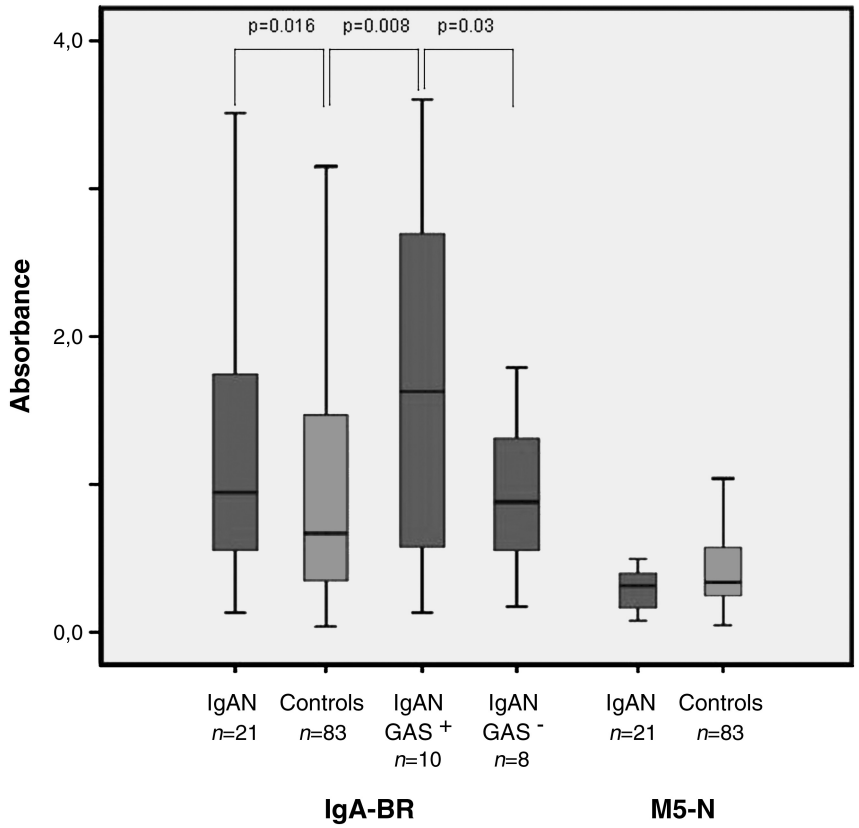

Fig. 1. Antibody levels to the IgA-BR of the M4, M22 and M60 proteins and the M5-N peptide in IgAN patients and controls. Levels are displayed as absorbance values obtained by ELISA. The line inside each box represents the median. The upper and lower limits of the box plot represent the interquartile range. The lower and upper limits represent the range. Higher antibody responses to the three IgA-BRs were found in IgAN patients than controls, especially if the subgroup of patients with evidence for recent GAS infection was compared with the controls. No difference between patients and controls was found in antibody levels to the non-IgA-binding M5-N.

U-test. Correlation of antibodies was evaluated using Spearman's rho test. P-values ≤ 0.05 were considered significant.

Results

Serum antibody response to the IgA-BRs of M4, M22 and M60

For all patients and age-matched controls, serum antibody levels to the IgA-BRs of the M4, M22 and M60 proteins were assayed for each protein separately and the results were combined (Figure 1). Antibody levels for each individual patient or control resulted in three separate observations, representing antibodies to the IgA-BRs of M4, M22 and M60 that were combined for comparison. Thus, results for IgAN patients (n=21) represent a combination of 63 observations, and the results for controls (n=83) represent 249 observations. The non-IgA-binding M5-N peptide, from the M5 protein, served as a control. Antibody levels to the IgA-BRs of the M4, M22 and M60 proteins were significantly higher in patients than in controls (P=0.016). This difference became even more significant when 10 sera of patients with evidence for recent GAS infection were compared with controls (P=0.008). Patients with evidence for recent GAS infection had higher levels of antibodies to IgA-BR than patients without (P=0.03).

Patient antibody levels to the IgA-BR of M4 correlated significantly with those to M4-N, derived from the most N-terminal region of M4 (r=0.683, P=0.001), suggesting an antibody response to a larger region of M4.

Discussion

In a previous study, we presented evidence that patients with IgAN have mesangial deposits of IgA in complex with a streptococcal M protein fragment that binds IgA-Fc, indicating that infection with an IgA-Fc-binding GAS strain contributes to the pathogenesis of IgAN [6]. The present study provides further support for this notion because patients with IgAN had significantly higher levels of IgG antibodies to streptococcal IgA-BRs. Although IgA-BRs vary in sequence among strains [15], sequence homology may cause cross-reactivity between different IgA-BRs, as described for M4, M22 and M60 [6]. Such cross-reactivity

may have also permitted demonstration of increased antibody levels in patients infected by a GAS serotype not included in our assay. Interestingly, analysis of the M4 system suggested that IgAN patients also have antibodies to the most N-terminal region of M4, suggesting an immunological response to a larger part of or the entire M protein in these patients. Together, these data provide evidence for a general prevalence of infections caused by IgA-binding GAS strains in children with IgAN.

Supplementary data

Supplementary data is available online at http://ndt.oxfordjournals.org.

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Conflict of interest statement. None declared.

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Antibody response to IgA-binding streptococcal M proteins in children with IgA nephropathy

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Supplementary data

Subjects

Patients with IgA nephropathy (IgAN, n=21, 6 girls and 15 boys, median age 13 years, range 3-19) treated at the Department of Pediatrics, Lund University, between 1994 and 2010 were included in the study. IgAN was clinically defined by the presence of glomerulonephritis with hematuria and/or proteinuria. All patients had proteinuria and all underwent renal biopsy showing mesangioproliferative glomerulonephritis with mesangial deposits of IgA. Thirteen of the 21 patients have been previously described [1]. Two patients were treated with steroids before blood samples were taken. No other immunosuppressive treatments had been given.

Control serum samples (n=83, 38 girls, 45 boys, median age 12 years, range 7-18 years) were available from pediatric outpatients suffering from diseases other than IgAN (asthma, growth retardation, diabetes mellitus type 1, status-post pyelonephritis, thrombotic thrombocytopenic purpura, neuroblastoma, neurological disease, opiate intoxication and children investigated for celiac disease in whom this diagnosis was ruled out).

Blood samples

Venous blood from patients and controls was collected in 4 mL vacutainer Hemogard SST tubes for serum or in 5 mL vacutainer tubes, containing 0.5 mL 0.129 mol/L sodium citrate for plasma (tubes from Becton Dickinson, Plymouth, UK). Samples were centrifuged at 2000 *g* for 10 min and frozen at −20°C until assayed.

Evidence for streptococcal infection in patients

Evidence for GAS infection was investigated by a microbiological method (throat culture at time of onset of IgAN) or serological methods (anti-streptolysin (ASO) and/or anti-DNaseB) assayed at routine hospital laboratories using kits from BioSystem S.A., Barcelona, Spain (ASO) and Dade Behring, Deerfield, IL (anti-DNaseB).

Serum samples were obtained and analyzed within 0-17 months, median 1.5 months, after clinical debut of IgAN. Seven patients with IgAN had a positive serologic assay and three had a positive throat culture for GAS.

ELISA for detection of IgG antibodies to the IgA-BR of M proteins serotype 4, 22, 60 and M4-N and M5-N

Microtiter wells (Maxisorp, Nunc, Roskilde, Denmark) were coated with 150 µl of Sap4, Sap22, Sap60, M4-N or M5-N peptides in solution, all at 2 µg/ml in 0.1 M $NaHCO_3$ pH 9.6 (Merck, Darmstadt, Germany), and incubated overnight at 4^{o}C. Wells were washed three times with 175 µl PBS-Tween 0.05% (PBS-T, Medicago, Uppsala, Sweden) and unspecific binding-sites blocked with 3% bovine serum albumin (BSA, MP Biomedicals, Irvine, CA) in PBS-T at rt for 2 h, followed by washing and incubation with 100 µl serum or plasma (1:100 v/v) in 0.1% BSA-PBS-T for 1 h at 37^{o}C. After washing, the secondary antibody (rabbit anti human-IgG:horse-radish-peroxidase (HRP, 100µl, Dako, Glostrup, Denmark, 1:2000 (v/v) in

0.1% BSA-PBS-T) was added and the wells were incubated for 1 h at 37°C. Wells were washed and the detection substrate O-phenylendiamine dihydrochloride tablets (Dako) suspended in dH_2O (3 ml/tablet) and 30% H_2O_2 (1.25 µl/tablet) was added for 6 min. The reaction was terminated by addition of 100 µl 1 M H_2SO_4 (Scharlau Chemie, Barcelona, Spain). Absorption was measured at $OD_{490\ nm}$.

Plate coating with the appropriate peptide was controlled for by ELISA as described above, but after the blocking step wells were incubated with rabbit sera anti-Sap or anti-N terminal peptide against the corresponding serotype 1/100 (v/v) in 0.1% BSA-PBS-T. As the secondary antibody goat-anti-rabbit IgG:HRP (Dako) 1/2000 (v/v) in 0.1% BSA-PBS-T was used.

Sap peptides could be expected to bind IgA in serum/plasma samples, resulting in reduced binding of IgG antibodies due to steric interference. The concentration of Sap used to coat the ELISA wells was therefore determined so that negligible IgA binding occurred. This was tested using microtiter wells coated with 150 µl Sap4, Sap22, or Sap60 in a dilution series from 0.5 to 15 µg/ml. After washing and blocking (as above) wells were incubated with 100 µl normal IgA (from human colostrum 50 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37°C. After washing, IgA-binding was measured using 100 µl rabbit anti human-IgA:HRP 1:2,000 (v/v, Dako) in 0.1 % BSA-PBS-T for 1 h at 37°C and detected as above. At a Sap concentration of 2 µg/ml, or less, the Sap peptides exhibited minimal IgA binding in this assay, but retained specific antigenicity for the corresponding rabbit anti-Sap antibodies as described above.

The specificity of IgG detection was demonstrated by IgG depletion of sera by passage through a Protein G-sepharose column (Amersham Biosciences, Uppsala, Sweden).

Immunoblotting to test the specificity of the secondary antibody

The IgG specificity of the secondary antibody (rabbit-anti-human-IgG:HRP) was tested by immunoblotting. Briefly, sera from two controls 1/50 (v/v) and IgG-depleted sera from the same controls 4/50 (v/v) in reducing sample buffer (4% (w/v) sodium dodecyl sulfate (SDS), Bio Rad, Hercules, CA, USA) in Tris buffer, pH 6.8 (ICN Biomedicals, Aurora, OH, USA) containing 10% (v/v) 2-mercaptoethanol (Kebo Lab, Spånga, Sweden) were separated by 10% SDS- polyacrylamide gel electrophoresis. The proteins were transferred onto a Protran nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) using a semi-dry electroblotter. The membranes were blocked with PBS-T containing 5% (w/v) dry milk powder (Semper, Sundbyberg, Sweden) and antibodies detected by rabbit anti-human IgG-HRP 1:2000 (v/v) or rabbit anti-human IgA-HRP 1:2000 (v/v) (both Dako) in blocking buffer. Labelling was detected by chemiluminiscence (Amersham ECL, GE Healthcare, Buckinghamshire, UK). Using anti-human-IgG:HRP IgG, but not IgA, was detected in serum from which IgG was not depleted. Anti-human IgA-HRP detected IgA in both IgG-depleted and non-depleted samples.

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Polymeric IgA_1 with galactose-deficient *O*-glycans binds to streptococcal IgA-binding M protein inducing IL-6 and C3 secretion by human mesangial cells: implications for the pathogenesis of IgA nephropathy

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Abstract

IgA nephropathy, the most common form of primary glomerulonephritis, is characterized by mesangial cell proliferation and matrix expansion with mesangial immune deposits containing predominantly polymeric IgA_1 with galactose-deficient *O*-glycans and complement C3 co-deposition. We have previously shown that IgA-binding regions of streptococcal M proteins co-localize with IgA in mesangial immune deposits in patients with IgA nephropathy. In the current study the IgA-binding M4 protein from group A streptococcus was found to bind to galactose-deficient polymeric IgA_1 with higher affinity than to other forms of IgA_1, as tested by surface plasmon resonance and a solid phase assay. Moreover, binding of M4 to mesangial cells was demonstrated by flow cytometry. When the mesangial cells were co-stimulated with M4 and galactose-deficient polymeric IgA_1 a significant synergistic effect on IL-6 synthesis and secretion was demonstrated by real-time PCR and ELISA, respectively. In addition, galactose-deficient polymeric IgA_1, but not M4, induced secretion of C3 from the cells. These results indicate that IgA-binding M4 protein binds preferentially to galactose-deficient polymeric IgA_1 and that these proteins induce excessive pro-inflammatory responses in human mesangial cells. Thus, tissue deposition of streptococcal IgA-binding M proteins may contribute to the pathogenesis of IgA nephropathy.

Introduction

IgA nephropathy (IgAN), the most common form of primary glomerulonephritis worldwide, is characterized by a proliferation of mesangial cells and matrix and deposits containing predominantly polymeric IgA_1 and C3 (1). The pathogenesis of IgAN has so far not been completely elucidated. IgA_1 differs from IgA_2 mainly by the presence of the hinge region, an 18 amino acid sequence between the $C\alpha_1$ and $C\alpha_2$ part of the heavy chains of IgA_1, with three to six attached *O*-glycans. The *O*-glycosylations consist of N-acetyl galactosamine (GalNAc), with the possible addition of galactose and up to two sialic acid residues. IgA_1 is considered galactose-deficient when the O-glycans lack terminal galactose (2). In sera from patients with IgAN a higher proportion of polymeric IgA_1 was found to be galactose-deficient when compared to healthy controls (3, 4) and galactose-deficient polymeric IgA_1 constitutes the major part of the IgA found in renal tissue deposits in IgAN (5, 6). Galactose-deficient polymeric IgA_1 was found to a have a higher affinity for human mesangial cells than normoglycosylated polymeric IgA_1 (7) and induced an inflammatory response and proliferation of these cells in vitro (8). However, as galactose-deficient IgA_1 is also found in healthy relatives of patients with IgAN and unrelated controls (3, 9) and deposits of IgA are also found in kidneys examined at autopsies of individuals without known kidney disease (10), other factors presumably contribute to the pathogenesis of IgAN.

The debut and exacerbations of IgAN are commonly preceded by infections, often affecting the upper respiratory tract. Thus various infectious agents have been investigated as possible triggers of IgAN (11-17). In particular, interest has been focused on group A streptococcus (GAS; *Streptococcus pyogenes*), a common cause of respiratory tract infections, which expresses surface-located M proteins. These proteins vary in amino acid sequence between strains, allowing division of GAS into more than 200 M serotypes (18). Many GAS serotypes

express M proteins that contain a region that binds human IgA (19-21). We have previously shown that children with IgAN are more likely than controls to have been infected with GAS expressing an IgA-binding M protein (22). Furthermore, we detected M protein-derived IgA-binding regions co-localizing with IgA in kidney samples of children with IgAN (14). These findings suggest a possible role of GAS M serotypes expressing IgA-binding regions in the etiology and pathogenesis of IgAN.

Interleukin 6 (IL-6) is a pro-inflammatory cytokine that most probably is of importance for the development of IgAN. The synthesis and secretion of IL-6 is up-regulated in renal tissues of patients with IgAN during active disease (23). The amount of IL-6 detectable in urine correlated with disease activity during IgAN (24). In vitro experiments have shown that IL-6 induces mesangial cell proliferation and matrix expansion, which are typical features of IgAN kidney pathology (25). IL-6 also induces the secretion of monocyte chemotactic peptide-1 (MCP-1), which results in the recruitment of lymphocytes, granulocytes and monocytes and subsequent inflammatory tissue damage (26). Interestingly, IL-6 synthesis in human mesangial cells is up-regulated by exposure to galactose-deficient polymeric IgA_1 (7).

Complement C3 is found in the mesangium in patients with IgAN. In IgAN complement activation in the kidney may occur via the alternative (27) or lectin pathways (28). Human mesangial cells have been shown to synthesize and secrete C3 in response to exposure to pro-inflammatory cytokines and immune complexes (29, 30) and mesangial C3 synthesis has been shown to be up-regulated in patients with IgAN (31).

Our previous studies demonstrated mesangial deposits of IgA-binding regions of GAS M proteins in the kidneys of IgAN patients. In the present study we hypothesized that IgA-

binding M proteins contribute to IL-6 and C3 release from human mesangial cells as inflammatory mechanisms contributing to IgA nephropathy. We investigated binding of IgA-binding M4 protein to galactosylated and galactose-deficient IgA_1 as well as to mesangial cells, and its capacity to induce IL-6 and C3 secretion from mesangial cells, alone and in combination with galactose-deficient IgA_1.

Materials and Methods

Streptococcal M proteins

M protein from group A streptococcus serotype 4 (M4, also known as Arp4) has been previously described and characterized (20, 21). The M4 protein binds human IgA-Fc due to the presence of an IgA-binding region in the semi-variable region of this M protein. The M4Δ451 mutant protein lacks this property due to a deletion of 18 amino acids within the IgA-binding domain (20) . The proteins were recombinantly synthesized in *Escherichia coli* and purified as described (20, 32). LPS contamination was ruled out using the Limulus amoebocyte lysate assay (Coatex AB, Gothenburg, Sweden). Rabbit anti-serum to the M4 protein was raised as described (33) and the IgG fraction was further purified using a Protein A-Sepharose column (Amersham Bioscience, Uppsala, Sweden).

Purification of galactosylated and galactose-deficient serum IgA_1

Venous blood was obtained from five healthy adults (median age 45 years) and from one boy (16 years old) with biopsy-proven IgAN. Blood sampling was performed with the informed written consent of adult controls and the patient as well as his parents and with the approval of the Ethics Committee of the Medical Faculty of Lund University. Samples were obtained in 4 mL vacutainer Hemogard SST tubes (Becton Dickinson, Plymouth, UK) and centrifuged, after blood-clotting, at 2000 *g* for 10 min. Sera from the five controls were pooled.

IgA_1 was purified from the sera (pooled normal sera or serum from the patient with IgAN) by ammonium sulphate precipitation (Merck, Darmstadt, Germany) followed by affinity chromatography using Jacalin immobilized on agarose (Vector labs, Burlingame, CA) (34).

Monomeric IgA_1 was separated from the polymeric form by size-exclusion chromatography on a Sephacryl S300-HR-column.

The galactosylated and galactose-deficient glycoforms of IgA_1 were separated using *N*-acetyl galactosamine (GalNAc)-specific lectin from *Helix aspersa* immobilized on agarose (Vector labs, San Mateo, CA) (35). The two fractions of IgA_1 (monomeric and polymeric) were separately processed using an overnight binding at 8°C. The unbound fraction (flow-through) contained galactosylated IgA_1. Galactose-deficient glycoforms of IgA_1 were eluted by addition of 0.05M GalNAc (Vector labs).

For cell stimulation experiments galactose-deficient IgA_1 was separated into monomeric and polymeric fractions by a centrifugal device with a spin-filter (Nanosep 300K Omega, Pall Norden AB, Lund, Sweden) followed by further purification by native gel electrophoresis under non-reducing conditions. After excision of gel bands corresponding to different molecular sizes, IgA fractions were eluted from the excised sections by one day's storage at rt in PBS (Medicago AB, Uppsala, Sweden) and concentrated using 10K centrifugal filter units (Millipore Corp., Billerica, MA). Purifications and size-differentiation were confirmed by silver-stained gels (Pierce Biotechnology Inc) and immunoblotting under non-reducing conditions using rabbit anti-human IgA:horseradish peroxidase (HRP, Dako Cytomation, Glostrup, Denmark) detected by chemiluminescence (ECL-Plus, GE Healthcare, Little Chalfont, UK).

Another form of galactose-deficient polymeric IgA_1 was isolated from the plasma of a patient with multiple myeloma using salt precipitation, IgG-depletion on an anti-IgG column and size-exclusion chromatography (36, 37). Heterogeneity of *O*-glycans of this IgA1 was fully characterized by high-resolution mass spectrometry (38).

Thus three sources of galactose-deficient polymeric IgA_1 were used in this study (from normal pooled sera, from a patient with IgAN and a well-characterized IgA1 myeloma protein (Table 1).

Binding of the M4 proteins to IgA_1 detected by surface plasmon resonance

The binding affinity of both M4 proteins to IgA_1 was determined by surface plasmon resonance using BIAcore technology. The IgA-binding M4 and non-IgA-binding M4Δ451 were diluted in 10 mM sodium acetate (pH 4) (Merck, Darmstadt, Germany) and immobilized via amine coupling on separate CM5 sensor chip chambers (GE Healthcare, Uppsala, Sweden) at moderate response levels (1500 response units) as previously described (39). A flow chamber subjected to the immobilization conditions but without addition of protein was used as a control (blank) for each experiment. Five IgA_1 fractions (galactose-deficient polymeric $IgA_{1\text{-Ale}}$ and $IgA_{1\text{-IgAN}}$, galactose-deficient monomeric IgA_1, galactosylated polymeric and monomeric IgA_1) were diluted in five dilution steps (31-500 nM) in running buffer (10 mM HEPES, 150 mM NaCl, 0.005% Surfactant P20, pH 7.5, BIAcore, Uppsala, Sweden) and injected separately over the surfaces. In between experiments the immobilized proteins were regenerated and washed as previously described (39). Experiments were performed on a BIAcore 2000 instrument (BIAcore). Binding curves were displayed and affinities (KD) calculated using BIAevaluation 4.1 software (BIAcore) as previously described (39).

Binding of the M4 protein to IgA_1 detected by ELISA

Microtiter wells (Maxisorp, Nunc, Roskilde, Denmark) were coated with M4 5 μg/ml in 0.1M $NaHCO_3$ pH 9.6 (Merck, Darmstadt, Germany) overnight at rt. Wells were washed three

times with phosphate buffered saline-Tween 0.05% (PBS-T, Medicago, Uppsala, Sweden) and unspecific binding sites blocked with 3% bovine serum albumin (BSA, MP Biomedicals, Irvine, CA) in PBS for 2 h at rt. Wells were washed and incubated with different fractions of IgA_1 (galactose-deficient polymeric $IgA_{1\text{-}Ale}$, galactosylated polymeric IgA_1, galactose-deficient and galactosylated monomeric IgA_1, the latter three fractions from normal pooled sera) at 20 µg/ml in 1% BSA-PBS for various times. The wells were then washed and rabbit anti human-IgA: HRP (Dako, Glostrup, Denmark) 1:2000 (v/v) in 1% BSA-PBS was added and incubated for 1 h at rt. After an additional wash binding was detected by incubation with O-phenylendiamine dihydrochloride tablets (OPD, Dako) for 15 min at rt. The reaction was terminated by addition of 0.5 M H_2SO_4 (Scharlau Chemie, Barcelona, Spain) and absorption measured at $OD_{490\ nm}$.

Binding of the M4 protein to human mesangial cells detected by flow cytometry

Flow cytometry experiments were conducted using primary human mesangial cells (Cell Systems Corp, Kirkland, WA) at passage 6-12 in DMEM medium (PAA Laboratory, Pasching, Austria) and a BD FACSCanto II cytometer with FACSDiva software (Becton Dickinson Immunocytometry Systems, San Jose, CA) as previously described (40). The M4 protein was incubated with mesangial cells for 60 min at 37^{o}C and binding was detected by rabbit-anti M4-IgG 10 µg/ml. As a control polyclonal rabbit IgG (AbD Serotec, Düsseldorf, Germany) was used. This was followed by the secondary antibody swine anti-rabbit-FITC (1/250, Dako Cytomation).

Culture and stimulation of human mesangial cells

Primary human mesangial cells at passage 5-7 were grown to near confluence in CS-C complete serum free medium (Cell Systems Corp) in 96-well culture plates (NUNC, Roskilde,

Denmark) at 21% O_2, 5% CO_2 and 37^{o}C. The cells were identified as mesangial by their typical stellate morphology and by immunofluorescence as previously described (41) showing positive staining for smooth muscle actin (monoclonal mouse anti-α SMA, Sigma, St. Louis, MO) and negative results for cytokeratin (monoclonal mouse anti-human cytokeratin, Dako Cytomations) and for von Willebrand factor (polyclonal rabbit anti-human VWF, Dako) excluding epithelial and endothelial cells, respectively.

Mesangial cells were washed twice with Dulbecco's PBS (D-PBS, PAA Laboratory). Cells were kept in resting condition for 36 h in CS-C serum-free maintenance medium. and then exposed to galactose-deficient polymeric $IgA_{1\text{-}ps}$ (100 µg/ml), M4 or M4Δ451 (both at 10 µg/ml) in CS-C serum-free maintenance medium, as well as M4 and M4Δ451 pre-incubated with galactose-deficient polymeric $IgA_{1\text{-}ps}$ for 1 h at rt. Cells were incubated with each of the agonists for 4 h, 24 h and 48 h. After stimulation supernatants were removed, Complete-Mini protease inhibitor (Roche Diagnostics, Mannheim, Germany) was added and supernatants were centrifuged at 200 xg for 5 min and stored at -80 ^{o}C until analyzed.

Viability of the mesangial cells was tested at the start of incubation, as well as at 4 h, 24 h, and 48 h using Trypan blue (Sigma). More than 95% of the cells were viable at all time points.

Detection of IL-6 mRNA in HMC cell lysates by real time-PCR

To obtain mesangial cell lysates the cells were dissolved in RLT buffer (Qiagen GmbH, Hilden, Germany) with 1% of mercapto-ethanol (Sigma). Cell lysates were stored at -80^{o}C until analyzed. Total RNA was extracted and complementary DNA synthesized using an RNeasy Kit (Qiagen) and Taqman Gold RT-PCR kit (Applied Biosystems, Foster City, CA)

according to the manufacturers' instructions. Real-time PCR was performed as described (41) using probes against IL-6 (Assay ID: Hs00985641_m1) and as a reference gene GAPDH (Assay ID Hs99999905_m1), Taqman Universal Master Mix (all from Applied Biosystems). No template was used as the negative control. Experiments were carried out on an ABI Prism 7000 machine (Applied Biosystems).

IL-6 in culture supernatants detected by ELISA

Supernatants from mesangial cell experiments were analyzed for IL-6 levels using an ELISA kit (R&D systems, Minneapolis, MN) according to the manufacturer's instructions.

C3 in culture supernatants detected by ELISA

Supernatants from mesangial cell experiments were analyzed for C3 levels using an ELISA as previously described (42), with minor modifications. Briefly, microtiter wells (Maxisorp, Nunc) were coated with polyclonal rabbit anti-human C3c (1:2000 v/v, Dako) in 0.1 M $NaHCO_3$, pH 9.6 (Merck). Wells were incubated with supernatants for 1 h at rt and bound C3 was labelled with goat anti-human C3c (1:1000 v/v, Sigma, St. Louis, MO). The secondary antibody was rabbit anti-goat immunoglobulin:horse radish-peroxidase (HRP, 1:1000 v/v, Dako).

Statistics

Differences in IgA binding to M4, IL-6 synthesis and IL-6 as well as C3 secretion were calculated by the Mann-Whitney U-test performed using SPSS version 20.0 (Chicago, IL). P values < 0.05 were considered significant.

Results

The IgA-binding streptococcal M4 protein preferentially binds to galactose-deficient *polymeric IgA$_1$*

Binding of IgA_1 dependent on size (monomeric vs polymeric) and O-glycosylation in hinge regions (galactosylated vs galactose-deficient) to M4 was investigated by two methods, surface plasmon resonance (BIAcore) and ELISA.

Results of the BIAcore analysis are presented in Figure 1A-F. Galactose-deficient polymeric IgA_1 (IgA1-$_{IgAN}$ and $IgA_{1\text{-}Ale}$) exhibited better binding to M4 than the other forms of IgA_1. Binding affinities were calculated by injecting five concentrations of each form of IgA_1 showing that galactose-deficient polymeric $IgA_{1\text{-}IgAN}$ and $IgA_{1\text{-}Ale}$ had a considerably lower KD and thus higher affinity (Figure 1G). None of the IgA preparations bound to the non-IgA-binding M4Δ451 mutant protein, as expected (data not shown).

Binding results were confirmed using an ELISA showing that galactose-deficient polymeric IgA_1 ($IgA_{1\text{-}Ale}$) bound significantly better to M4 than galactosylated polymeric IgA_1 (Figure 2). Both of the polymeric fractions of IgA_1 bound significantly better to M4 than monomeric IgA_1.

Binding of M4 protein to human mesangial cells

Binding of the M4 protein to human mesangial cells was investigated by flow cytometry as binding would be a prerequisite for the induction of a cellular response. M4 was detectable on 32% of the human mesangial cell population (Figure 3).

Binding of the M4 protein to human mesangial cells induced IL-6 synthesis

Human mesangial cells were stimulated with M4, the non-IgA-binding mutant protein M4Δ451, galactose-deficient polymeric $IgA_{1\text{-ps}}$, or a combination of either of the M proteins with galactose-deficient polymeric $IgA_{1\text{-ps}}$. Results using real-time PCR for analysis of mRNA from mesangial cell lysates are shown in Figure 4. At 0 h and after 4 and 24 h unstimulated mesangial cells synthesized minute amounts of IL-6 mRNA. For all stimulated samples, IL-6 synthesis was noted after 4 h stimulation and, with the exception of M4, exceeded IL-6 synthesis after 24 h stimulation. Stimulation with a combination of galactose-deficient polymeric IgA_1 and M4 induced significantly more IL-6 synthesis than any of the M proteins alone, but not than IgA_1 alone after 4 h. A similar trend was noted at 24 h but at this time-point there was significantly more IL-6 synthesis in the co-stimulated sample compared with IgA_1 alone, but not with M4 alone. Co-stimulation with galactose-deficient polymeric IgA_1 and M4Δ451 resulted in a significantly higher IL-6 synthesis than either of the stimulants alone at both time-points. At 4 h stimulation M4 did not induce more IL-6 synthesis than the non-IgA binding M4Δ451 variant suggesting that early induction of IL-6 synthesis in the cells was not associated with the IgA binding site on M4.

Galactose-deficient *IgA_1 induced IL-6 and C3 secretion from human mesangial cells is enhanced when co-stimulated with M4*

IL-6 was measured in supernatants from human mesangial cells stimulated with M4, the non-IgA-binding mutant protein M4Δ451, galactose-deficient polymeric $IgA_{1\text{-ps}}$, or a combination of either of the M proteins with IgA_1 (Figure 5). After 4 and 24 h stimulation all agonists induced a significant increase in IL-6 secretion compared to unstimulated mesangial cells. Even after 48 hr a significant increase in IL-6 was detected for all stimulants compared with unstimulated cells, with the exception of cells stimulated with M4Δ451. Co-stimulation with

galactose-deficient polymeric IgA_1 and M4 or M4Δ451 resulted in significantly higher IL-6 secretion than stimulation with either of the stimulants alone at all three time points. There was no difference in IL-6 secretion between cells co-stimulated with galactose-deficient polymeric IgA_1 and either of the two M proteins, with the exception of stimulation for 24 h, when M4-IgA_1 induced a higher IL-6 secretion than M4Δ451-IgA_1. Unstimulated human mesangial cells also exhibited a certain degree of IL-6 secretion after 24 h and 48 h incubation.

Stimulation of mesangial cells with galactose-deficient polymeric $IgA_{1\text{-}ps}$ induced C3 secretion from mesangial cells whereas M4 and M4Δ451 did not (Figure 6). However, co-stimulation with $IgA_{1\text{-}ps}$ and M4 or M4Δ451 increased the stimulatory effect of $IgA_{1\text{-}ps}$ on mesangial C3 secretion. C3 secretion was studied at the protein level but not at the mRNA level.

Discussion

IgAN is a chronic progressive form of glomerulonephritis characterized by mesangial deposits of galactose-deficient IgA_1 and C3 (1, 5). In a previous study we demonstrated that mesangial IgA deposits co-localized with streptococcal IgA-binding regions from M proteins and suggested that these may contribute to the pathogenesis of disease (14). Here we show that the IgA binding M protein from GAS serotype 4, one of the most common serotypes among clinical GAS isolates (43), bound preferentially to galactose-deficient polymeric IgA_1 and that co-stimulation of human mesangial cells with galactose-deficient polymeric IgA_1 and M4 induced excessive IL-6 secretion from the cells. Furthermore, we show that galactose-deficient polymeric IgA_1 induced C3 secretion from mesangial cells although the combined effect with M4 protein was less pronounced. The secretion of IL-6 and C3 from mesangial cells could contribute to the inflammatory process occurring in the renal cortex during IgAN.

During upper respiratory tract infection caused by GAS, the surface-localized M proteins will encounter IgA in the saliva and along the mucous membrane. Dimeric and polymeric IgA_1 are the most prevalent forms of antibodies in saliva (44) whereas the predominant form of IgA in the circulation is monomeric IgA_1 (2). Binding to IgA will probably protect the bacteria from phagocytosis and enable survival (45). However, M proteins, or parts thereof, may also detach from the bacterial cell wall due to the effect of streptococcal or neutrophil granulocyte-derived proteases (46, 47), a process that may occur during the course of infection. These detached M proteins (or M protein fragments) presumably gain access to the circulation and reach the kidney (14). The results presented here indicate that M4 binds with higher affinity to polymeric than to monomeric IgA_1 and preferentially binds to galactose-deficient polymeric IgA_1 ($IgA_{1\text{-Ale}}$ and $IgA_{1\text{-IgAN}}$). The galactose-deficient polymeric IgA_1 will presumably

circulate bound to M4 or its IgA-binding region and thus reach the renal circulation as an M4-IgA_1 complex. Galactose-deficient IgA_1 has indeed been detected in the circulation and kidneys of patients with IgAN and shown to bind preferentially to human mesangial cells (3, 5, 6). Thus we speculate that the M protein-IgA_1 complex will reach the mesangium and exert an inflammatory response.

The increased binding of M4 to galactose-deficient compared to galactosylated polymeric IgA_1 may have several explanations related to alterations in the tertiary structure of IgA, when the hinge region is galactose-deficient, or in charge, due to the expression of sialic acid residues. Current models of IgA_1 show the immunoglobulin in a T-like structure with a 90^o angle between the hinge region and $C\alpha_2$ (2). This would situate the hinge region in proximity of the Fc-binding streptococcal M proteins, which bind at the $C\alpha_2$-$C\alpha_3$ interface (48). A reduction in glycosylation in the hinge region as well as polymerization of IgA_1 could influence the tertiary structure of the IgA molecule and thus the binding affinity of streptococcal IgA-binding M proteins possibly due to reduced steric hindrance. As the increased binding was only observed for galactose-deficient polymeric IgA_1 but not the galactose-deficient monomeric form of IgA_1 it seems that the critical change within IgA_1 is caused by polymerization and that this effect may be enhanced by galactose-deficient *O*-glycans of hinge residues.

IL-6 has been implicated in the pathogenesis of IgAN, as up-regulation of IL-6 mediates mesangial cell proliferation, matrix expansion and contributes to inflammatory cell infiltration in the kidney (23-26). In the current study we show that the streptococcal IgA-binding M4 protein binds to human mesangial cells and enhances their IL-6 synthesis and secretion. Induction of IL-6 secretion was also induced by the non-IgA binding mutant variant M4Δ451

suggesting that this effect was not dependent on IgA binding to M4. In addition, we found a synergistic effect on IL-6 secretion from mesangial cells, when these cells were co-stimulated with a combination of M4 and galactose-deficient polymeric IgA_1. A similar synergistic effect was detected when cells were co-stimulated with the non-IgA binding mutant variant M4Δ451 and galactose-deficient polymeric IgA, which suggests that IgA binding to M4 is not directly involved in the stimulatory effect on IL-6 secretion of human mesangial cells. We envisage that IgA circulates in vivo bound to M proteins, or their IgA-binding domain, and, upon reaching the mesangial cells each agonist exerts a separate stimulatory effect regardless if they are in complex or not. These findings suggest that streptococcal M proteins deposited in the mesangial region contribute to the inflammatory response and could therefore be of importance in the pathogenesis of IgAN.

To mimic the conditions within the glomerulus we used maintenance medium to minimize cell proliferation. During the stimulation period unstimulated mesangial cells synthesized and secreted a minimal amount of IL-6, which could reflect starvation due to extended exposure to maintenance medium. Nevertheless, the IL-6 synthesis and secretion of cells stimulated with streptococcal M4 protein or galactose-deficient polymeric IgA_1 exceeded that of unstimulated cells significantly. As expected, the induction of IL-6 mRNA synthesis preceded that of protein secretion from mesangial cells and the pattern of IL-6 upregulation reflected that of the measured IL-6 amounts in the corresponding supernatants for each stimulant. There was a significantly higher rate of IL-6 mRNA synthesis after 4 h stimulation than after 24 h stimulation with all stimulants with the exception of M4 for which the same degree of IL-6 synthesis persisted. The reduced rates of synthesis after 24 h stimulation may, however, be sufficient to maintain elevated levels of IL-6 in the cell culture supernatants.

Mesangial deposits of C3 are commonly detected in kidney samples from patients with IgAN (1). C3 may originate from the circulation (hepatic and extra-hepatic synthesis) or mesangial cell secretion (31). The finding that galactose-deficient polymeric IgA induces C3 secretion by mesangial cells is novel and may explain why C3 is deposited in the mesangium during IgA. Mesangial stimulation with M4 did not trigger C3 secretion. Co-stimulation with M4 and galactose-deficient polymeric IgA_1, however, resulted in an enhanced C3 secretion compared to galactose-deficient polymeric IgA alone. The difference in response of mesangial cells to M proteins and galactose-deficient polymeric IgA_1 with regard to IL-6 and C3 secretion suggest that these agonists may utilize different signal pathways to exert their effect on mesangial cells.

The manner by which IgA binds to mesangial cells is still a matter of debate. Two IgA-receptors have been described on human mesangial cells, the transferrin receptor CD71 and the $Fc_{\alpha/\mu}$ receptor. The latter receptor is probably not involved in the pathogenesis of IgAN as mesangial immune deposits usually do not contain significant amounts of IgM (49). However, CD71 on mesangial cells has been shown to have high affinity for galactose-deficient polymeric IgA and its binding to CD71 induces an inflammatory response (50, 51). In the current study we found that the M4 protein bound to mesangial cells and that binding to mesangial cells induced an increased synthesis and secretion of IL-6. The putative receptor for M proteins on human mesangial cells remains to be elucidated and will be the subject of future studies, which may shed further light on the pathogenetic mechanisms of IgAN and have therapeutic potential if the M protein binding receptor on mesangial cells is identified.

We have previously shown that patients with IgAN were more likely than age-matched controls to have been infected with GAS serotypes expressing IgA-binding M proteins. We

suggest, based upon results of our previous (14, 22) and current studies, that during GAS infection M proteins, or parts thereof, will bind preferentially to galactose-deficient polymeric IgA_1 prevalent in the serum of patients with IgAN and thus circulate as an IgA_1-M protein complex. IgA-binding M proteins in complex with IgA will thus deposit in the mesangial area (14). Streptococcal IgA-binding M proteins may thus partake in the initiation and propagation of an inflammatory response in the kidney during IgAN.

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Figure 1: Binding of IgA to M4 detected by surface plasmon resonance

IgA_1 in five different forms; A: galactose-deficient polymeric IgA_1 ($IgA_{1\text{-}Ale}$), B: galactose-deficient polymeric IgA_1 ($IgA_{1\text{-}IgAN}$), C: galactosylated polymeric IgA_1, D: galactosylated monomeric IgA_1, E: galactose-deficient monomeric IgA_1 were injected at five different concentrations (31 – 500 nM) onto M4 immobilized on CM5 sensor chip chambers. Panel F shows the comparative effect of the highest concentration (500 nM) of each IgA_1 form on association and dissociation. Arrow indicates time point of injection of IgA. Due to high response buffer bulk refraction spikes, resonance data in Panel B were cut for a short time-frame at the injection start and stop, respectively. G: the binding affinities (in nM) of the different IgA1 fractions for M4. $gdpIgA_1$: galactose-deficient polymeric IgA_1; $gpIgA_1$: galactosylated polymeric IgA_1; $gmIgA_1$: galactosylated monomeric IgA_1; $gdmIgA_1$: galactose-deficient monomeric IgA_1.

Figure 2: Binding of IgA to M4 measured by ELISA

M4 immobilized in ELISA wells was incubated with either of four different IgA_1 preparations: $gdpIgA_1$: galactose-deficient polymeric IgA_1 ($IgA_{1\text{-}Ale}$), $gpIgA_1$: galactosylated polymeric IgA_1, $gdmIgA_1$: galactose-deficient monomeric IgA_1, $gmIgA_1$: galactosylated monomeric IgA_1. Results represent absorption measured by spectrophotometry from three independent experiments with duplicate wells (box-plots representing medians, interquartal ranges and ranges). * $p < 0.05$, ** $p < 0.01$; ns: not significant. Galactose-deficient polymeric $IgA_{1\text{-}Ale}$ exhibited better binding to M4 than normoglycosylated polymeric IgA_1. The difference was not significant after 2 min incubation ($p=0.055$).

Figure 3: M4 protein binding to human mesangial cells

M4 binding to human mesangial cells detected by flow cytometry. M4 was detected on 32 % of the human mesangial cell population after subtraction of values obtained with the control antibody.

Figure 4: IL-6 synthesis in human mesangial cells stimulated with the streptococcal M4 protein and galactose-deficient **polymeric IgA_1**

IL-6 mRNA in human mesangial cell lysates from three independent experiments with duplicate wells per stimulant is displayed as the ratio of IL-6/GAPDH. Box-plots represent medians, interquartal ranges and ranges. * $p < 0.05$, ** $p < 0.01$, ns: not significant. $gdpIgA_1$: galactose-deficient polymeric $IgA_{1\text{-}ps}$.

Figure 5: IL-6 secretion from human mesangial cells stimulated with M4 and galactose-deficient **polymeric IgA_1**

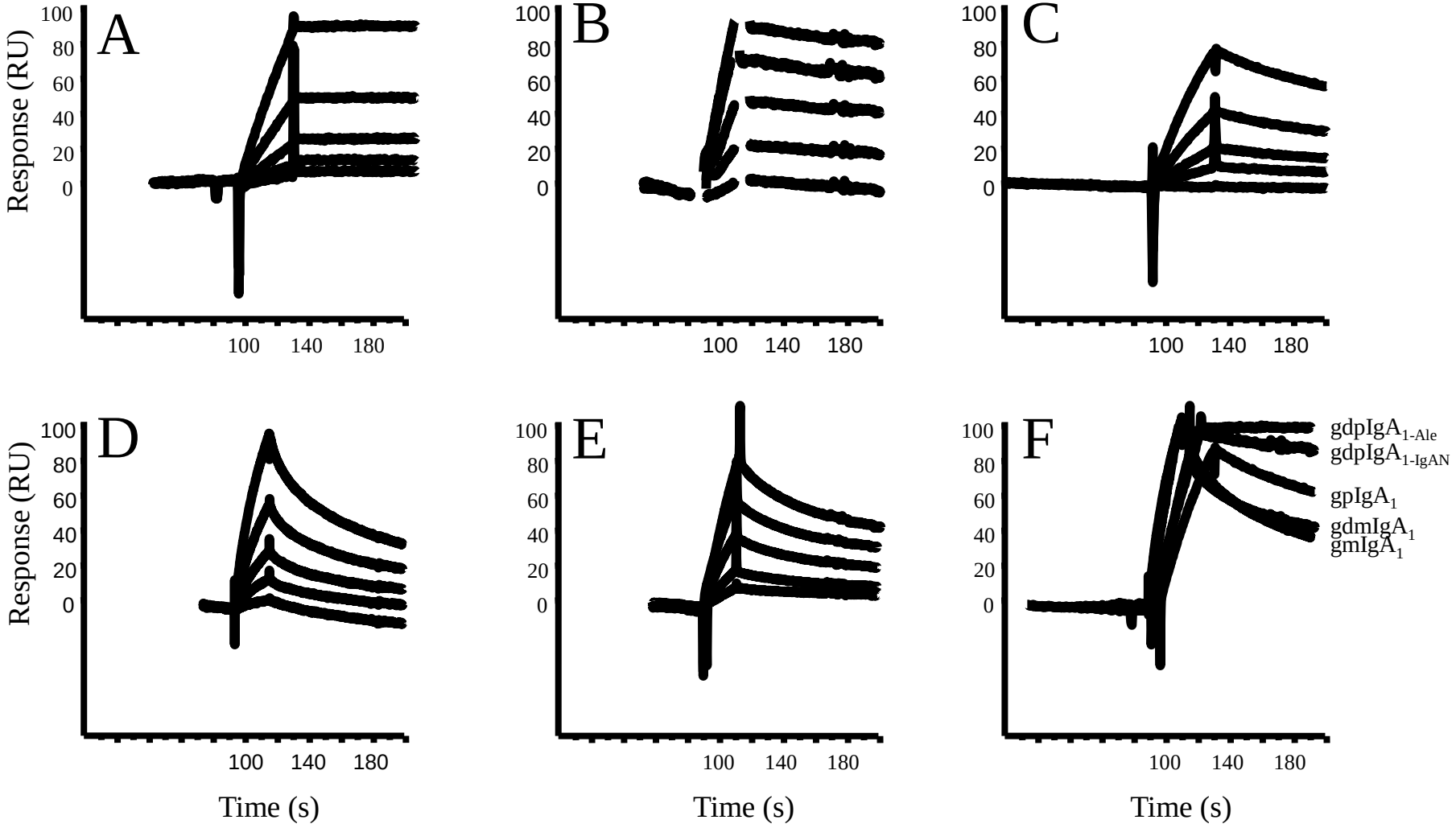
IL-6 in human mesangial cell culture supernatants from three independent experiments with duplicate wells per stimulant are shown. Box plots represent medians, interquartal ranges and ranges. * $p < 0.05$, ** $p < 0.01$, ns: not significant. $gdpIgA_1$: galactose-deficient polymeric $IgA_{1\text{-}ps}$. The Y-axis is displayed in logarithmic scale. Outliers have been removed.

Figure 6: C3 secretion from human mesangial cells stimulated with M4 and galactose-deficient **polymeric IgA_1**

C3 levels in human mesangial cell culture supernatants from three independent experiments with duplicate wells per stimulant are shown. Box plots represent medians, interquartal ranges and ranges. * $p < 0.05$, ** $p < 0.01$, ns: not significant. $gdpIgA_1$: galactose-deficient polymeric $IgA_{1\text{-}ps}$. Outliers have been removed.

Table 1: IgA_1 proteins used in this study

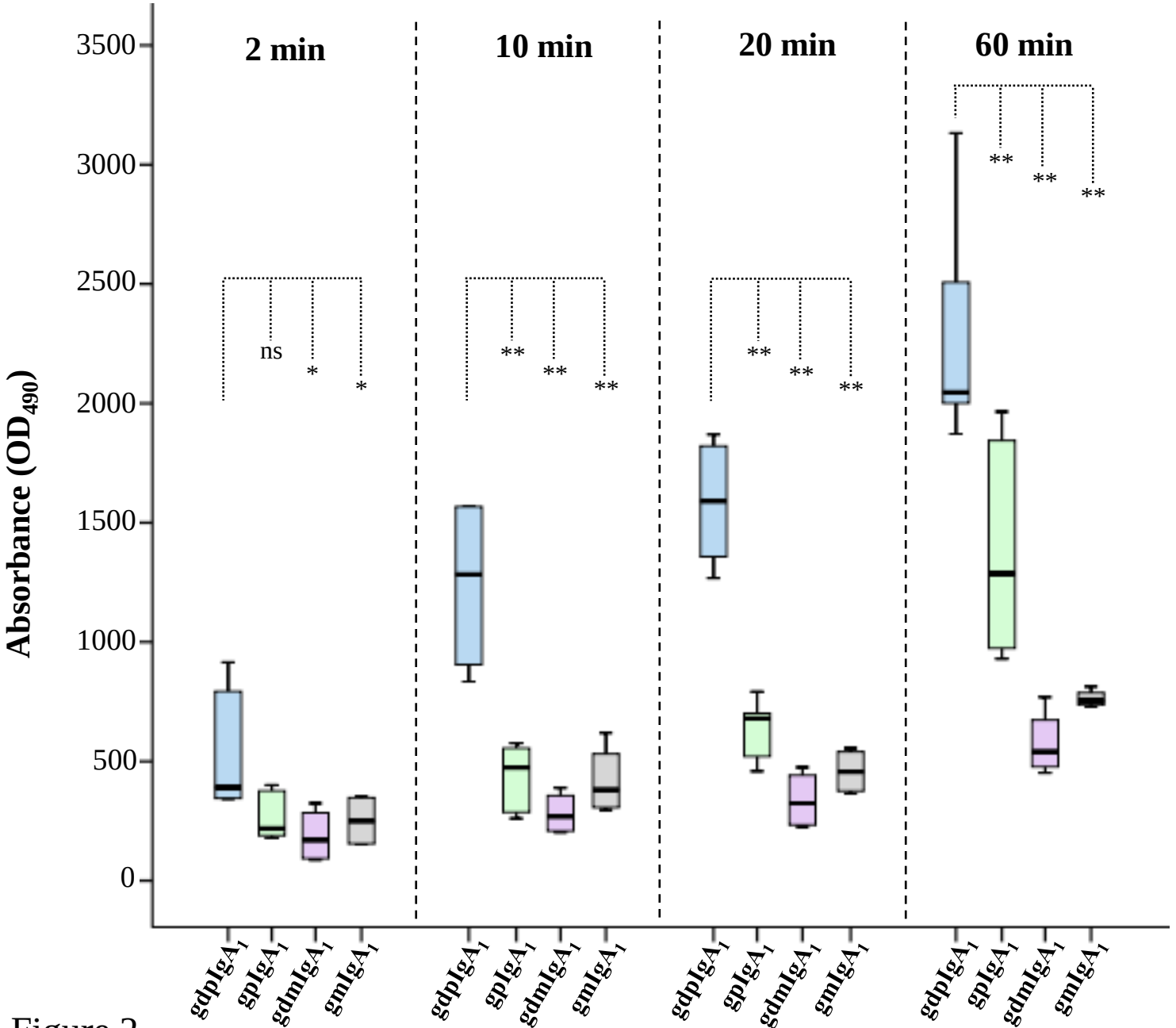
IgA	Source	Reference
Galactose-deficient polymeric $IgA_{1\text{-ps}}$	Purified from pooled normal sera	This study
Galactose-deficient polymeric $IgA_{1\text{-IgAN}}$	Purified from a patient with IgAN	This study
Galactose-deficient polymeric $IgA_{1\text{-Ale}}$	Polymeric IgA_1 myeloma protein (Ale)	(38)
Galactosylated polymeric IgA1	Purified from pooled normal sera	This study
Galactose-deficient monomeric IgA_1	Purified from pooled normal sera	This study
Galactosylated monomeric IgA_1	Purified from pooled normal sera	This study



G

Panel	A	B	C	D	E
Type of IgA_1	$gdpIgA_{1\text{-}Ale}$	$gdpIgA_{1}$-IgAN	$gpIgA_1$	$gmIgA_1$	$gdmIgA_1$
KD nM	0.54	0.89	6.9	32	50

Figure 1


2 min
10 min
20 min
60 min
Absorbance (OD490)
3500
3000
2500
2000
1500
1000
500
0
ns
*
*
**
**
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**
**
**
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gdpIgA1
gpIgA1
gdmIgA1
gmIgA1

Figure 2

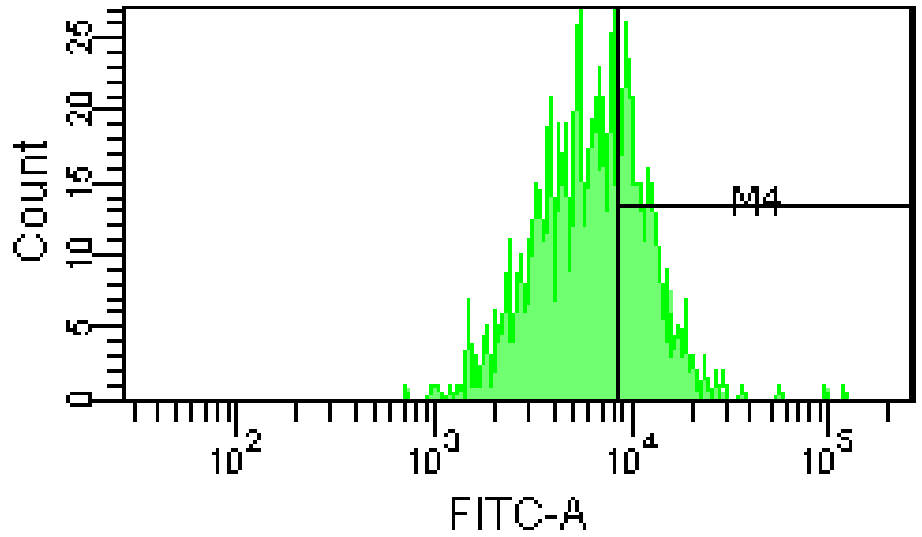


Figure 3

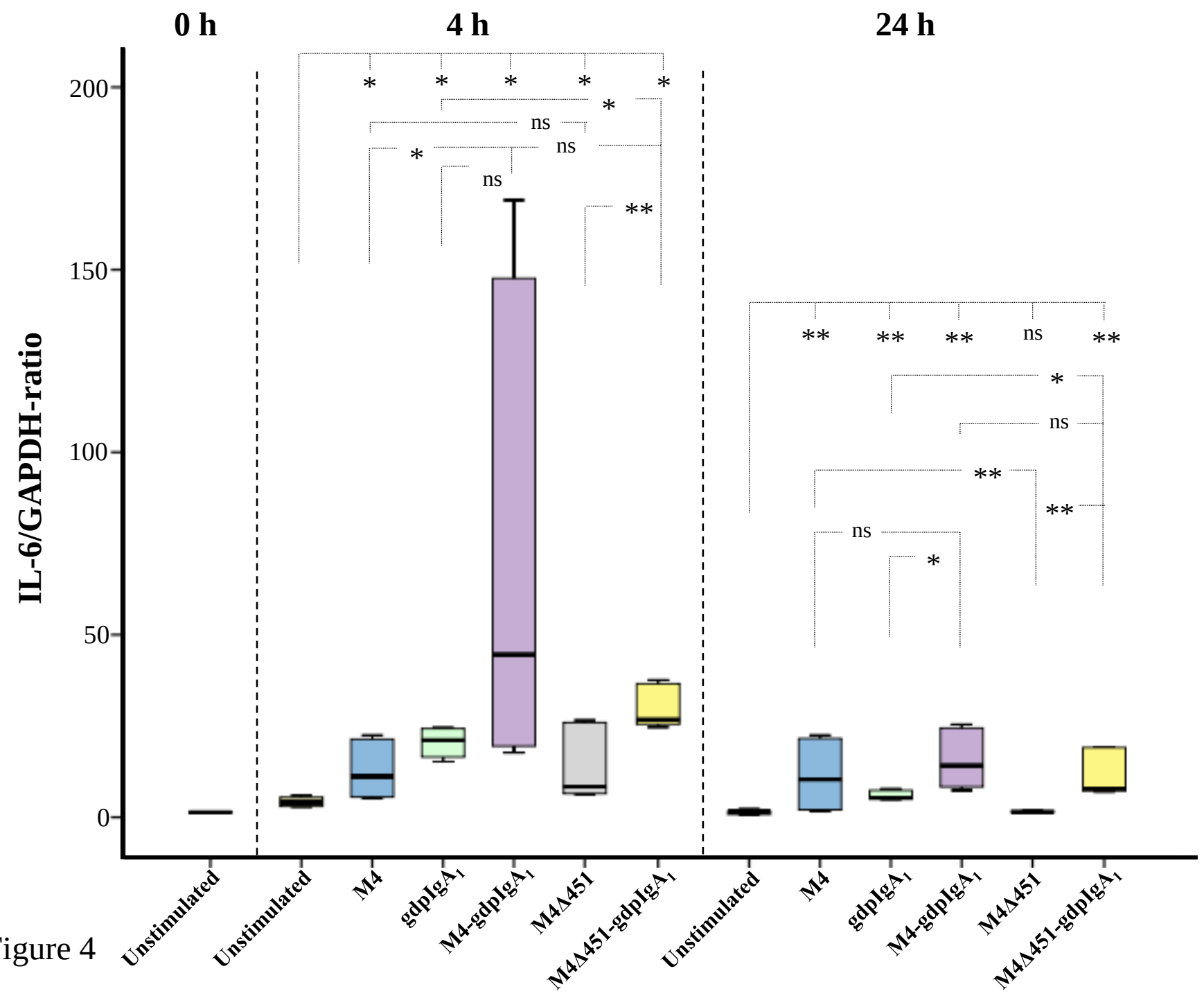

0 h
4 h
24 h
IL-6/GAPDH-ratio
200
150
100
50
0
Unstimulated
Unstimulated
M4
gdpIgA1
M4-gdpIgA1
M4Δ451
M4Δ451-gdpIgA1
Unstimulated
M4
gdpIgA1
M4-gdpIgA1
M4Δ451
M4Δ451-gdpIgA1

Figure 4

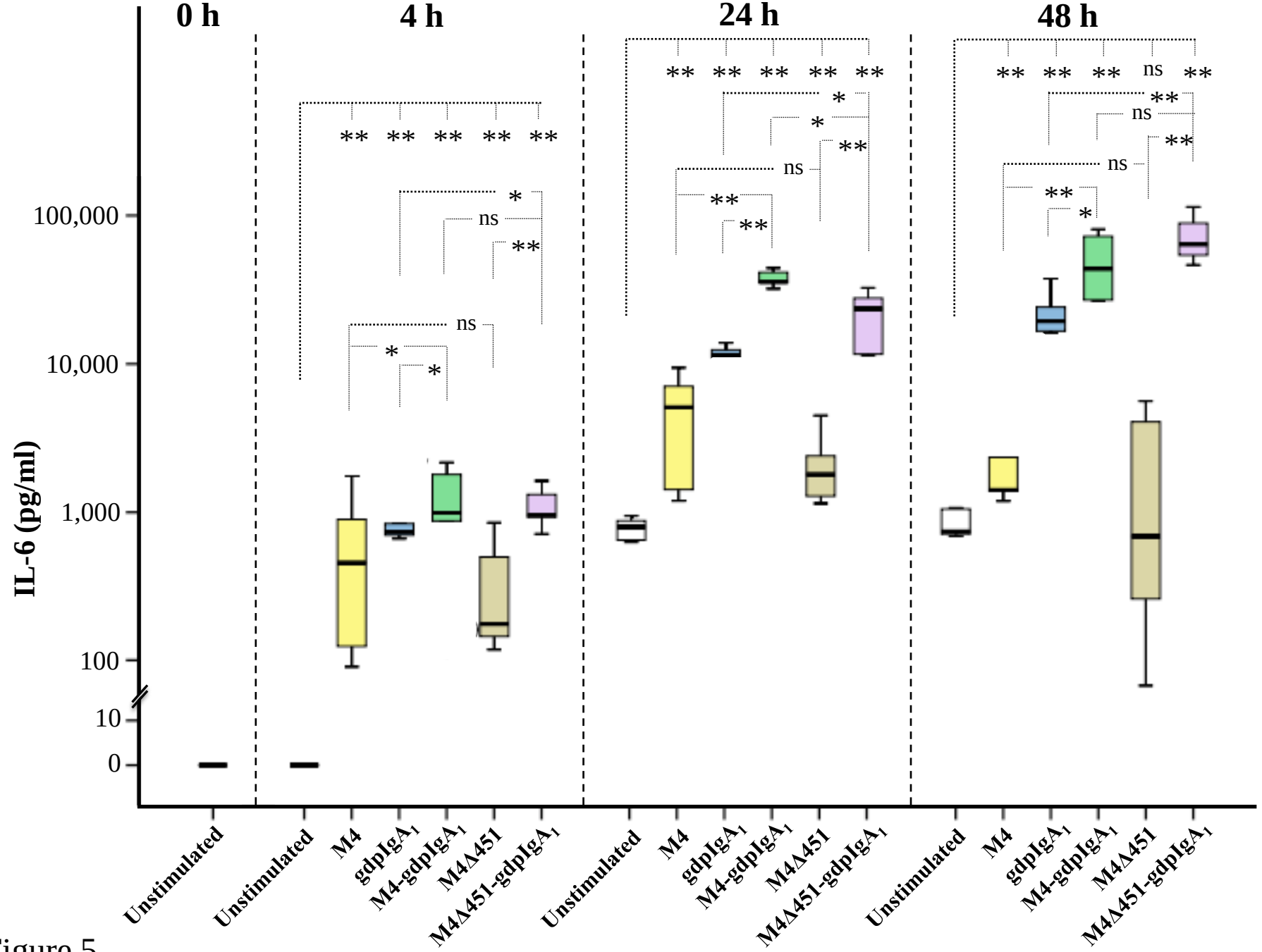
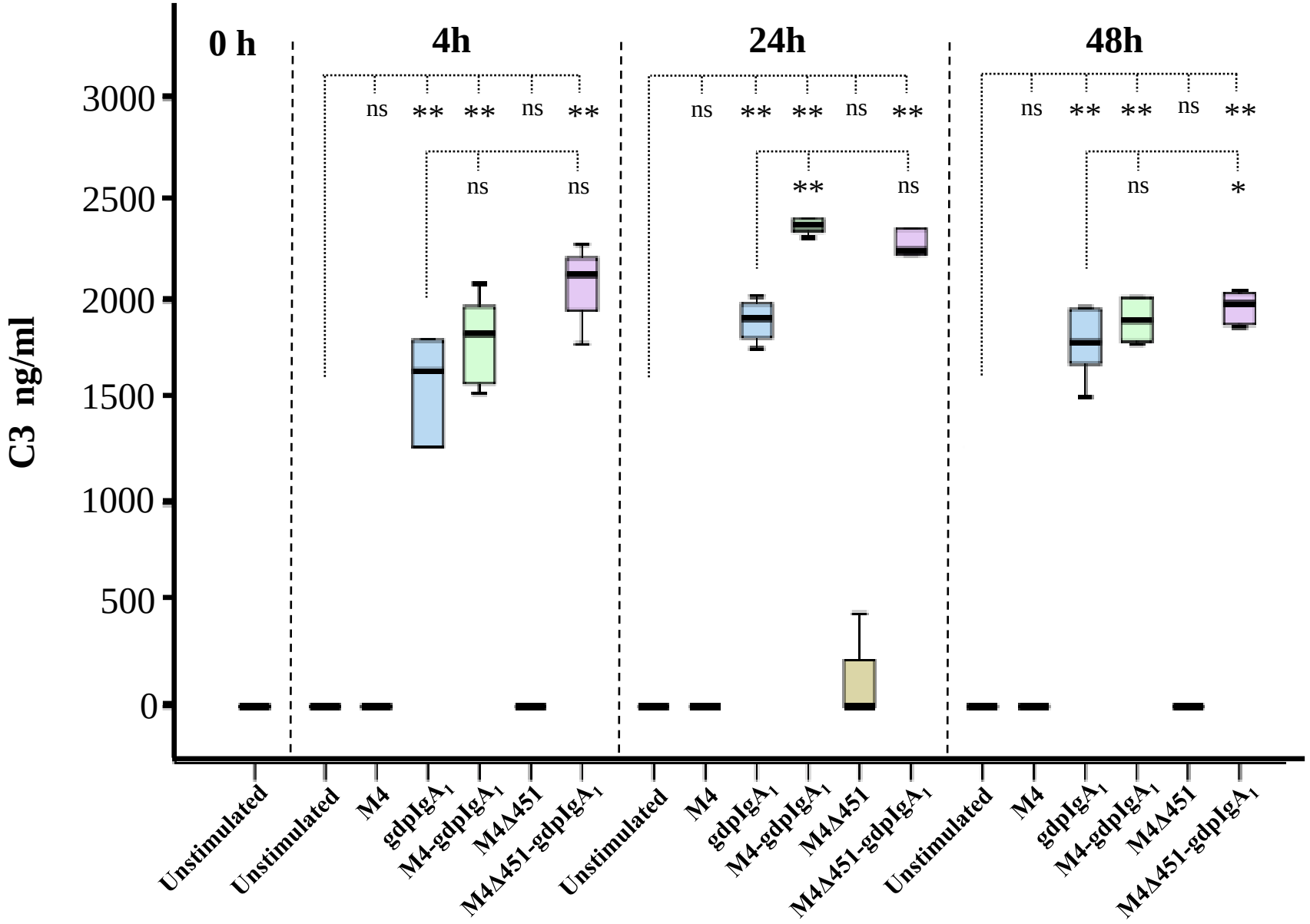

0 h
4 h
24 h
48 h
IL-6 (pg/ml)
100,000
10,000
1,000
100
10
0
Unstimulated
M4
gdpIgA1
M4-gdpIgA1
M4Δ451
M4Δ451-gdpIgA1
**
*
ns

Figure 5


0 h
4h
24h
48h
C3 ng/ml
3000
2500
2000
1500
1000
500
0
ns
**
**
ns
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ns
ns
ns
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Unstimulated
Unstimulated
M4
gdpIgA1
M4-gdpIgA1
M4Δ451
M4Δ451-gdpIgA1
Unstimulated
M4
gdpIgA1
M4-gdpIgA1
M4Δ451
M4Δ451-gdpIgA1
Unstimulated
M4
gdpIgA1
M4-gdpIgA1
M4Δ451
M4Δ451-gdpIgA1

Figure 6

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SHORT REPORT

IgA nephropathy associated with a novel N-terminal mutation in factor H

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Abstract Most patients with IgA nephropathy exhibit complement deposition in the glomerular mesangium. Certain cases of IgA nephropathy have been associated with reduced levels of complement factor H. A recent study could not demonstrate mutations at the C-terminal of factor H. We describe a novel heterozygous mutation in factor H, position A48S (nucleotide position 142 G>T, alanine> serine), detected in exon 2 of a 14-year-old girl with IgA nephropathy. The patient exhibited reduced levels of C3 and factor H, the latter suggesting that the mutation affected factor H secretion. The patient developed initial signs and symptoms of glomerulonephritis at the age of 9 years but presented again at the age of 14 years with weight gain, renal failure, nephrotic-range proteinuria and malignant hypertension. Blood tests suggested the development of microangiopathic hemolytic anemia (MAHA) but the renal biopsy was mostly indicative of chronic changes associated with IgA nephropathy as well as vascular changes associated with malignant hypertension. Immunofluorescence exhibited deposits of IgA, C3, and IgM. Screening of the factor H gene revealed, in addition to the mutation, three heterozygous hemolytic uremic syndrome -associated risk polymorphisms (−257 c/t, 2089 a/g, and 2881 g/t) which may have increased the patient's susceptibility to the occurrence of MAHA triggered by malignant hypertension. The combined clinical picture of IgA nephropathy and MAHA may have been partly related to the alterations in factor H.

Keywords IgA nephropathy · Factor H · Complement · Child

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Introduction

IgA nephropathy (IgAN) is characterized by glomerular deposits of aberrantly glycosylated IgA1 and complement proteins [10, 17]. Complement components deposit mainly in the mesangium and include C3, C4d, C4-binding protein, factor H, mannose-binding lectin, C5b-9, and properdin [1, 7, 8, 13, 15, 17, 20, 21]. Polymeric IgA may activate both the alternative and the lectin pathways of complement [11, 16, 17] and studies have suggested that complement activation during IgAN involves the alternative and lectin pathways [17]. Mechanisms by which complement activation occurs during IgAN are not fully understood. It has been suggested that inadequate complement regulation could lead to complement activation in vivo and progressive glomerular disease [7].

Factor H is the main fluid phase regulator of the alternative pathway of complement. Dysfunction of factor H has been associated with certain renal diseases

such as atypical hemolytic uremic syndrome (HUS) and membranoproliferative glomerulonephritis as well as the ophthalmological condition termed age-related macular degeneration (reviewed in [27]). Factor H is deposited in the kidneys during IgAN [1]. Urinary levels of factor H have been found to be increased and related to disease activity [26]. A recent study investigated 46 patients with IgAN and found normal factor H levels and no mutations in the C-terminal of factor H, the region responsible for host cell recognition [6].

In this study we present a girl who primarily developed IgAN followed several years later by malignant hypertension and microangiopathic hemolytic anemia (MAHA). The latter led us to investigate the patient's factor H levels which were found to be low. A novel mutation was detected at the N-terminal of factor H. In addition to the mutation, three polymorphisms, associated with increased risk for HUS, were detected in the factor H gene.

Patient and family member

A currently 18-year-old Caucasian girl was admitted to Karolinska University Hospital at the age of 9 years with macroscopic hematuria and proteinuria after a severe tonsillitis. Her serum creatinine was slightly elevated at 67 μmol/L (normal reference value <60 μmol/L). Within the following 2 months, she recovered with persistent microscopic hematuria but no proteinuria after which she was lost to follow-up. She was readmitted at the age of 14 years with a history of weight gain during a few months as well as progressive fatigue, weakness, headache, and blurred vision during the days before admission. There was no history of diarrhea. Upon admission her blood pressure was 250/150 mmHg, she had altered sensorium and was in respiratory distress. She exhibited severe oliguric renal failure with high levels of creatinine (1360 μmol/L, reference value <90) and BUN. In addition, laboratory values showed low serum albumin (31 g/l; reference value, 40–51 g/L), hemolytic anemia (hemoglobin, 65 g/L (110–160); lactate dehydrogenase, 16.5 μkat/L (<6.2)) and thrombocytopenia (69×10^9/l, 150–400). C3 was low 0.52 g/L (0.67–1.43), C3dg elevated 11.5 (<5 mg/L), and C4 normal. Urinalysis revealed microscopic hematuria and nephrotic-range proteinuria.

Serologic analysis for anti-nuclear antibodies, anti-double stranded antibodies, anti-phospholipid antibodies, anti-neutrophil cytoplasmic antibodies, anti-glomerular basement membrane antibodies, hepatitis B and C, and HIV were all negative. Fundoscopic exam revealed papilledema, exudates and retinal hemorrhages. She was treated with continuous veno-venous hemofiltration, anti-hypertensive medications, and pulses of methyl-prednisolone. Ophthalmologic and cardiovascular involvement as well as hemolytic anemia remitted and blood pressure normalized.

Renal biopsy showed 17 glomeruli, 11 exhibited total sclerosis (Fig. 1a), and two crescents (Fig. 1b). The remaining exhibited mesangial proliferation (matrix and cells). There was no evidence of thickening or double contours of the glomerular basement membrane. Tubules showed marked atrophy with mononuclear infiltrates in the interstitium. There were no visible thrombi in the renal blood vessels, but arterioles displayed myointimal proliferation in a concentric pattern typical for "onion-skin" lesions (Fig. 1c). Immunofluorescence showed intense mesangial deposits of IgA (Fig. 1d) and to a lesser degree IgM and C3 (not shown). The electron microscopy sample did not contain glomeruli. She did not regain renal function and underwent a successful renal transplant donated by her father 16 months later. She has not had a recurrence of IgA nephropathy or MAHA since transplantation in June 2007.

Serum and whole blood in EDTA tubes were obtained from the patient and her father. The project was performed with the informed written consent of the patient and her parents and the approval of the Ethics committee of the Medical Faculty, Lund University.

Materials and methods

Factor H levels and mutation analysis

Factor H levels were measured by rocket immunoelectrophoresis as previously described [22]. Factor H size was detected by immunoblotting [22]. Extraction of genomic DNA and sequencing of the factor H gene were performed as described [22].

ADAMTS13

ADAMTS13 activity in plasma was detected by a modified collagen binding assay as previously described [9].

Results

Factor H levels were repeatedly low at 50% (reference value, 69–154) upon admission at the age of 14 years, and 52% 3 years later, after transplantation. Immunoblotting revealed a weak factor H band at 150 kD (data not shown) indicating normal size.

Genomic DNA from the patient and her father were screened for mutations in the factor H gene. A novel heterozygous mutation in exon 2, corresponding to short consensus repeat (SCR) 1, was found at G142T leading to replacement of alanine by serine: A48S. In addition, three

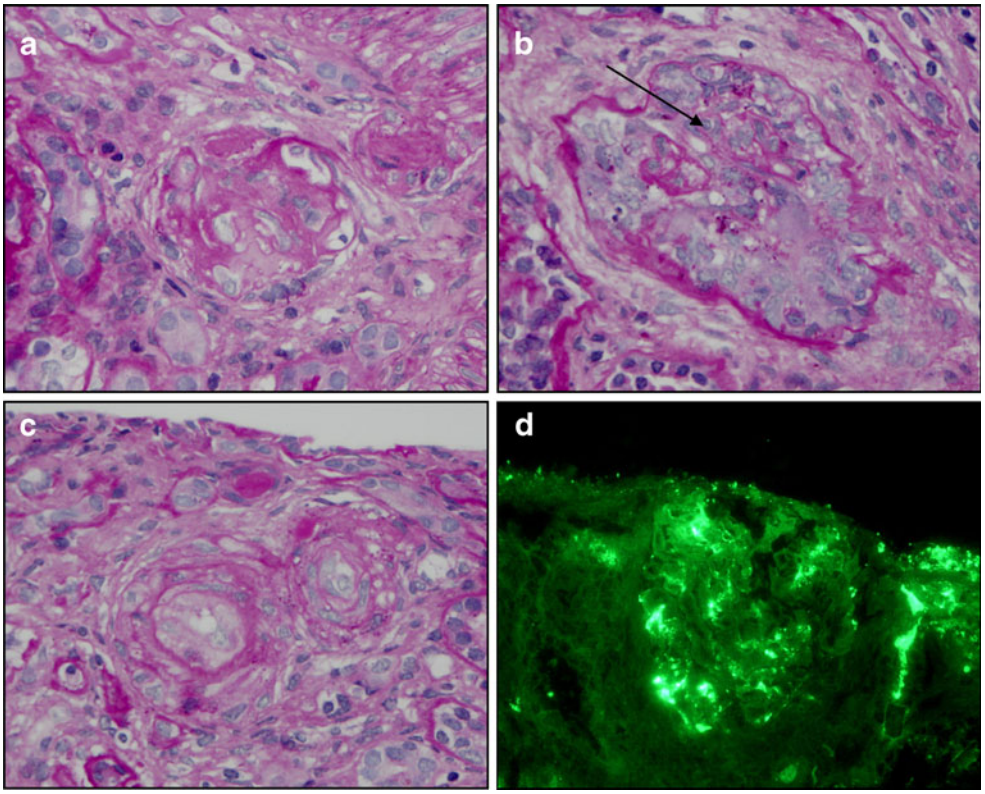

Fig. 1 Histopathologic findings in the patient's renal biopsy. Renal biopsy showed global sclerosis in 11/17 glomeruli (**a**), crescents in two glomeruli (see *arrow* in (**b**) and "onion-skin" lesions in arterioles (**c**). Immunofluorescence showed mesangial deposits of IgA (**d**)

heterozygous polymorphisms were identified in the factor H gene: −257 c/t (promoter region), 672 a/g A2089G in exon 14 (silent), and G2881T: E936D in exon 19. These polymorphisms have been previously described as risk-associated with HUS [3]. The patient's father did not bear the mutation but had all three heterozygous polymorphisms. DNA was not available from the patient's mother. ADAMTS13 function was normal.

Discussion

A novel mutation at the N-terminal of factor H is described in a girl with evidence of IgAN and one episode of MAHA in conjunction with malignant hypertension. The mutation is located in SCR 1 of factor H. Factor H is a co-factor of factor I in cleaving C3b. The co-factor and complement-regulating domain of factor H is ascribed to SCRs 1–4 which bind C3b. This region is active in decay acceleration, displacing factor B from the C3 and C5 convertase [12]. Low levels of factor H have been previously described in certain patients with IgAN [23–25]. The patient exhibited low levels of factor H at separate time points suggesting that the heterozygous mutation interfered with secretion of the product of the mutated allele. The mutation is in proximity of a cysteine residue at codon 52 (http://www.fh-hus.org/) possibly altering a disulphide bridge and/or the stability of SCR1. Thus we suggest that the mutation may affect complement regulatory functions and may partially block secretion of factor H from cells as has been demonstrated for other N-terminal mutations in factor H, mostly associated with membranoproliferative glomerulonephritis [5, 22].

The pathological findings were indicative of IgAN due to intense mesangial deposition of IgA. Membranoproliferative glomerulonephritis was ruled out due to lack of typical changes such as glomerular basement membrane thickening with double contours. The clinical history, with a glomerulonephritis in association with pharyngeal infection at the age of 9 years, indicates that the primary lesion was IgAN. The combined clinical picture of IgAN and HUS has been reported [4, 14] in association with chronic advanced IgA nephropathy and malignant hypertension as was evident in our patient. Malignant hypertension in itself has been associated with MAHA (reviewed in [2]). This may be due to endothelial cell injury with narrowed microvasculature and enhanced shear stress [18]. MAHA may have developed in this patient secondary to progressive IgAN and malignant hypertension but the presence of three HUS-associated polymorphisms in factor H [3] could have contributed to this process. Recently patients with IgAN have been investigated regarding allele frequency and these three polymorphisms were not associated with IgAN [6]. However, the presence of these polymorphisms in an IgAN patient with malignant hypertension and vascular damage may be a predisposing factor reducing complement regulation and precipitating MAHA. This raises the ethical issue of if a

patient with a factor H mutation, and three factor H polymorphisms associated with increased risk to develop HUS, should undergo renal transplant. In patients with HUS and factor H mutations the risk of HUS recurrence after renal transplant, leading to graft loss, is high [19]. The primary diagnosis in the patient described herein was IgAN and she developed MAHA as a secondary phenomenon due to malignant hypertension. As N-terminal factor H mutations have not been explored in a larger cohort of IgAN patients it is, as yet, unclear if these genetic alterations can increase the risk of IgAN recurrence after renal transplantation.

There may be several mechanisms for complement activation in IgAN via both the alternative and lectin pathways. Although we describe only one patient with an N-terminal mutation in factor H, we suggest that the mutation and the three polymorphisms in factor H may have contributed to complement dysregulation and C3 deposition in the glomeruli.

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Conflicts of interest The authors declare that they do not have any conflicts of interest and no financial relationships that might have influenced the present work.

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“If you can’t be with the one you love, love the one you’re with!”

Billy Preston (1946-2006)