# Complement activation in renal microangiopathies

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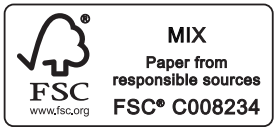

# COMPLEMENT ACTIVATION

# IN RENAL MICROANGIOPATHIES

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“学而不厌,诲人不倦”— 孔子《论语》

“Learning without satiety and teaching others without weariness”

— Confucius (Analects)

Voor mijn ouders, mijn zusjes en mijn geliefde

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# PREFACE

## The human immune system

It is remarkable to be alive and it is astonishing to be healthy. Our environment contains an impressive number of life-threatening viruses, bacteria, fungi, parasites, and other pathogens that may cause disease.[1] In addition, our body hosts a great number of processes that can cause life-threatening diseases, if homeostasis is disturbed.[2-5] Fortunately, throughout evolutionary history, the human body has developed an equally remarkable series of barriers and defenses to protect itself from these perils: the immune system.

## Two immune systems

The immune system is a network of molecules, cells, and tissues that protects the body by detecting pathogens and responding to eliminate them.[6] It is traditionally divided into two distinct, but interconnected systems: the innate immune system and the adaptive immune system.[6]

The innate immune system is a primitive system that is already present at birth – hence the name – in healthy individuals. It has two main goals: to quickly detect the presence of pathogens and to recruit effector mechanisms that eliminate them.[7] When a pathogen succeeds in breaching the anatomic barriers of the body, the innate immune system is the first line of defense. It is activated within seconds of the encounter with a pathogen. Enzymes and peptides break down the pathogen's cell walls and cell membranes, and a cascade of highly orchestrated proteins cooperate swiftly in recruiting effector mechanisms that eliminate the pathogen. Thus, the innate immune system is a non-specific, fast system that is effective against all sorts of pathogens at an early stage.[6]

In contrast, the adaptive immune system is a more specific, but a much slower immune system that is required once the hosts' innate immune system is compromised, evaded, or overpowered.[6, 7] In several days to weeks after the encounter with a pathogen, the adaptive immune system strikes with full force using a tailored immune response to the particular pathogen.[6, 7] The adaptive immune system is organized around two types of cells, T- and B-lymphocytes, that adapt to the threat by proliferating, dividing and differentiating into highly specialized cells, such as cytotoxic T cells and antibody-producing B cells.[6, 8] After the battle is won, some of these cells become long-lived memory cells that

enable the immune system to respond faster and more vigorously whenever a particular pathogen is encountered again.[6]

A powerful effector mechanism of the innate immune system, and the linking pin between the innate and the adaptive immune systems, is the complement system.

# GENERAL INTRODUCTION

## Overview of the complement system

### Complement evolution

The complement system in vertebrates is estimated to be at least 600 million years old,[9] pre-dating the existence of our human predecessors by approximately 597 million years.[10, 11] Although genes encoding complement proteins are not present in all animals,[9] even primitive species such as arthropods, corals, and sea urchins have a functioning complement system. Over millions of years, the complement system has evolved into an efficient and highly versatile system that can respond within seconds according to a hard-wired, 'standard protocol'.[12] This response is possible because of the architectural backbone of the complement system: a proteolytic cascade.[13] In such a cascade, many proteins are zymogens, inactive precursors of enzymes that are activated by proteolytic cleavage. The complement zymogens are widely distributed throughout the body without any adverse effect.[14] When the zymogen is activated locally, it forms an active complement enzyme that cleaves its substrate – another complement zymogen – thereby activating the next zymogen in the cascade, and so on. This enzyme-triggered cascade ensures that activation of a small number of complement proteins at the start of the pathway results in a rapidly amplified complement response.[14]

### Complement discovery

A series of experiments in the late 19$^{th}$ century led to the first recognition of the complement system, as was reviewed previously.[15, 16] In 1874, Traube and Gscheideln showed that microorganisms injected into the circulation were killed quickly and that blood remained sterile.[17] In 1884, Grohmann demonstrated that microorganisms could be killed by cell-free serum *in vitro*, indicating that a component in the serum was bactericidal.[18] In 1888, Nuttall discovered that this bactericidal activity could be destroyed by heating blood serum above 55°C.[19] One year later, Buchner demonstrated that the bactericidal activity in serum was caused by a heat-labile substance in serum which he named '*Alexin*' (derived from the Greek word 'αλεξειν', or '*alexein*', which means to defend).[20] But the discovery of the complement system is generally credited to Bordet

for performing the critical experiments needed to identify the complement system.[21] In 1895, he inactivated the bactericidal activity of serum from an immunized animal by heating the serum. Next, he added fresh serum from a non-immunized healthy control to this inactivated serum, which restored the bactericidal activity in the serum. Thus, he concluded that bactericidal activity in serum was caused by two different factors. The first was a heat-stable factor that was only present in immunized serum and which he termed '*sensitizer*' (now known as antibodies). The second was a heat-labile, lytic factor, that was present in normal serum and which he thought to be Alexin. In 1899, Ehrlich was responsible for replacing the term 'Alexin' with 'complement'.[22] He hypothesized that immune cells had receptors on their surface that could recognize antigens and that after immunization with the antigen, these receptors were shed from the cells as '*amboceptors*' (now known as antibodies). He introduced the term '*das Komplement*' to emphasize that antibodies have a site for recognizing the antigen and a different site that attaches to the heat-labile serum factor which 'complemented' or aided the bactericidal effect of the antibodies. Today, the term '*complement*' is widely accepted even though it is now known that the functions of complement proteins exceed merely 'complementing' the antibody-mediated response.

**Complement system: main functions and proteins**

More than a century after its discovery, a wide variety of functions have been attributed to the complement system, which by far exceed the effector arm of the innate immune system. As was reviewed previously,[13, 23-27] these functions include: recognizing and clearing foreign pathogens and antigens, stimulating phagocytosis of opsonized targets, promoting humoral immune responses, modulating cellular immune responses, clearing self-antigens derived from apoptotic processes, facilitating immune complex transport, promoting the auto-inflammatory response to injured self-tissue following recognition of neo-epitopes by natural antibodies, shaping the composition of the natural antibody repertoire, regulating the growth of inflammatory tumors, and enhancing tissue regeneration. Furthermore, several complement proteins are able to elicit responses from different cell types and different tissues that are not directly related to host-defense but rather bridge immunity and developmental biology.[13, 24, 27]

The complement system has a large number of soluble and membrane-bound proteins that are found in the circulation and in tissues.[13] In general, the complement proteins have one of three functions in the complement system.[27] The first function is activating the complement cascade. Proteins with this function are often present as zymogens and serve as proteolytic enzymes, enzyme cofactors, or enzyme substrates. The second function is regulating the complement cascade. Complement regulatory proteins typically inhibit enzymes and inactivate peptides, to ensure that the extent of complement activation is proportional to the required amount and duration. The third function is serving as a cellular receptor for complement proteins and their fragments. Some proteins overlap these functional categories and several proteins have additional functions in other physiological systems.[24]

**Complement nomenclature**

The nomenclature of the complement proteins is not the most amiable aspect of the complement system. It follows the chronological order of discovery, which has led to inconsistencies that may form an important stumbling block in the understanding of the complement system. Thus, before discussing the complement cascade in more detail, some comments are given on the nomenclature of the complement system. These comments are based on the recent recommendations made by the International Complement Society and the European Complement Network to harmonize the complement nomenclature while making minimal changes to long-established conventions.[28] Nevertheless, for several proteins consensus has not been reached and some inconsistencies remain.

The processes that initiate complement activation are traditionally divided into three pathways: the classical pathway, the lectin pathway and the alternative pathway (Figure 1). These three pathways converge at a final common pathway: the terminal complement pathway. The classical complement pathway was the first to be discovered, and the first eleven proteins that constitute this pathway are designated by the capital letter C, followed by a number. Unfortunately, these proteins were numbered in the order of discovery instead of the sequence of reactions which is: C1, C4, C2, C3, C5, C6, C7, C8, and C9.[14] Furthermore, C1 is a complex of three distinct proteins, termed C1q, C1r, and C1s; the letters q, r, and s designate their elution order on ion exchange

chromatography.[28] Proteolytic cleavage fragments of the native complement protein are designated by adding a lower-case letter, in which the smaller fragment is designated a, and the larger fragment is designated b (e.g. C4a and C4b). C2a is the exception to this rule; C2a originally indicated the activated C2 fragment in the C3 and C5 convertases of the classical pathway, but was found to be larger than C2b.[28] Consensus has not yet been reached, thus in some literature C2b is used to describe the smaller, inactive C2 fragment, whereas C2b is used in other literature, including this thesis, to describe the larger active C2 fragment. Inactive proteins or inactive protein fragments are designated by the lowercase letter i (e.g. iC3b).

The alternative pathway was the second pathway to be discovered and was named 'alternative' for being an alternative to the already established 'classical pathway'. Newly discovered proteins in the alternative pathway were first designated by a 'factor' and a capital letter (e.g. factor H), and are now also designated by their abbreviation (e.g. FH is factor H). Properdin is the exception to this rule, and debate exists on whether to rename this protein to factor P or FP.[28] Just as in the classical pathway, the cleavage products of the proteins in the alternative pathway are designated, by adding a lower case letter (e.g. Ba and Bb). Spontaneous hydrolysis of C3 is an important part of the alternative pathway, and hydrolyzed C3 is designated by adding $H_2O$ in parenthesis (e.g. $C3(H_2O)$).

The lectin pathway was the most recently discovered pathway and was originally termed so for being organized around the protein mannose-binding lectin. Proteins that were discovered in the lectin pathway are designated by trivial names or their abbreviations (e.g. ficolins; MBL, for mannose-binding lectin; MASP, for MBL-associated serine protease).[27]

The terminal complement pathway has protein complexes consisting of multiple proteins. Each complex is designated by a hyphen between the first and last protein of the complex (e.g. C5b-9 is the membrane attack complex which consists of the proteins C5b, C6, C7, C8, and C9). The soluble variant of C5b-9 is designated by the lowercase letter s (i.e. sC5b-9).

Some final remarks: convertases are designated by the active fragments from which they are composed (e.g. C3bBb is the C3 convertase of the alternative pathway, which consists of the protein fragments C3b and Bb). Receptors are usually designated by the capital letter R; four complement receptors are

designated by CR, followed by the numbers 1-4 (e.g. CR1), the remaining receptors are designated by the protein or protein fragment to which they bind, followed by the capital letter R (e.g. C1qR).[27] Membrane-bound proteins also have been assigned a CD number (e.g. DAF is also known as CD55). Proteins with trivial names may have other trivial names (e.g. Ficolin-1 was formerly known as M-Ficolin).

**Complement activation**

Although the proteins involved in the complement system have many functions and are structurally diverse, in general, complement activation is characterized by striking operational simplicity.[27] Complement activation can be divided into four main phases (Figure 1): 1) initiation of complement activation, 2) C3 convertase activation and amplification, 3) C5 convertase activation and 4) the assembly of the membrane attack complex (C5b-9) in the terminal pathway.[13]

*Phase 1: Initiation of the three main complement pathways*

The processes that initiate complement activation are traditionally divided into three pathways: the classical, the lectin, and the alternative pathway.[13] The classical complement pathway is typically activated by immune complexes, but this pathway can also be activated in an antibody-independent manner, for instance by C reactive protein (CRP), viruses and Gram-negative bacteria.[13, 25] Classical pathway activators are recognized by C1q, a protein with six pattern-recognizing globular heads (Figure 2A). The binding of initiating activators to C1q induces a conformational change in C1q which activates C1r, which in turn activates C1s. Activated C1s then cleaves C4 into C4a and C4b. C4b attaches to the activator surface and binds to C2. C2 is then cleaved by C1s into C2a and C2b. In the presence of magnesium ions, C2a binds to C4b, which was already bound on the activator surface, thereby creating the C4b2a complex that serves as the classical pathway C3 convertase.[27]

The lectin pathway is initiated when e.g. carbohydrates on microbial surfaces are recognized via mannose-binding lectin (MBL), ficolins 1–3, and collectin 11 (CL-11) (Figure 2B).[23] Other processes can also activate the lectin pathway, such as the direct recognition of self-proteins, the binding of MBL to autoantibodies containing agalactosyl (G0) carbohydrates, or the binding of MBL to pathogenic natural IgM antibodies.[23, 29] After recognition, activation

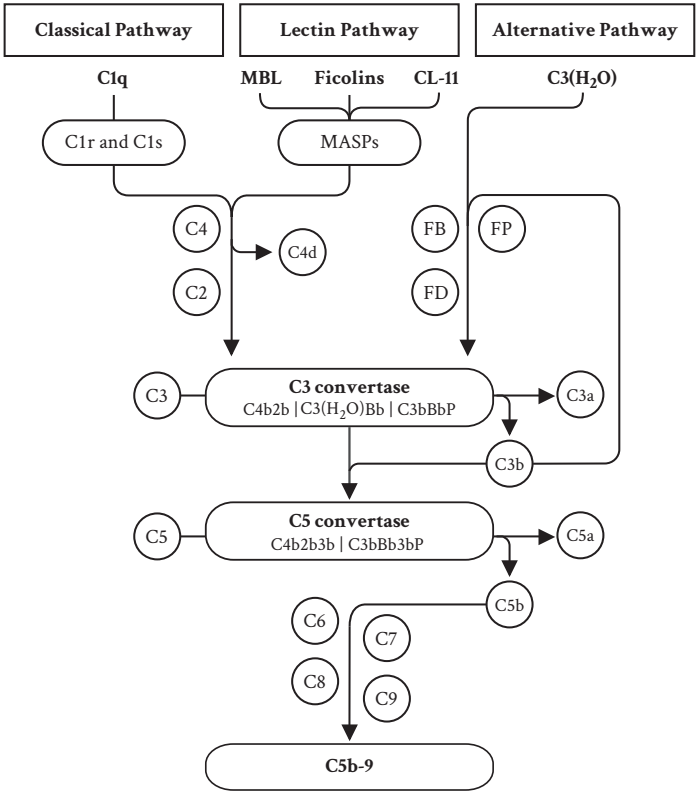


**Figure 1. Simplified flowchart of complement activation**

of the lectin pathway proceeds through the activities of MBL-associated serine proteases (MASP) that cleave and activate C4 and C2, into a C3 convertase, quite like the cleavage of C4 and C2 in the classical pathway.[23]

The alternative pathway does not require specific activation and is spontaneously and constantly activated under physiological circumstances in a process called '*tick over*' (Figure 2C).[30] The alternative pathway starts with the hydrolysis of C3 into C3($H_2O$). C3($H_2O$) can bind to factor B which, in the presence of magnesium ions, can be cleaved by factor D into two fragments: Ba and Bb.[27] The Bb fragment remains bound to C3($H_2O$), thereby forming the initiation C3 convertase, C3($H_2O$)Bb. Under physiological circumstances, this initiation C3 convertase has a short half-life and is continuously inhibited by

regulatory proteins. However, this spontaneous activation is readily amplified if C3b binds to cellular surfaces such as that of virally infected cells, bacteria, parasites, and fungi. Following the binding of properdin to a C3bBb complex, a stabilized C3 convertase C3bBbP is formed and the half-life is extended from 1.5 minutes to approximately 8-15 minutes.[31-33] In addition to activation by spontaneous hydrolysis, the alternative pathway can be activated via repeating polysaccharides, endotoxin, IgA-containing immune complexes, C3 nephritic factor, and immunoglobulin light chains.[23]

In conclusion, there are three main pathways of complement activation with different pattern-recognition proteins that recognize the surface structures of the different activators. Following activation of a pathway, a cascade of highly orchestrated protein-protein interactions and proteolytic cleavages can lead to the generation of a C3 convertase.

*Phase 2: C3 convertase and amplification*

Depending on the nature of the initiating activator, different pathways are activated and different proteins are used to form a C3 convertase. All C3-convertases however, cleave C3 into its two active fragments: C3a and C3b. C3a is an anaphylatoxin that recruits and activates the effector cells of the innate immune system. C3b amplifies the cascade and coats microbial or apoptotic surfaces, thereby opsonizing or "marking" the attached target as distinct molecules for phagocytosis. On the surface membranes of intact self-cells, regulators prevent further complement activation, whereas on the surfaces of microbes or modified self-cells, activation proceeds.[13]

*Phase 3: C5 convertase*

If complement activation proceeds, an additional C3b molecule binds to the C3 convertase, which transforms the protein complex into a C5 convertase (Figure 2D).[13] Thus the C5 convertase for the alternative pathway is C3bBbC3b and the C5 convertase for the classical and lectin pathways is C4bC2bC3b. Complement can also be activated by additional pathways that act independently of C3 and may bypass the C3 convertase, such as thrombin acting on the C5 convertase.[34] The C5 convertases cleave C5 into C5a and C5b. C5a is a powerful anaphylatoxin and chemotactic factor with many biological functions.[35] C5b is necessary for the assembly of the membrane attack complex (C5b-9).[35]

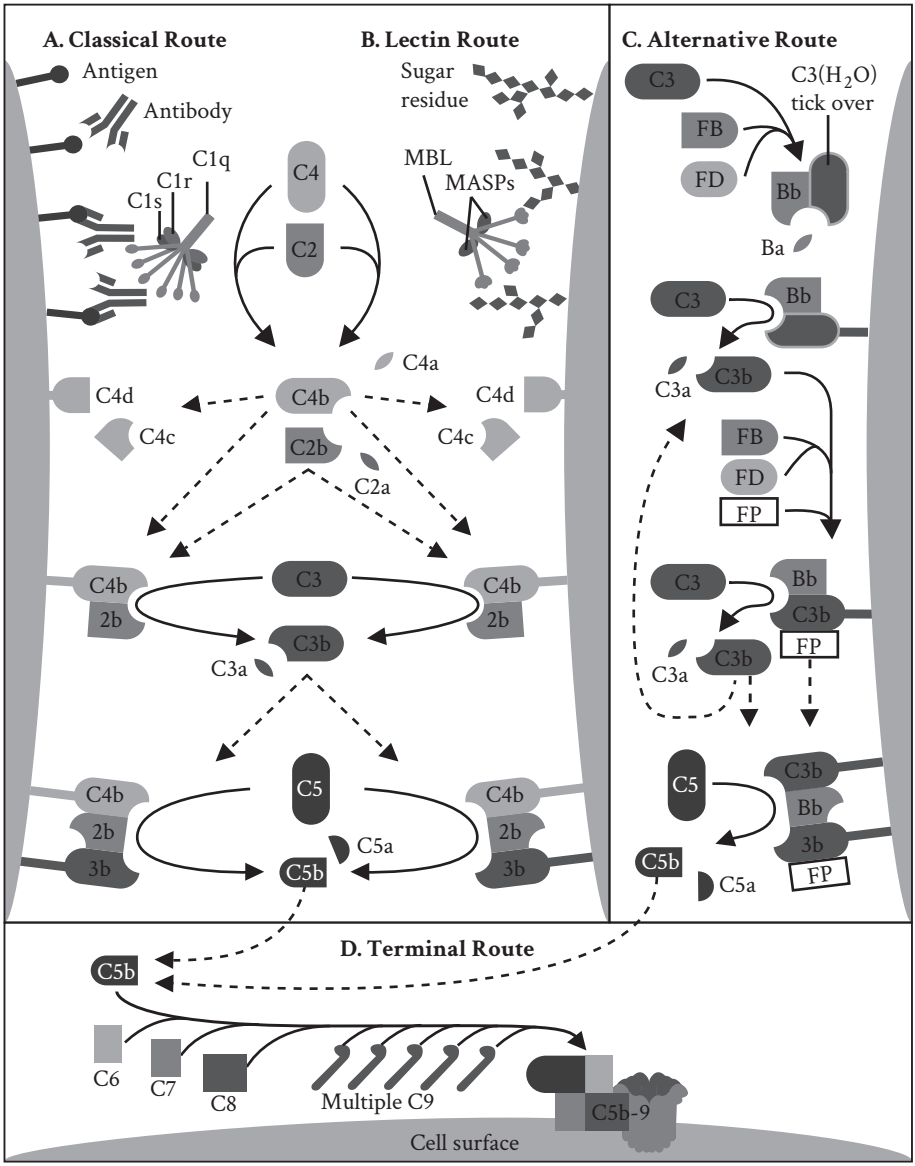


**Figure 2. Schematic view of complement activation**

*Phase 4: Formation of the membrane attack complex*

The formation of the membrane attack complex begins with the binding of C5b to C6, and the sequential binding to C7, C8, and several molecules of C9 (Figure 2D).[27] During these reactions, hydrophobic domains become exposed on the surface.[27] The assembly and conformational changes generate C5b-9, a lipophilic, membrane-inserting, and pore-forming complex, that can lead to cell lysis.[13]

**Complement regulation**

A powerful system needs powerful regulation to protect the hosts' own tissues from harmful effects. Complement regulatory proteins ensure that the extent of complement activation is proportional to the required amount and duration of complement activation.[27] It is therefore not surprising that the number of regulators that inhibit complement activation is far greater than the number of regulators that stimulate complement activation.[23] Complement regulators are categorized into three groups: soluble regulators, membrane-bound regulators that are attached to the surface of host cells, and complement clearance receptors.[13] Currently known regulators of complement activation include factor H, factor I, C1-inhibitor (C1-INH), C4b binding protein (C4BP), Vitronectin, Clusterin, Membrane Cofactor Protein (MCP; CD46), Decay-Accelerating Factor (DAF; CD55), Complement Receptor 1 (CR1), Thrombomodulin, and CD59.[13, 23, 30] Interestingly, several regulators have additional activities beyond complement-mediated host defense, such as mediating cell adhesion, interacting with the extracellular matrix, or linking the complement cascade with other important physiological systems, such as the coagulation cascade.[13, 23, 24]

## Complement activation in disease

The importance of the complement system is best illustrated when it is defective. A clinically relevant concept in the pathogenesis of complement-mediated diseases is a disturbance in the balance between insufficient and excessive complement activation. Insufficient complement activation results in diseases such as severe infections and lupus-like disease.[13, 25, 26, 36] These diseases reflect defective physiologic functions of the complement system, such as protecting the host from infection, clearing immune-complexes, and removing debris. On the other hand, excessive complement activation that is caused by inadequate regulation, excessive stimulation, or both, can result in rare, but life-threatening systemic diseases such as atypical hemolytic uremic syndrome and paroxysmal nocturnal hemoglobinuria.[13, 25, 26] These diseases reflect tissue damage that occurs when the powerful mechanisms that are focused on recognition and clearance of foreign pathogens and antigens, are directed to self-tissues. Thus, while complement activation is essential for protection and repair, it can also cause detrimental inflammation and cell injury. Much like the concept of Yin and Yang, it is the balance of these seemingly opposite forces within the same system that determines health or disease. Although the complement system can affect many organs, the kidney is frequently affected and is, therefore, the main focus of this thesis.

# The kidney

According to the theory of evolution, life began in the seas. The cells of the first primitive organisms had an interior milieu that was similar to the salt water that surrounded them. For the transition to life on land, these primitive organisms required kidneys.[37]

**Gross anatomy**

The kidneys are paired organs that lie in the retroperitoneal space of the abdominal cavity and extend from the twelfth thoracic vertebra to the third lumbar vertebra (Figure 3A).[38] To produce urine, they filter more than 180 L of fluid from the blood plasma, every day. Urine is conducted through the ureters to the urinary bladder and exits the body via the urethra. The shape of the kidney is oval with a convex border and a concave border. This shape is common in nature and is so characteristic that several languages have a single word to describe objects with this shape (e.g. "*reniform*" in English and Dutch, "*reniformia*" in Italian, and "*reniforme*" in French and Spanish), derived from the Latin words for kidneys "*renes*" and shape "*forma*". The size of a normal kidney (approximately 11 x 6 x 3 cm) is comparable to a human fist, and the mass of a normal kidney (approximately 125-170 grams in adult males, and 115-155 grams in adult females) is comparable to a small apple.[39] A fibrous capsule covers the surface of the kidney. The concave medial border of the kidney contains the renal hilum, a vertical fissure that serves as a portal for structures that enter or exit the kidney, such as the renal artery, vein, nerve, and ureter.

Gross examination of a cross-sectioned kidney shows two distinct regions: the cortex and the medulla, collectively termed the renal parenchyma (Figure 3B).[40] The cortex is the brown-reddish outer layer between the medulla and the fibrous cap. It contains most of the initial blood-filtering structures; more than 90% of the blood that passes the kidney flows through the cortex. The medulla is the lighter-colored middle part of the kidney, which only receives approximately 10% of the blood that passes through the kidney. The medulla contains two distinct regions: renal pyramids and renal columns. The renal pyramids resemble inverted pyramids and help transport urine towards the ureters. The apex of the pyramid is called the papilla, and each papilla has perforated openings of the collecting ducts through which urine is excreted.

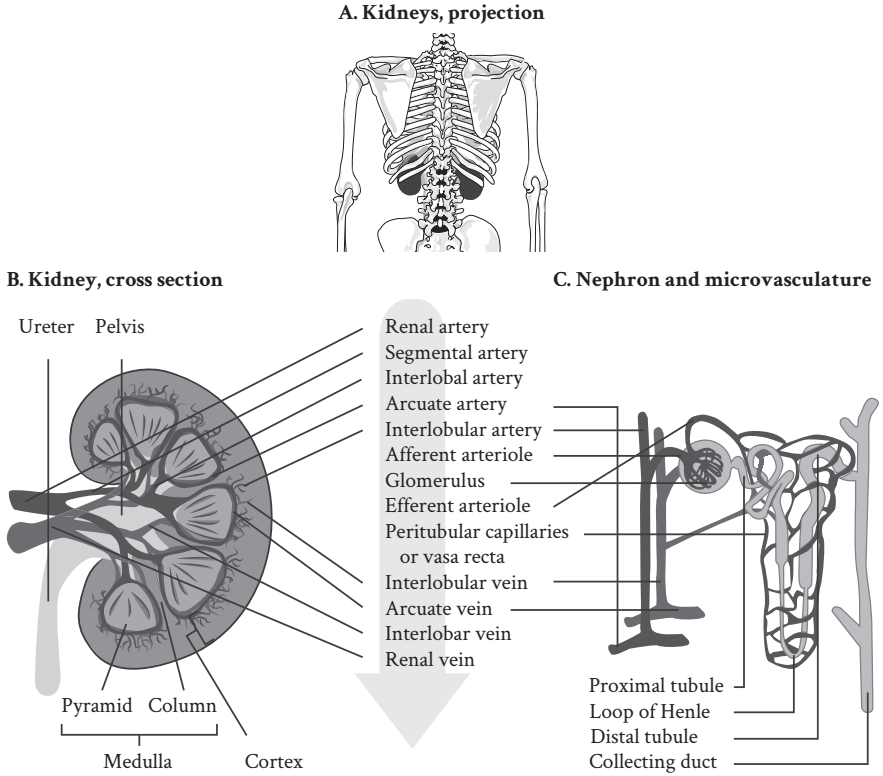


**Figure 3. The kidney, from gross to ultrastructural examination**

The papilla empties urine into a minor calyx; minor calyces empty urine into a major calyx, and major calyces empty urine into the renal pelvis, the funnel-shaped beginning of the ureter. Projections of cortical tissue lie between the renal pyramids in the medulla; these projections are called renal columns.

**The nephron**

Over the course of evolution, the human kidneys developed into a pair of organs that serves crucial functions: filtering toxins and metabolic products from the blood and excreting them through the urine, maintaining homeostasis by regulating the body's extracellular fluid status, electrolyte balance, and acid-base balance, contributing to the metabolism of glucose, and serving as an endocrine organ by producing hormones that are involved in erythropoiesis, calcium metabolism, and the regulation of blood pressure and blood flow.[39, 41] The nephron is the smallest functional unit of the kidney that converts blood to urine (Figure 3C). Each kidney has approximately one million nephrons, which can be classified based on their location into superficial cortical nephrons, mid-cortical nephrons, and juxtamedullary nephrons.[42] The nephron consists of the glomerulus (also known as the renal corpuscle), the tubules, and the collecting ducts.[42]

The glomerulus is the initial blood-filtering component of the nephron and consists of a small ball-shaped cluster of capillaries, known as the glomerular tuft, and a surrounding capsule, known as Bowman's capsule (Figure 3D). Blood that enters the glomerular capillaries is forced to pass the glomerular filtration barrier in a process called ultrafiltration. The determinants of glomerular ultrafiltration are hydraulic and colloid osmotic pressure differences, and the permeability of the glomerular filtration barrier.[43] The glomerular filtration barrier consists of three components that blood must pass (Figure 3E): the fenestrated glomerular endothelium and its glycocalyx, the glomerular basement membrane with three distinct layers (lamina rara interna, lamina densa, and lamina rara externa), and the slit diaphragm created by the visceral epithelial cells (podocytes) and their foot processes.[40, 43] The glomerular filtration barrier can prevent the passage of particles according to their molecular size, electrical charge, and stereotypical configuration: large and negatively charged molecules have more difficulty in passing the glomerular filtration barrier than small particles with an electroneutral or positive charge.[44] As a result, blood cells and

large proteins, such as antibodies and albumin, remain in the blood, whereas small waste products are filtered out of the blood to form the ultrafiltrate. In addition to the endothelial cells and the podocytes, the glomerulus contains two other main resident cell types: parietal epithelial cells and mesangial cells.[40] Parietal epithelial cells are simple squamous cells that form Bowman's capsule. They are continuous with the visceral epithelial cells. Mesangial cells are positioned between the capillary loops and have direct contact with the fenestrated endothelium. This position enables the mesangium to stabilize the glomerular endothelium, alter the intra-glomerular capillary flow and ultrafiltration surface area, and clear filtration residue through phagocytosis by mesangial cells or macrophages located within the mesangium.[45] The space between the parietal and the visceral layers of Bowman's capsule is termed Bowman's space. Bowman's space receives the ultrafiltrate and drains into the renal tubules at the urinary pole of the glomerulus. Tubuloglomerular feedback is regulated through the juxtaglomerular apparatus, which consists of the macula densa, juxtaglomerular cells, and extraglomerular mesangial cells.[40] After ultrafiltration, the remaining blood proceeds through the efferent arteriole, whereas the ultrafiltrate flows from Bowman's space into the tubules.

The tubules modify the glomerular ultrafiltrate and can be subdivided into the proximal tubules, the loop of Henle, and the distal tubules (Figure 3C).[42] The proximal tubule is lined with epithelial cells containing microvilli and mitochondria. In the proximal tubules most of the water, electrolytes, and other nutrients in the ultrafiltrate are reabsorbed from the lumen into the peritubular capillaries.[46] Reabsorption ensures that important substances that pass the glomerular filtration barrier, such as glucose and amino acids, are not lost by urinary excretion. The proximal tubule is also important for active solute secretion and hormone production.[46, 47] Substances that are secreted include hydrogen ions (for pH homeostasis), potassium ions (for salt homeostasis), ethanol, toxins, drugs, and other "foreign" substances. Following the proximal tubules, the ultrafiltrate flows into the loop of Henle, which encompasses the thin descending limb, the thin ascending limb, and the thick ascending limb.[40] The main function of the loop of Henle is to create an osmolality gradient within the renal medullary interstitium, enabling the downstream nephron segments to concentrate urine.[48, 49] The loop of Henle is also responsible for maintaining calcium, magnesium, bicarbonate, and ammonium homeostasis.[48] Following the

loop of Henle, the ultrafiltrate flows into the distal tubules, which include the distal convoluted tubules and the connecting tubules.[50] The distal tubules are responsible for sodium, potassium, calcium, and magnesium homeostasis, and are regulated by hormones.[50]

The distal tubules of several nephrons drain into a collecting duct (Figure 3C).[42] Collecting ducts run through the osmotic gradient in the medulla and reabsorb water under the influence of hormones. Collecting ducts merge into larger collecting ducts, which are also known as the ducts of Bellini.[40] The end result is concentrated urine, which leaves the collecting ducts via the renal papilla, renal pelvis, and ureter, respectively.

**The renal microvasculature**

The kidneys are highly vascularized organs and receive approximately 20-25% of the cardiac output, which constitutes approximately 1 L of blood per minute.[51] Considering that the volume of each kidney is only approximately 200 mL,[52] each kidney is perfused with more than 2.5 times the total volume, every minute. This high renal blood flow is required to ensure that sufficient plasma is delivered to the glomeruli for filtration.

Each kidney receives blood from the renal artery, a direct branch of the abdominal aorta.[40] After entering the hilum of the kidney, the renal artery branches into interlobar arteries, followed by arcuate and interlobular arteries, which in turn give rise to the afferent arterioles that feed the glomerular capillaries (Figure 2B and C).[51] Most capillary beds in the body convey blood between arterioles and venules, enabling the exchange of gases, nutrients, and other substances with the surrounding tissue. In contrast, the glomerular capillaries are sandwiched between two arterioles: the afferent and efferent arteriole. The main function of the afferent and efferent arterioles is to regulate the glomerular blood flow and the glomerular filtration rate.[51] Both arterioles can constrict or dilate separately, thereby influencing the hydrostatic pressure gradient in the glomerular capillaries. Blood flows out of the glomerular capillaries and into the efferent arteriole, which drains into the peritubular capillaries in cortical nephrons, or the vasa recta in the juxtamedullary nephrons.[51] The peritubular capillaries and the vasa recta are both important for the delivery of oxygen and nutrients to the surrounding cells.[51] Additionally, the peritubular capillaries are essential for the tubular modification of urine in the cortex, whereas the

vasa recta are particularly important for the maintenance of the medullary concentration gradient.[51] The peritubular capillaries and the vasa recta progress into venules, followed by a series of veins that parallel the arterial system, and drain into the inferior vena cava.[51]

The endothelium that lines the interior surface of the renal microvasculature plays an important role in health and disease.[53] Under physiological circumstances, endothelial cells ensure appropriately regulated blood flow and express an anti-inflammatory phenotype, inhibiting platelet aggregation, coagulation, and inflammation which includes resistance to complement activation.[54-56] In a physiologic response to a disruption of homeostasis, such as in injury or infection, the endothelial cells become activated and cause the opposite: they promote vasoconstriction, platelet aggregation, coagulation, and inflammation, which may help resolve the disorder. The kidney has a remarkably heterogeneous population of endothelial cells, each with structural and functional features, as was reviewed previously.[51, 53, 57] The glomerular endothelial cells are an essential component of the glomerular filtration barrier. These cells are highly specialized and have a unique morphology: they are characterized by a flattened shape and contain many fenestrae with a diameter of approximately 60 nm that lack a classic diaphragm. Moreover, they are covered by the glycocalyx, a layer of negatively-charged macromolecules that contribute to permselectivity and repel blood cells from the vascular wall.[51, 53] There is cross-talk between glomerular endothelial cells, mesangial cells, and podocytes to maintain the function and morphology of the glomerular filtration barrier in relation to the local microenvironment.[58] As a result, the glomerular microvasculature may be affected in glomerular diseases by direct injury to the glomerular endothelial cells, but also by injury or disturbance of the glomerular microenvironment.[51] The endothelium of peritubular capillaries also contains fenestrations but these fenestrae have a thin diaphragm that modulates the filtration property. Because these post-glomerular capillary beds do not have a collateral circulation,[51] events in the glomerular capillary bed may have downstream influences. For example, inflammatory mediators released in the glomerular capillaries can activate the endothelium of the peritubular capillaries.[51, 59] The afferent and efferent arterioles are phenotypically distinct.[51, 60] The proximal section of the afferent arteriole has fenestrated endothelial cells that face the extraglomerular mesangial cells, whereas the distal section of the

afferent arteriole, along with the efferent arteriole, does not contain fenestrae.[51] The afferent arteriole and the proximal section of the efferent arteriole have a mono-layer of smooth muscle cells, the distal section of the efferent arterioles, along with the peritubular capillaries, is covered by pericytes.[51] These properties help in regulating blood flow and filtration.

While the larger arteries are important for delivering blood from the systemic circulation into the kidney, the renal microvasculature is important because it supplies the renal cells with oxygen and nutrients, and maintains renal function by providing an adequate glomerular filtration rate, modulating the urinary composition, and sustaining the medullary concentration gradient.[51, 57] Consequentially, microvascular endothelial injury and dysfunction are central in the pathogenesis of various kidney diseases.

# Complement in renal microangiopathies

This thesis comprises studies on complement proteins in the kidneys from patients with various renal microangiopathies. Renal microangiopathies are diseases of the renal microvasculature. The term 'microangiopathy' is derived from the Ancient Greek words 'μικρόν' (micron) which means small, 'αγγείο' (angeios) which means blood vessel, and 'πάθος' (pathos) which means suffering or disease. Renal microangiopathy refers to a status of injury or dysfunction of the renal microvascular endothelium. This may occur in the context of common diseases such as diabetes mellitus, hypertension, and preeclampsia, or in rare diseases such as atypical hemolytic uremic syndrome, primary glomerulonephritides, systemic vasculitides with renal manifestations, and rejection of the kidney allograft.[53] The renal endothelium can be injured directly by a variety of factors (such as toxins, hyperglycemia, complement proteins, autoantibodies, alloantibodies, immune cells, and cytokines) or by defects in protective mechanisms (such as complement dysregulation or an imbalance between pro-angiogenic and anti-angiogenic factors).[53] The microvasculature of the kidney is particularly vulnerable to complement-mediated injury, which is reflected by the broad range of renal diseases that have been linked to abnormal complement activation and the predominant renal manifestations of systemic diseases caused by a dysfunctional complement system.[13, 25, 26, 30] The reason for this susceptibility is not fully elucidated, but it has been suggested that it results from the kidney's unique microvascular bed,[30, 53] which is subjected to high levels of shear stress and a wide range of substances in the bloodstream that can activate the complement system, such as immune complexes, pathogens, toxins, and cytokines. Several diseases relevant to this thesis will be discussed.

### Glomerulonephritides

Glomerulonephritides are a relatively heterogeneous group of rare diseases characterized by glomerular inflammation, either as part of a primary renal disease (such as IgA nephropathy) or as a manifestation of a systemic disease (such as anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, or systemic lupus erythematosus).[61] They often affect young people, are mostly incurable, and can lead to chronic kidney disease with progression to end-stage renal disease and the need for dialysis or kidney transplantation.

In several glomerulonephritides, complement has been well studied as an important mediator of renal injury, causing activation of granulocytes and platelets, chemotaxis of leucocytes via the anaphylatoxins C3a and C5a, and direct cytotoxicity via the assembly of C5b-9.[62] As was reviewed previously,[62, 63] glomerular pathology in glomerulonephritis often results from immune complexes that activate the classical pathway, although recent studies show that the lectin and alternative pathways are also involved in the pathogenesis of several glomerulonephritides. The consequences of complement activation in glomerulonephritis depend on various factors. For example, the human immunoglobulin isotypes differ in their ability to cause classical pathway activation: IgM, IgG1, and IgG3 are strong complement activators, whereas IgG2 is a relatively weak complement activator; IgG4, IgA, IgD, and IgE are incapable of activating the classical pathway.[64] Moreover, complement activation can take place in different glomerular compartments, following passive entrapment of pre-formed immune complexes, or in situ immune complex formation; antibodies may bind to antigens that are either intrinsic constituents of glomerular structures or to soluble antigens that are taken up by the mesangium or glomerular capillary wall.[62] As a result, different cell types may be affected, causing different histopathological lesions and immune staining patterns (Figure 4).[62, 63] For example, subendothelial deposits are accessible to circulating cells, such as neutrophils and platelets, and may cause endothelial cell injury, hemostasis, coagulation, and exudative lesions.[62] Mesangial deposits can activate and injure mesangial cells, causing them to proliferate and produce growth factors, cytokines, and extracellular matrix.[62] Linear immune complex deposits indicate the binding of antibodies to autoantigens in the glomerular basement membrane.[62] Subepithelial deposits can cause complement-mediated podocyte injury without an extensive inflammatory reaction; subepithelial immune complexes are separated from the circulation by the glomerular basement membrane, and the ultrafiltration flow carries mediators towards the urine, rather than toward the circulation.[62] A pauci-immune staining pattern indicates an absence of immune complex deposition.[63]

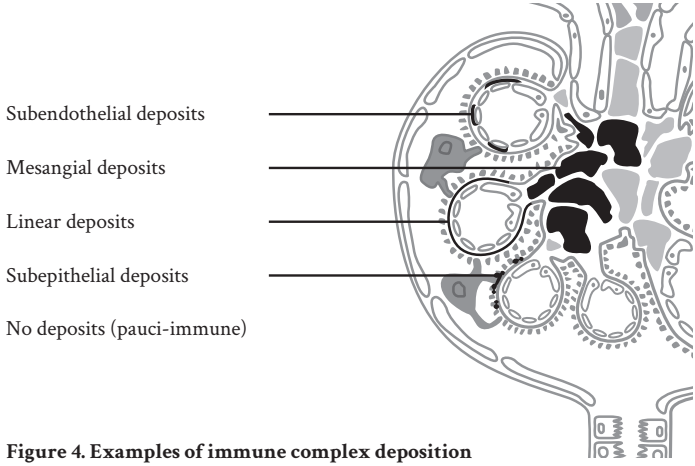


**Figure 4. Examples of immune complex deposition**

## Thrombotic microangiopathies

Thrombotic microangiopathy (TMA) may result from various disorders that are characterized by extensive endothelial cell injury (Table 1), but it is historically connected to two disorders: thrombotic thrombocytopenic purpura (TTP), and hemolytic uremic syndrome (HUS). An improved understanding of the pathogenesis of HUS and TTP has changed the way in which these historically defined terms are now used.[65] Therefore, a short summary of the first descriptions of TTP and HUS and how these terms developed in medical history is given below.

*The first description of TTP*

The first clinical and pathological description of TMA is generally attributed to Moschcowitz who presented a case history of TMA to the New York Pathological Society in New York, on the 7th of February 1924.[66] He gave a detailed account of a 16-year old girl who presented with acute fever, petechiae, pallor, and anemia, followed rapidly by paralysis, coma, and death. Her urine showed marked traces of albumin with hyaline and granular casts, but renal failure was absent. At autopsy, hyaline thrombi were observed in the arterioles and capillaries of the heart, liver, spleen, and kidney. It took more than 10 years before Baehr, Klemperer, and Schifrin published the clinical and morphological findings of four cases with '*Moschcowitz syndrome*' in 1936.[67] They suggested

that the microthrombi were composed of platelets and that the associated thrombocytopenia was caused by the excessive consumption of these platelets. In 1947, Singer et al. introduced the term '*thrombotic thrombocytopenic purpura*' and in 1952, Symmers introduced the term '*thrombotic microangiopathy*', brief for '*thrombotic microangiopathic haemolytic anaemia*'.[68, 69] Symmers considered '*TMA*' a better diagnostic term to describe the syndrome than Moschcowitz's syndrome, TTP, and other contemporary terms, because it was not eponymous, remained appropriate for patients without thrombocytopenia or purpura, and described the most striking histological lesions.[69]

*The first description of HUS*

The first publications suggestive of HUS include a case-report on a soldier who died in 1918 following gastroenteritis, and the descriptions of severe cases during an epidemic of gastroenteritis due to *Escherichia coli* in 1955.[70, 71] In 1955, von Gasser et al. introduced the term "*Hämolytisch-urämische Syndrome*" in their description of five children with bilateral necrosis of the renal cortex, and four characteristic clinical features: acquired hemolytic anemia, acute renal failure, thrombocytopenia, and cerebral symptoms.[72] There were several reasons why HUS was considered distinct from TTP.[72, 73] First, the condition primarily affected children, whereas TTP was considered a disease of the adults. In addition, the children presented with acute renal failure and had histopathological lesions of bilateral renal cortical necrosis, which were not considered typical of TTP at the time. Interestingly, von Gasser et al. suggested the existence of multiple syndromes, instead of one hemolytic uremic syndrome. The German word "*Syndrome*" translates to the plural form 'syndromes', in contrast to the singular "*Syndrom*", which translates to 'syndrome'. This distinction seems to have gone lost in translation in the subsequent papers but appears to be accurate in retrospect, as the reported patients may have had various syndromes, such as TTP and pneumococcal-associated HUS. The lesions of HUS were subsequently defined by Habib et al. who found that cortical necrosis was not a requirement for HUS and that the lesions observed in kidneys from patients with TTP and HUS were similar.[74, 75] Habib et al. used the term '*TMA*', with permission from Symmers, and TMA became used to denote a morphological manifestation in addition to a clinical syndrome.[73, 76] From the initial description of HUS, the diagnostic triad of thrombocytopenia, microangiopathic hemolytic anemia, and acute renal injury remained, and are still in use today.[77]

**Table 1. Differential diagnosis of TMA**

**TTP:** caused by a functional deficiency of ADAMTS-13
- Genetic abnormalities in ADAMTS-13 (Upshaw Schulman Syndrome)
- Acquired autoantibodies against ADAMTS-13

**Infectious-TMA**
- STEC-HUS caused by Shiga-toxin or Shiga-like toxins such as in E. Coli or Shigella dysenteriae type-1
- Pneumococcal-HUS
- Human immunodeficiency virus (HIV)-associated TMA
- Influenza-TMA
- TMA following other infections

**Atypical HUS**
- Complement-mediated TMA
  - Genetic abnormalities in complement regulatory proteins or complement effector proteins
  - Acquired autoantibodies against complement regulatory proteins
- Broad term for all other causes than TTP or STEC-HUS

**DGKE-TMA:** caused by mutations in diacylglycerol kinase epsilon (DGKE)

**CblC deficiency associated TMA:** caused by a functional deficiency in cobalamin C (CblC)

**Secondary TMA:** TMA secondary to non-infectious clinical conditions
- Solid organ transplantation
- Hematopoietic stem cell transplantation
- Post-radiation
- Post-surgery
- Drug-induced
  - *Chemotherapy* (mitomycin, cisplatin, bleomycin, and gemcitabine)
  - *Immunosuppressive drugs* (cyclosporine, tacrolimus, OKT3, IFN, and quinidine)
  - *Antiplatelet agents* (ticlopidine and clopidogrel)
  - *Anti-VEGF therapy (*bevacizumab, sunitinib)
- Pregnancy
  - *Preeclampsia/HELLP syndrome* (hemolysis, elevated liver enzymes, and low platelets)
  - Other TMA following pregnancy
- Malignancy
- Pancreatitis
- Severe and malignant hypertension
- Glomerular diseases
  - *IgA nephropathy*
  - *Membranous nephropathy*
  - *C3 glomerulopathy/ Membranoproliferative glomerulonephritis (MPGN)*
  - *Focal segmental glomerulosclerosis (FSGS)*
  - *Diabetic nephropathy*
- Systemic autoimmune diseases
  - *Systemic lupus erythematosus*
  - *Antiphospholipid syndrome*
  - *Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis*
  - *Systemic sclerosis*

Summary of diseases, disorders, and syndromes that may manifest with systemic or local TMA. Recent publications listed multiple causes and differential diagnoses for TMA.[65, 135, 140, 141, 151-155] Some of these TMAs are entities with a specific pathophysiology. Other TMAs are described in association with clinical conditions that often cause endothelial cell injury or dysfunction, but the pathophysiological mechanisms that drive these processes are poorly understood. For many of these TMAs, it is unknown if TMA is a separate entity, or related to a specific cause that can only be found if it is meticulously looked for. Defects in the complement system may contribute to various of these TMAs.

*Advancements in the understanding of HUS and TTP*

In 1966, Amorsi and Ultmann published a case-series of 16 patients with TTP and reviewed the literature of 255 previously described cases.[78] They concluded that TTP had a dramatically high mortality rate of 90% in the era before effective therapy. Moreover, they established the diagnostic criteria for TTP consisting of the 'pentad' of hemolytic anemia, thrombocytopenia, renal abnormalities, neurological abnormalities, and fever. Given the overlap with the diagnostic triad of criteria for HUS, the classification of patients with TMA became based on clinical features: TTP for patients with predominantly neurological manifestations, and HUS for patients with predominantly renal manifestations. However, this distinction was complicated by the significant overlap in clinical features: both TTP and HUS could have severe neurological, renal, or multi-organ involvement, both could present in children and adults, and there were case-reports of patients with recurrent episodes that had alternating phenotypes of HUS and TTP, as well as case-reports of family members in which some individuals had HUS and others had TTP.[79-90] For more than 40 years since the von Gasser publication, controversy remained as to whether HUS and TTP were separate disease entities or different clinical expressions of the same disease.[90-93] As the clinical and histopathological findings of both syndromes were often indistinguishable and the underlying pathophysiology was largely unknown, in 1987, Remuzzi introduced the term *'HUS/TTP'* to describe patients with thrombocytopenia, microangiopathic hemolytic anemia, and renal injury.[90] Others suggested that the term TMA could be used to encompass the spectrum of TTP and HUS, or that TMA could be appropriate for cases that could not unequivocally be considered as having either TTP or HUS.[94, 95]

Subsequent discoveries were useful for untangling the HUS/TTP knot. In the field of TTP, major discoveries include the description of familial TTP in 1975 and the association between TTP and large von Willebrand factor (vWF) multimers leading to platelet agglutination.[96-99] Plasma-exchange therapy was found to be effective for patients with TTP,[100] reducing mortality from 90% to approximately 25%.[101] The efficacy of plasma-exchange therapy for patients meant that more inclusive diagnostic criteria were required to allow rapid initiation of treatment: in the clinical trial that demonstrated the efficacy of plasma-exchange therapy in patients with TTP, only microangiopathic hemolytic anemia and thrombocytopenia, without an apparent alternative cause, were required for the

diagnosis of TTP.[101, 102] In 1997, two siblings with TTP were described who were deficient in a protease that cleaves vWF.[103] In 2001, this protease was identified as ADAMTS-13, short for *A Disintegrin-like And Metalloprotease with ThromboSpondin type 1 motif, member 13*, and mutations in ADAMTS-13 were discovered in families with TTP.[104] These studies supported the hypothesis that patients with TTP are deficient in ADAMTS-13, preventing adequate cleavage of vWF and leading to ultra large vWF multimers and increased platelet aggregation.

In the field of HUS, the distinction between the most common form (termed typical HUS), and other variants (termed atypical HUS) became relevant.[85, 105-109] After the introduction of HUS, diarrhea became recognized as a prodromal feature, and this diarrhea-associated HUS (D+ HUS or typical HUS) was associated with epidemics of *Escherichia coli* (E. coli) infection.[71, 105, 109, 110] Following the discovery of verocytotoxigenic E. coli (VTEC) strains, also known as enterohemorrhagic E. coli (EHEC),[111-113] Karmali et al. showed in 1983 that D+HUS could be caused by the Shiga-like verotoxins produced by these strains.[108] Subsequently, it was recognized that most cases of D+ HUS were caused by infection with strains of Shiga toxin-producing E. Coli, and the term STEC-HUS became common.

In contrast, it was observed that a minority of HUS patients had an atypical, diarrhea-negative course, for whom the term 'D- HUS' or 'atypical HUS' was used.[105, 114] Atypical HUS was often described in families and patients with atypical HUS were found to have evidence of complement activation as determined by serological testing and deposition of complement proteins in renal biopsies.[115-126] Subsequently, loss-of-function mutations were found in genes encoding important regulators of the complement system, such as factor H, factor I, and MCP; gain-of-function mutations were found in genes encoding effector proteins factor B, and C3; and auto-antibodies were discovered that lead to a functional deficiency of factor H.[127-135] In 2013, the efficacy of terminal complement inhibitor eculizumab was demonstrated in patients with atypical HUS.[136] The criteria for atypical HUS in the clinical trials of eculizumab were designed to exclude patients with TTP and patients with STEC-HUS: evidence of hemolysis (e.g., lactate dehydrogenase level at or above the upper limit of the normal range, haptoglobin level below the lower limit of the normal range, or the presence of schistocytes) and impaired renal function (creatinine level at or above the upper limit of the normal range), without plasma ADAMTS13 activity below 5% or STEC; identification of

complement gene mutations or factor H autoantibodies was not required.[136] At least half of the patients with atypical HUS are now known to have an inherited or acquired abnormality in the complement system, causing endothelial injury.[137] The availability and efficacy of complement-inhibiting therapy in patients with atypical HUS, including those with and without proven mutations in complement regulatory genes, has led to a significant reduction in morbidity and mortality in these patients.[138, 139]

*Morphologic TMA*

TMA is also used as a morphologic term for disorders of structure and function with microvascular lesions that reflect endothelial cell injury (such as microthrombi) but lack the microvascular inflammatory cell infiltrate that defines vasculitis (morphologic TMA).[65, 140, 141] Morphologic TMA is a histopathological diagnosis for a local pattern of lesions and can occur in a wide range of clinical settings.[140] As was reviewed previously,[65, 137, 140-142] morphologic TMA in the kidney may manifest with acute and chronic lesions (Table 2). These lesions may result from extrinsic causes, such as Shiga-like toxins and drugs, or from intrinsic causes that lead to endothelial dysfunction and injury (Table 1), but often the underlying cause is unknown.[143] Typically, a patient with systemic TMA, such as atypical HUS, has morphologic TMA on the renal biopsy. However, morphologic TMA can also be observed in the biopsies of patients who lack thrombocytopenia and/or microangiopathic hemolytic anemia but who presented with other symptoms for which a renal biopsy was performed (local TMA).[65, 140, 141, 144-147] In these cases, TMA in the renal biopsy may, for example, 'unmask' patients with atypical HUS caused by complement dysregulation, or demonstrate TMA in the setting of drug toxicity. Unfortunately, the precise etiology of the underlying disease cannot be distinguished by renal biopsy alone.[140] When TMA persists, chronic lesions become predominant and there may be a paucity or even absence of microthrombi.[140] Recently, it was suggested to replace the term '*TMA*' with '*microangiopathy with or without thrombosis*', to account for biopsies with microangiopathic lesions in the absence of microthrombi.[137] Moreover, microangiopathic lesions consistent with morphologic TMA may co-exist with other kidney diseases, or overlap with lesions seen in other disorders, such as a duplicated glomerular basement membrane in the setting of transplant glomerulopathy, or glomerular endotheliosis in the setting of preeclampsia, complicating histopathological diagnosis.[140, 146, 148, 149]

**Table 2. Morphologic features of microangiopathy with or without thrombosis**

| Acute lesions | Chronic lesions |
|---|---|
| **Glomeruli:**<br>• Fibrin or platelet thrombi in capillary lumina, the mesangium, and the subendothelium<br>• Endothelial swelling and subendothelial widening<br>• "Bloodless glomeruli" with capillary luminal narrowing<br>• Mesangiolysis<br>• Fragmented erythrocytes in the subendothelium and mesangium<br>• Glomerular capillary tuft collapse, with predominant arterial involvement | **Glomeruli:**<br>• Duplicated glomerular basement membrane with variable proliferation<br>• Mesangiolysis<br>• Glomerular sclerosis |
| **Arterioles and arterial branches:**<br>• Fibrin thrombi in capillary lumina<br>• "Mucoïd" intimal hyperplasia in arteries<br>• Fibrinoid necrosis | **Arterioles and arterial branches:**<br>• Arterial intimal fibrosis<br>• Organization and recanalization of luminal thrombi<br>• Concentric myointimal proliferation (onion-skinning) |

Summary of the histopathological lesions that can be observed in the renal biopsies of patients with microangiopathy, with or without thrombosis. These lesions have been reviewed previously.[65, 137, 140-142]

*Contemporary view on TTP, HUS, and TMA*

Currently, the term 'TTP' is used to diagnose patients with severe ADAMTS-13 deficiency, although it is recognized that a number of patients meet the clinical criteria for TTP but only have mildly reduced ADAMTS-13 activity levels.[150] Although the term 'HUS' is still typically used to describe STEC-HUS, it is also used for all patients with microangiopathic hemolytic anemia, thrombocytopenia, and acute kidney injury, and can be classified according to primary and secondary causes.[77] Atypical HUS is used by some to describe patients with a primary defect in the complement system (also described as complement-mediated TMA or complement-mediated HUS) and by others to roughly describe all other forms of TMA than TTP or STEC-HUS, including patients with mutations in diacylglycerol kinase epsilon (DGKE), defective cobalamin C metabolism, and TMA secondary to various diseases and therapies that cause endothelial cell injury or dysfunction (Table 1).[65, 135, 140, 151-158] As TTP has connotations with beneficial effects of plasma exchange, HUS with an infectious component that is treated with conservative

therapy, and atypical HUS with mutations in genes affecting complement proteins, for which complement inhibition may be beneficial, some prefer to use the more value-neutral term TMA in all instances.[141] Consequentially, this may lead to miscomprehension as TMA is used as a term for syndromes that are characterized by thrombocytopenia, microangiopathic hemolytic anemia, and evidence of end-organ injury (systemic TMA),[135, 159, 160] for a histopathological pattern of lesions (morphologic TMA) that can also be observed in the absence of thrombocytopenia or microangiopathic hemolytic anemia (local TMA),[65, 137, 140, 147] and as an all-embracing term for disorders that can present with evidence of severe endothelial injury and microvascular thrombosis (the thrombotic microangiopathies; TMAs).[141, 146, 150, 154] As was reviewed recently,[139, 140, 158, 161] case reports and small studies suggest that complement activation is an important contributing factor for many etiologies of TMA, but the pathophysiological mechanisms that lead to microangiopathic lesions are incompletely understood and the exact role of complement in this process remains to be determined.

**Antibody-mediated rejection and transplant glomerulopathy**

Kidney transplantation is the treatment of choice for patients with end-stage renal disease because it has superior outcomes in terms of morbidity, mortality, and quality of life, in comparison to dialysis.[162] As was reviewed previously,[53, 57, 163, 164] the renal microvasculature is the primary target of several acute and chronic immunologic processes directed against the transplanted kidney, causing allograft rejection. Antibody-mediated rejection is a distinct form of allograft rejection in which donor-specific antibodies (DSAs) from the recipient are directed against the antigens of the donor kidney.[57, 165] The DSAs are most commonly directed towards class I or class II human leukocyte antigens (HLAs) that are expressed in the endothelial cells of the renal microvasculature.[57] They cause endothelial injury by activating endothelial cells directly, recruiting inflammatory cells, and activating the complement system. Complement activation can cause endothelial cell injury via various mechanisms that were reviewed previously.[53, 163, 164, 166] For example, the Fc regions of IgM and IgG DSAs can bind to C1q, activating the classical complement pathway and resulting in endothelial injury.[53] In addition, anaphylatoxins C3a and C5a cause inflammation, and C5b-9 can lead to apoptosis, activation, and lysis of endothelial cells, as well as activation of T cells. Moreover, the transplantation process can cause ischemia-reperfusion

injury, which promotes local activation of complement via lectin and alternative pathways. Endothelial cell activation leads to increased expression of adhesion molecules, inflammatory cell recruitment, and a pro-coagulative state, causing further allograft injury. Complement split product C4d forms after C4 cleavage and can attach covalently to endothelial cells and basement membranes.[167] This property makes C4d a stable, target-bound biomarker that can reveal complement activation even though the initiating factors or the subsequent complement proteins have gone into solution.[168] Although several studies have shown that a proportion of patients with antibody-mediated rejection are C4d-negative, the presence of C4d deposition in the peritubular capillaries is both sensitive and specific for antibody-mediated rejection, and is part of the diagnostic criteria for active antibody-mediated rejection.[166-170]

Over the past three decades, there has been an enormous improvement in renal allograft survival in the first year after transplantation.[171] In contrast, the rate of long-term renal allograft loss has remained almost unchanged.[171] Improving long-term allograft survival is one of the major unmet needs in renal transplantation, but progress is limited by an incomplete understanding of the causes of long-term allograft loss. Chronic active antibody-mediated rejection is an important cause of long-term allograft failure. It is thought to result from undetectable, low-titer DSAs or de-novo antibodies generated after transplantation, which can bind to microvascular endothelial surfaces and activate the complement and coagulation cascades.[172] According to the most recent Banff criteria, chronic active antibody-mediated rejection is diagnosed by identifying evidence of DSAs, interaction of the antibody with vascular endothelium (such as C4d positivity), and morphologic evidence of chronic tissue injury (such as peritubular capillary basement membrane multilayering, arterial intimal fibrosis, and transplant glomerulopathy).[170] Transplant glomerulopathy is a morphologic description of glomerular basement membrane duplication ('tram tracking') by light or electron microscopy, that is associated with poor graft survival.[173] The clinical course of patients with transplant glomerulopathy is often insidious, with progressive, unexplained loss of renal function, minor proteinuria, and mild hypertension, although nephrotic range proteinuria has also been documented.[148, 173] In addition to glomerular basement membrane duplication, renal biopsies may show mesangial matrix expansion, mesangial hypercellularity, and glomerulitis.[148, 165, 173] Ultrastructural analysis

shows circumferential multilayering of the glomerular basement membrane, which is frequently accompanied by multilayering of the basement membrane of peritubular capillaries.[173] Glomerular capillaries also show subendothelial widening with mesangial cell interposition, and podocyte foot process effacement may be seen in patients with proteinuria.[173]

Transplant glomerulopathy is considered a morphologic manifestation of chronic antibody-mediated rejection; it is associated with DSAs, most notably against HLA class II antigens, prior episodes of antibody-mediated rejection, glomerulitis, and C4d deposition in peritubular capillaries.[173, 174] However, transplant glomerulopathy is not pathognomonic for chronic antibody-mediated rejection; other etiologies include hepatitis C virus infection, TMA, and T cell-mediated rejection.[149, 173-175] In addition to C4d staining in peritubular capillaries, transplant glomerulopathy is also associated with C4d deposition in glomerular capillaries.[176, 177] This glomerular staining pattern is frequently considered a sign of chronic antibody-mediated rejection, but the significance of glomerular C4d deposition in transplant glomerulopathy is unclear, particularly in absence of other signs of antibody-mediated rejection, such as concomitant C4d in peritubular capillaries.

**Preeclampsia**

Preeclampsia is a pregnancy-specific microangiopathy, complicating approximately 5% of all pregnancies.[178, 179] It is characterized by hypertension and proteinuria occurring after 20 weeks of gestation.[179] A more recent definition broadened the diagnostic criteria: preeclampsia can now also be diagnosed in the absence of proteinuria if hypertension occurs after 20 weeks together with any new onset maternal organ dysfunction (including renal insufficiency, impaired liver function, neurologic complications, or hematological complications), or uteroplacental dysfunction as evidenced by fetal growth restriction.[180] Severe preeclampsia may progress into eclampsia which is characterized by the development of tonic-clonic seizures, or present as HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets), a life-threatening variant of preeclampsia.[180] The current treatment is supportive with the aim of reducing blood pressure levels, preventing the progression of systemic disease, and prolonging the pregnancy in order to maximize fetal development.[180] There is no cure other than the delivery of the placenta, which causes significant

fetal morbidity and mortality, depending on the gestational age. As a result, preeclampsia remains one of the leading causes of maternal and neonatal mortality in the world.[179]

The lesions observed in the renal biopsy or autopsy samples of patients with preeclampsia have been reviewed previously.[146] Light microscopic examination of the kidneys from women with preeclampsia can reveal 'bloodless glomeruli', in which swelling of the glomerular endothelial cells (glomerular endotheliosis) causes the occlusion of the glomerular capillaries. Glomerular volume is typically increased, but glomerular hypercellularity or arteriolar involvement is unusual. In severe cases, microthrombi and features of chronic TMA and transplant glomerulopathy can also be observed. In some cases, it is impossible to distinguish preeclampsia from other TMAs.[146, 152]

The pathogenesis of preeclampsia is incompletely understood but is considered to be multifactorial.[179, 181] The prevailing hypothesis is that abnormal placentation causes placental hypoxia, triggering the release of placenta-derived circulating factors, which leads to an imbalance between pro-angiogenic factors (such as vascular endothelial growth factor (VEGF) and placental growth factor), and anti-angiogenic factors (such as soluble Fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin).[57, 178, 179, 181] For example, sFlt-1 can bind to VEGF, leading to a reduced biological availability for VEGF-receptor signaling. This relative deficiency in VEGF can then lead to endothelial dysfunction, endothelial cell injury, and the disruption of the glomerular filtration barrier, reflected by proteinuria and hypertension. Several findings support this hypothesis. For example, serum levels of sFlt-1 are elevated in women with preeclampsia,[182] and animal models with increased expression of sFlt-1 develop a clinical and morphological phenotype that resembles preeclampsia.[183, 184] Moreover, patients who receive anti-VEGF drugs for the treatment of malignancies may develop morphologic TMA and a preeclampsia-like phenotype.[185] Preeclampsia can be distinguished in two stages: placental preeclampsia, caused by placental dysfunction in the first half of pregnancy, and maternal preeclampsia, in which an exaggerated maternal inflammatory response can cause the clinical phenotype of preeclampsia in the second half of pregnancy.[178, 181] The inflammatory response is amplified in women with other risk factors for endothelial dysfunction such as hypertension, obesity, and diabetes.[186] Recently, genetic defects in complement regulation were found to predispose to the development of preeclampsia and

our group has shown that patients with preeclampsia have evidence of classical pathway activation in the placenta.[186-188] These data suggest that abnormal complement activation in the placenta or the kidney could be involved in the pathogenesis of preeclampsia, but it remains to be determined whether this process takes place in the kidney; the molecular mechanisms of complement activation in preeclampsia have not yet been identified.

**Diabetic nephropathy**

Diabetes mellitus is a pandemic disease that affects more than 400 million people worldwide.[189] It is characterized by hyperglycemia, resulting from defective insulin secretion, insufficient insulin action, or both.[190,191] The two main forms of diabetes are type 1 and type 2, with type 2 diabetes accounting for more than 85% of the total diabetes prevalence.[192] Type 1 diabetes is caused by an autoimmune destruction of the insulin-producing β-cells of the pancreas; type 2 diabetes results from a combination of insulin resistance and a progressive loss of insulin secretion.[191] Prolonged hyperglycemia causes macrovascular and microvascular injury and is associated with complications in various organs, including the eyes, nerves, kidneys, and the heart. Diabetic nephropathy is the renal microvascular complication of diabetes mellitus type 1 and type 2. Currently, it is the leading cause of end-stage renal disease in high-income countries.[193, 194] The clinical diagnosis of diabetic nephropathy is based on persisting microalbuminuria or macroalbuminuria, or an estimated glomerular filtration rate ≤ 60 mL / 1.73 $m^2$, along with clinical features, such as diabetes duration and the presence of diabetic retinopathy.[193-195] Renal biopsy is the gold standard for the diagnosis of diabetic nephropathy, but the majority of diabetic patients with renal involvement are not biopsied because it is an invasive procedure with limited benefits in an otherwise uncomplicated patient.[196] Nevertheless, the renal biopsy can be useful to discern diabetic nephropathy from non-diabetic kidney disease, or a superimposed non-diabetic condition on underlying diabetic nephropathy.[194, 195, 197, 198] Moreover, the renal biopsy can be used to classify lesions by various degrees of severity, guiding therapeutic management and outcome prediction.[194, 195, 198, 199]

Diabetic nephropathy is characterized by mesangial matrix expansion, which may be nodular (Kimmelstiel-Wilson nodules) and hyaline arteriolosclerosis in afferent and efferent arterioles by light microscopy, as well as thickening of the glomerular basement membrane by electron microscopy.[200]

Other light microscopical lesions include glomerular hypertrophy, segmental or global glomerulosclerosis, mesangial cell proliferation, mesangiolysis, capillary microaneurysms, hyaline deposits in Bowman's capsule (capsular drop), interstitial fibrosis, tubular atrophy, and arteriolar intimal sclerosis. By electron microscopy, other ultrastructural lesions include podocyte loss, foot process effacement, glomerular fibrillar extracellular matrix deposition, tubular basement membrane thickening, and subendothelial or transmural hyaline deposits in the small arteries and arterioles.[200]

Despite the high global burden of disease, 60-70% of patients with diabetes mellitus type 1 or 2 do not develop diabetic nephropathy and only a proportion of the patients with diabetic nephropathy develop advanced stages of glomerular or arteriolar injury. Risk factors for diabetic nephropathy include susceptibility factors (such as age, gender, ethnicity, and genetic predisposition), initiation factors (such as hyperglycemia and acute kidney injury) and progression factors (such as hypertension and dietary intake).[193] As was reviewed previously,[193, 201-206] different molecular processes may cause endothelial dysfunction or injury in diabetic nephropathy, including the accumulation of reactive oxygen species and advanced glycation end products, increased flux through the polyol and hexosamine pathways, and activation of protein kinase C, with downstream effects on various proteins, growth factors, and inflammatory mediators. Two recent reviews highlighted that complement activation may also be involved in the macrovascular and microvascular complications of diabetes mellitus,[207] and in the development of diabetic nephropathy specifically.[208] For example, hyperglycemia can induce the glycation of complement regulatory proteins, leading to a dysfunction of their regulatory capacity and complement-mediated injury. Moreover, diabetes-induced alterations in glycoproteins may stimulate complement activation through the binding of MBL to neo-epitopes. Gene expression analysis of microdissected human renal glomeruli and tubule samples showed that various complement regulators and proteins of the classical pathway were upregulated in patients with diabetic nephropathy.[209] However, complement deposition along the renal microvasculature of patients with diabetic nephropathy has not yet been thoroughly characterized and the involvement of complement activation in the development or progression of diabetic nephropathy is incompletely understood.

## Aims and outline of this thesis

This thesis is focused on the clinicopathologic significance of complement deposits along the renal microvasculature of patients with renal microangiopathies. Special attention is paid to C4d, a cleavage product of C4 activation, which remains covalently bound to the surrounding tissue long after the complement-pathway initiating factors have dissociated. C4d is used as a biomarker for complement activation worldwide.[168]

The specific aims of this thesis are:

- to determine the prevalence, localization, distribution, and staining pattern of complement deposits in kidneys from patients with morphologic TMA, in the setting of various underlying clinical conditions;
- to determine the clinicopathologic significance of microangiopathy with or without thrombosis and complement deposits in the kidneys of patients with IgA nephropathy and IgA vasculitis with nephritis;
- to determine the relationship between glomerular C4d deposits and glomerular basement membrane remodelling in native and transplanted kidneys;
- to determine the clinicopathologic significance of complement activation in glomeruli of patients with preeclampsia; and
- to determine the clinicopathologic significance of complement deposits in kidneys of patients with diabetic nephropathy.

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# Chapter 2

## Complement factor C4d is a common denominator in thrombotic microangiopathy

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# ABSTRACT

Complement activation has a major role in thrombotic microangiopathy (TMA), a disorder that can occur in a variety of clinical conditions. Promising results of recent trials with terminal complement-inhibiting drugs call for biomarkers identifying patients who might benefit from this treatment. The primary aim of this study was to determine the prevalence and localization of complement factor C4d in kidneys of patients with TMA. The secondary aims were to determine which complement pathways lead to C4d deposition and to determine whether complement activation results in deposition of the terminal complement complex. We examined 42 renal sections with histologically confirmed TMA obtained from a heterogeneous patient group. Deposits of C4d, mannose-binding lectin, C1q, IgM, and C5b-9 were scored in the glomeruli, peritubular capillaries, and arterioles. Notably, C4d deposits were present in 88.1% of TMA cases, and the various clinical conditions had distinct staining patterns within the various compartments of the renal vasculature. Classical pathway activation was observed in 90.5% of TMA cases. C5b-9 deposits were present in 78.6% of TMA cases and in 39.6% of controls (n=53), but the staining pattern differed between cases and controls. In conclusion, C4d is a common finding in TMA, regardless of the underlying clinical condition. Moreover, C5b-9 was present in >75% of the TMA samples, suggesting that terminal complement inhibitors may have a beneficial effect in these patients. C4d and C5b-9 should be investigated as possible diagnostic biomarkers in the clinical work-up of patients suspected of having complement-mediated TMA.

# INTRODUCTION

Thrombotic microangiopathy (TMA) is a devastating disorder characterized by the development of multiple vascular microthrombi, endothelial cell damage, thrombocytopenia, and hemolysis.[1] TMA is mostly systemic, with the kidney and brain most commonly affected.[2] Renal biopsies can reveal the presence of microthrombi even in the absence of systemic manifestations. TMA can occur in a wide range of diseases.[3, 4] Given the heterogeneity of TMA-associated diseases, identifying mechanistic pathways common in most cases has diagnostic and therapeutical value.

Since the discovery that defects in complement regulatory genes are the leading cause of atypical hemolytic uremic syndrome (aHUS), evidence has accumulated rapidly that complement activation is important in the pathogenesis of TMA.[5] Complement hyperactivation—*via* excessive stimulation, inadequate regulation, or both—can cause TMA in a variety of underlying clinical conditions: up to 60% of patients with aHUS have mutations in complement regulatory genes.[6] Recent evidence showed that in Shiga toxin-producing *Escherichia coli*-associated HUS (STEC-HUS), Shiga toxins can directly activate the complement system, causing severe endothelial damage and ultimately leading to TMA.[7] An association between complement activation and TMA has also been reported in patients with thrombotic thrombocytopenic purpura, systemic lupus erythematosus (SLE), antiphospholipid syndrome, and antibody-mediated kidney allograft rejection.[8-11]

The efficacy of eculizumab treatment in patients with TMA in different clinical settings provides further evidence that complement activation is involved in the mechanistic pathway leading to TMA.[12-17] Eculizumab is a monoclonal antibody that selectively inhibits the cleavage of complement C5, thereby preventing the generation of C5a and the terminal complement complex, C5b-9. Results from the first systematic trial of eculizumab in patients with aHUS show that eculizumab improved renal function, increased platelet counts, and prevented thrombotic microangiopathic events in most patients.[18]

Given the heterogeneity of clinical settings under which TMA can occur, biomarkers would be useful to identify which patients would benefit from terminal complement inhibitors. We previously reported that for the diagnosis TMA, glomerular C4d staining is a useful biomarker in patients with SLE with

or without antiphospholipid syndrome.[9] The aim of this study was to investigate the prevalence and localization of C4d deposits in renal biopsy specimens from patients with TMA in the setting of various underlying clinical conditions, in combination with the prevalence and localization of C5b-9.

# METHODS

### Patients and controls

We retrospectively searched the database of our hospital's Department of Pathology for patients with a diagnosis of TMA who underwent renal biopsy, nephrectomy, and/or autopsy from 1991 through 2010 at the Leiden University Medical Center. Search terms included 'HUS', 'microthrombi', 'thrombi', 'TMA', 'TTP', 'microangiopathy' and 'thrombocytopenia'. Cases were reviewed by an experienced nephropathologist (I.M.B.). Histologically confirmed TMA was defined as the presence of one or more platelet thrombi obstructing vessel lumens on renal biopsy, with or without thickening of the arterioles and capillaries, endothelial swelling and detachment, or widening of the subendothelial space.

Healthy control samples without TMA (n=9) were obtained from Eurotransplant donor kidneys that were unsuitable for transplantation because of technical deficits. Diseased control samples without TMA (n=44) included the following three groups: native renal biopsy specimens from patients with FSGS (n=19), native renal biopsy specimens from patients with Alport syndrome (n=5), and kidney transplant biopsy specimens showing a variety of lesions other than antibody-mediated rejection (n=20). This kidney transplant control group included interstitial fibrosis and tubular atrophy (n=6), T-cell mediated rejection (n=7), no apparent lesions (n=1), calcineurin inhibitor toxicity and/or hyalinosis (n=4), and recurrent disease (n=2). Two independent investigators (J.C. and L.vE.) analyzed the clinical data and correspondence, which were retrospectively obtained from the medical records and included the underlying clinical diagnosis; serum LDH; hemoglobin; thrombocytes; ADAMTS13; urea and creatinine; and the presence of schistocytes, neurological symptoms, and/or renal dysfunction at the time of tissue sampling.

### Native and transplanted kidney case groups

Renal samples were obtained from 28 patients with native kidney disease and 8 kidney transplant recipients. Patients with native kidney disease were classified into one of six groups: aHUS (n=11), including patients with and without demonstrated mutations in complement regulatory genes; diarrhea associated HUS (n=1), defined as HUS following gastro-enteritis caused by E. coli; HSCT-TMA (n=6); SLE with or without antiphospholipid syndrome (n=8); IgA nephropathy (n=1), and ANCA-associated vasculitis (n=1). Kidney transplant recipients were classified into one of three groups: recurrent aHUS (n=3), defined as cases with recurrence of microthrombi in the renal allograft, with or without known mutations in complement regulatory genes; drug toxicity (n=2) defined as cases who received high-dose cyclosporine and/or tacrolimus that improved clinically after a change in therapeutic regimen, and rejection (n=3), defined as cases with TMA that developed in association with a rejection episode.

### Histopathology

Renal tissue was fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded sections were stained with hematoxylin and eosin, periodic-acid Schiff, silver-stain, and phosphotungstic acid-hematoxylin.

### Immunohistochemistry

To investigate complement activation, immunohistochemical staining was performed for various components of the complement system. Paraffin-embedded sections were cut at 4-μm thickness, deparaffinized, and subjected to antigen retrieval. After blocking endogenous peroxidases, the sections were incubated in the relevant primary antibody for 1 hour. Binding of the primary antibody was visualized using the appropriate horseradish peroxidase-labeled secondary antibodies and diaminobenzidine as the chromogen. Finally, the sections were counterstained with hematoxylin. The primary antibodies included antibodies against C4d (BI-RC4d; Biomedica Gruppe, Vienna, Austria; 1:50), C1q (A0136; Dako, Glostrup, Denmark; 1:800), mannose-binding lectin (MBL) (HPA002027; Sigma-Aldrich, St Louis, MO; 1:500), and sC5b-9 (A239; Quidel, San Diego, CA; 1:150). C4d is a cleavage product of C4 that binds covalently to the target tissue. The deposition of C4d can result from activation of the classical pathway (represented by C1q) or from activation of the lectin pathway (represented by

MBL). Activation of any of the complement pathways can lead to deposition of the terminal complement complex, C5b-9. The optimum antibody dilution and incubation time were determined empirically for each antibody by performing a titration experiment on positive control sections.

For C4d staining, a renal specimen obtained from a patient with antibody-mediated allograft rejection and C4d-positive peritubular capillary staining (confirmed by immunofluorescence) was used as a positive control. Negative controls were obtained by incubating the positive control samples and TMA cases in negative control rabbit immunoglobulin fraction (X0936; Dako). For MBL staining, a section of healthy liver was used as the positive control, and omitting the primary antibody served as the negative control. For C1q staining, a section of healthy tonsil served as a positive control and negative control was obtained by adding the negative control rabbit immunoglobulin fraction (X0936) to the positive control section. For C5b-9 staining, a renal specimen obtained from a patient with antibody-mediated allograft rejection and confirmed C5b-9-positive staining served as the positive control, and the negative control was obtained by incubating the positive control section in the negative control mouse IgG2b antibody (X0944; Dako).

To investigate the presence of IgM deposits, direct immunofluorescence staining was performed on renal paraffin sections of 4-μm thickness. After deparaffinization, the sections were incubated in Protease 24 for 1 hour at at 37°C. The sections were then incubated for 1 hour with a fluorescein isothiocyanate (FITC)- conjugated Fc-specific F(ab')2 mouse anti-human IgM antibody (1:40; Protos Immunoresearch, Burlingame, CA).

**Evaluation of histopathologic and immunohistochemistry results**

Stained sections were evaluated by two investigators (I.M.B. and D.C.) who were blinded with respect to the clinical data. The sections were scored according to the presence of microthrombi in the glomeruli and arterioles, the presence of fibrin in the glomeruli, thickening and double contours of the glomerular basement membrane, glomerular fibrinoid necrosis, arterial and arteriolar endothelial swelling, and microaneurysms. Endothelial changes were scored on the basis of proliferation and foamy changes. Immunohistochemical staining patterns in peritubular capillaries was scored in accordance with the Banff 2007 criteria for C4d staining.[31] Immunohistochemical staining patterns in nonsclerotic segments

of glomeruli were scored as absent, focal, or diffuse. Positivity in glomeruli typically followed a global staining pattern along the glomerular capillary walls. For immunohistochemical staining patterns of arterioles, positive staining was defined as circumferential staining along the vessel lumina. Positivity in the proportion of the total number of arterioles in the tissue specimen resulted in a scoring of absent, focal or diffuse. Finally, an overall staining score comprising the scorings of peritubular capillaries, glomeruli and arterioles was given.

On the basis of absence or presence of the various complement components, the cases were categorized into five groups of complement activation. "Complement-negative" was defined as the absence of C1q, MBL, C4d, and C5b-9 deposits; "classical pathway" was defined as the presence of C1q deposits and the absence of MBL deposits or the presence of both IgM and C4d deposits and the absence of MBL deposits; "lectin pathway" was defined as the presence of MBL deposits and the absence of C1q deposits; "both classical and lectin pathways" was defined as the presence of both C1q and MBL deposits; and "unknown complement pathway" was defined as the presence of C4d and/or C5b-9 deposits in the absence of C1q, IgM and MBL deposits.

Two cases had insufficient tissue samples remaining for IgM and C1q staining. The first case had ANCA-associated vasculitis, and the renal biopsy was negative for C4d, MBL, and C5b-9 deposits in every scored location (i.e., the glomeruli, peritubular capillaries, and arterioles); this case was therefore classified as 'complement-negative'. The second case with insufficient tissue sample remaining had aHUS, and the native renal biopsy specimen contained C5b-9 deposits in the arterioles. Overall, this case was classified as "unknown complement pathway"; when subcategorizing for each location, this case was regarded as "complement-negative" in the glomeruli and peritubular capillaries and as "unknown complement pathway" in the arterioles.

**Statistical analysis**

Categorical variables were compared using the chi-squared test (or Fisher's exact test if the sample size was small). SPSS software, version 20.0 (IBM, Armonk, NY) was used to perform all analyses. Differences with a p value $<0.05$ were considered statistically significant.

### Ethics

All tissue samples were coded and then handled and analyzed anonymously in accordance with the Dutch National Ethics Guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies). This national code enables researchers to use human material that became available within the framework of patient care; this human material can be used for research purposes if properly coded and handled anonymously.

## RESULTS

### Patients and histopathology

Our search strategy resulted in 145 renal samples with a diagnosis of TMA (110 biopsies, 18 autopsies, 17 nephrectomies). We excluded 103 cases mainly because of missing and/or inadequate clinical data or tissue samples. We eventually included 42 renal tissue samples with histologically confirmed TMA that were obtained from 36 patients. Thirty samples were obtained from 28 patients with native kidney disease and 12 samples were obtained from 8 kidney transplant recipients. Five patients had follow-up biopsies. Table 1 shows patient characteristics. At the time of renal sampling, 10 patients had neurologic symptoms and 4 had a history of malignant hypertension. All 36 patients had renal dysfunction. Donor-specific antibodies were detected in all 3 kidney transplant recipients with TMA due to rejection. Microthrombi were localized in the glomeruli of 36 TMA samples (85.7%) and in the arterioles of 22 samples (52.4%). Additional histopathologic lesions are summarized in Table 2; typical examples are shown in Figure 1.

### Prevalence and localization of C4d and C5b-9 deposits

C4d deposits were present in 37 of the 42 TMA samples (88.1%) (Table 3). C4d deposits were localized in the glomeruli (76.2% of the samples), peritubular capillaries (9.5%), and arterioles (59.5%) (Supplemental Table 1). C4d staining in glomeruli was focal in 50.0% of the samples and diffuse in 26.2%. C4d staining in arterioles was focal in 33.3% of the samples and diffuse in 26.2%. C4d deposits were present in the same renal vascular structure as the microthrombi in 78.6% of the TMA samples. C5b-9 deposits were present in 78.6% of the samples (Table 3) and were colocalized with C4d deposits in 59.5%. In glomeruli, C5b-9 deposits were mainly focally distributed (Supplemental Table 2). In peritubular capillaries,

**Table 1. Patient characteristics**

| Characteristic | Patients with TMA in kidney allograft (n=8) | | | Patients with TMA in diseased native kidney (n=28) | | | | | | All TMA (n=36) |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Recurrent aHUS (n=3)* | *Drug toxicity (n=2)* | *Rejection (n=3)* | *aHUS (n=11)* | *STEC-HUS (n=1)* | *SLE/APS (n=8)* | *HSCT-TMA (n=6)* | *IgAN (n=1)* | *AAV (n=1)* | |
| Age at time of first renal sampling | 34.8 (6.4-37.2) | 43.4 (40.0-46.9) | 66.4 (31.6-72.0) | 46.0 (22.4-77.2) | 13.5 (NA) | 29.5 (16.6-49.4) | 37.7 (17.5-54.2) | 32.1 (NA) | 35.3 (NA) | 37.7 (6.4-77.2) |
| Women | 1 (33.3) | 2 (100.0) | 2 (66.7) | 9 (81.8) | 0 (0.0) | 8 (100.0) | 3 (50.0) | 0 (0.0) | 0 (0.0) | 25 (69.4) |
| Neurologic symptoms | 0 (0.0) | 0 (0.0) | 0 (0.0) | 3 (27.3) | 0 (0.0) | 3 (37.5) | 3 (50.0) | 0 (0.0) | 1 (100.0) | 10 (27.8) |
| History of malignant hypertension | 1 (33.3) | 0 (0.0) | 0 (0.0) | 1 (9.1) | 0 (0.0) | 0 (0.0) | 1 (16.7) | 0 (0.0) | 1 (100.0) | 4 (9.5) |
| Renal tissue samples | 6 (100.0) | 2 (100.0) | 4 (100.0) | 11 (100.0) | 1 (100.0) | 10 (100.0) | 6 (100.0) | 1 (100.0) | 1 (100.0) | 42 (100.0) |
| Biopsy | 5 (83.3) | 2 (100.0) | 4 (100.0) | 6 (54.5) | 1 (100.0) | 8 (80.0) | 2 (33.3) | 1 (100.0) | 1 (100.0) | 30 (71.4) |
| Autopsy | 0 (0.0) | 0 (0.0) | 0 (0.0) | 5 (45.5) | 0 (0.0) | 2 (20.0) | 4 (66.7) | 0 (0.0) | 0 (0.0) | 11 (26.2) |
| Nephrectomy | 1 (16.7) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (2.4) |

Age is expressed in years as median (range); all other values are expressed as number (%). TMA, thrombotic microangiopathy; HUS, hemolytic uremic syndrome; aHUS, atypical HUS; STEC-HUS, Shiga toxin-producing *Escherichia coli*-associated HUS; Drug toxicity refers to cases with TMA in the renal allograft caused by toxicity of immunosuppressive therapeutics; Rejection refers to cases with TMA in the renal allograft during a rejection episode; HSCT-TMA, TMA associated with hematopoietic stem cell transplantation; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome; IgAN, immunoglobulin A nephropathy; AAV, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis.

C5b-9 deposits were not observed in any sample. In arterioles, C5b-9 deposits were present in 69.0% of the samples. C5b-9 deposits were present in the same renal vascular structure as the microthrombi in 61.9% of the TMA samples.

### Complement pathways

Classical pathway activation was observed either solely (61.9%) or in combination with the lectin pathway (28.6%), in total representing 90.5% of TMA cases (Table 4). The complement pathways are summarized per renal vascular structure in Supplemental Table 3. In glomeruli, classical pathway activation was observed in 90.5% of cases. In peritubular capillaries, complement deposits were predominantly absent (76.2%). In arterioles, classical pathway activation was observed in 69.0% of the samples. IgM deposits were present in 77.5% of samples. The presence of glomerular IgM deposits was significantly associated with the presence of glomerular C4d deposits ($p<0.05$). Figure 2 summarizes colocalization patterns of C4d, C5b-9, C1q, and MBL deposits in glomeruli, peritubular capillaries and arterioles.

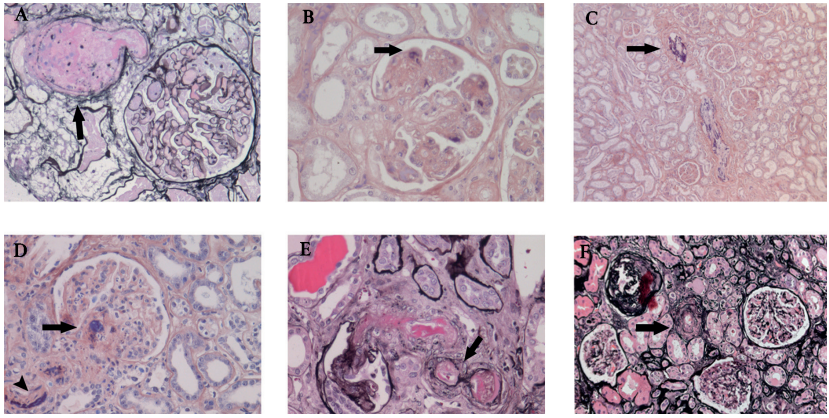


**Figure 1. Typical examples of renal histopathology in patients with TMA.** (A) Microaneurysm (arrow), silver stain, (original magnification, x100). (B) Fibrin microthrombi in glomerular capillaries (arrow), phosphotungstic acid-hematoxylin (PTAH) stain, (original magnification, x100). (C) Fibrin microthrombi in an artery (arrow), PTAH stain, (original magnification, x40). (D) Fibrin microthrombi in an arteriole (arrowhead) and the vascular pole of the glomerulus (arrow), PTAH stain, (original magnification, x100). (E) Organizing microthrombi with recanalization (arrow), silver-stained sample, (original magnification, x100). (F) Arteriolar onion ring (arrow), silver stain, (original magnification, x60).

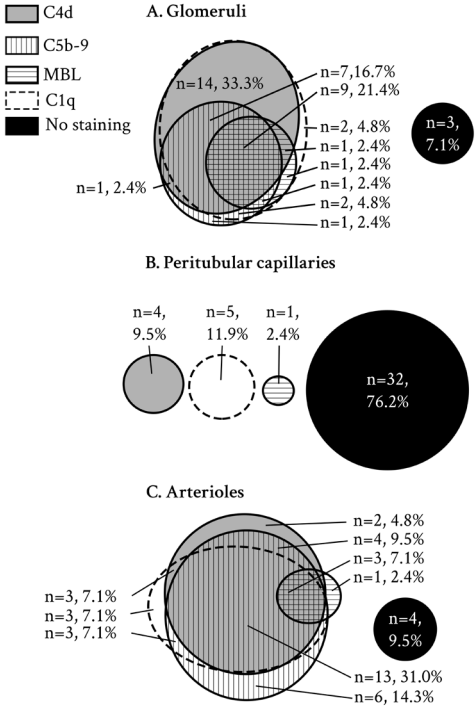


**Figure 2. Evidence for classical pathway activation is frequently observed in glomeruli and arterioles.** Values represent the number of renal sections stained as indicated. Deposition frequency is shown for the (A) glomeruli, (B) peritubular capillaries, and (C) arterioles. In both glomeruli and arterioles, C4d deposits are frequently colocalized with C1q deposits, reflecting activation of the classical pathway. If present, MBL deposits are frequently colocalized with C4d and/or C1q deposits, reflecting that the lectin pathway was occasionally activated, but often together with the classical pathway.

**Table 2. Histopathologic features of kidney sections from patients with TMA**

| Histopathological parameter | All TMA (n=42) |
|---|---|
| Microthrombi | 42 (100.0) |
| Glomerular microthrombi | 36 (85.7) |
| Arteriolar microthrombi | 22 (52.4) |
| Glomerular fibrin deposits | 13 (31.0) |
| Glomerular basement membrane thickening | 6 (14.3) |
| Glomerular basement membrane duplication | 8 (19.0) |
| Arteriolar swelling | 17 (40.5) |
| Endothelial changes | |
| No proliferation, no foamy changes | 12 (28.6) |
| Proliferation, no foamy changes | 8 (19.0) |
| Foamy changes, no proliferation | 5 (11.9) |
| Foamy changes and fibrosis | 17 (40.5) |
| Microaneurysms | 18 (42.9) |
| Fibrinoid necrosis | 2 (4.8) |

Values are expressed as number (%).

## Native kidneys

Thirty of 42 TMA samples were derived from diseased native kidneys. C4d deposits were present in 25 of these samples (83.3%) (Table 3). Specifically, C4d deposits were present in the glomeruli, peritubular capillaries and arterioles of 23 (76.7%), 1 (3.3%), and 15 (50.0%) of these samples, respectively (Supplemental Table 1). C5b-9 deposits were present in 76.7% of the native kidneys.

*SLE and antiphospholipid syndrome*

TMA cases associated with SLE with or without antiphospholipid syndrome were characterized by glomerular C4d staining (90.0%), which was diffuse in 50% of the samples. C4d deposits were less prevalent in arterioles (50.0%). C5b-9 deposits were less prevalent than C4d deposits (80.0%) and were localized in glomeruli (60.0%) and in arterioles (60.0%). Figure 3, A-D, shows typical examples of these stains.

*aHUS and STEC-HUS*

Cases with aHUS had C4d deposits in glomeruli (54.5%) and arterioles (54.5%). If present, arteriolar C4d stained predominantly in a diffuse pattern. C5b-9 deposits were observed in most aHUS samples (81.8%) and were predominantly localized in arterioles. Figure 3, E-H, shows typical examples of these stains. One patient had STEC-HUS: classical pathway activation was observed, C4d deposits were focally present in glomeruli and C5b-9 deposits were absent.

*TMA associated with hematopoietic stem cell transplantation*

The complement staining pattern in TMA associated with hematopoietic stem cell transplantation (HSCT-TMA) resembled the staining pattern of aHUS cases. C4d deposits were present in all six samples and were predominantly localized in the glomeruli (100.0%) and in the arterioles (66.7%). C5b-9 deposits were observed in all samples. C5b-9 deposits were localized in the arterioles of all samples and stained predominantly in a diffuse pattern. Furthermore, classical pathway activation was observed in all cases. Four patients had a history of graft-versus-host disease; three of these patients had both C4d and C5b-9 deposits in the arterioles. Figure 3, I-P, shows typical examples of these stains.

**Table 3. Immunohistochemistry results of TMA kidney sections from patients with various underlying clinical conditions**

| Immuno-histochemical stain | Kidney allografts (n=12) | | | Native kidneys (n=30) | | | | | | All TMA (n=42) |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Recurrent aHUS (n=6)* | *Drug toxicity (n=2)* | *Rejection (n=4)* | *aHUS (n=11)* | *STEC-HUS (n=1)* | *SLE/APS (n=10)* | *HSCT-TMA (n=6)* | *IgAN (n=1)* | *AAV (n=1)* | |
| C4d | 6 (100.0) | 2 (100.0) | 4 (100.0) | 8 (72.7) | 1 (100.0) | 9 (90.0) | 6 (100.0) | 1 (100.0) | 0 (0.0) | 37 (88.1) |
| C5b-9 | 5 (83.3) | 2 (100.0) | 3 (75.0) | 9 (81.8) | 0 (0.0) | 8 (80.0) | 6 (100.0) | 0 (0.0) | 0 (0.0) | 33 (78.6) |
| MBL | 5 (83.3) | 0 (0.0) | 1 (25.0) | 3 (27.3) | 0 (0.0) | 2 (20.0) | 1 (16.7) | 0 (0.0) | 0 (0.0) | 12 (28.6) |
| C1q | 6 (100.0) | 2 (100.0) | 4 (100.0) | 9/10 (90.0)[a] | 1 (100.0) | 8 (80.0) | 6 (100.0) | 1 (100.0) | NA[a] | 37/40 (92.5)[a] |
| IgM | 6 (100.0) | 2 (100.0) | 2 (50.0) | 6/10 (60.0)[a] | 1 (100.0) | 7 (70.0) | 6 (100.0) | 1 (100.0) | NA[a] | 31/40 (77.5)[a] |

Overall staining in biopsy specimens, defined as the presence of staining along the glomeruli, peritubular capillaries, and/or arterioles. Values are expressed as number (%). TMA, thrombotic microangiopathy; HUS, hemolytic uremic syndrome; aHUS, atypical HUS; STEC-HUS, Shiga toxin-producing *Escherichia coli*-associated HUS; Drug toxicity refers to cases with TMA in the renal allograft caused by toxicity of immunosuppressive therapeutics; Rejection refers to cases with TMA in the renal allograft during a rejection episode; HSCT-TMA, TMA associated with hematopoietic stem cell transplantation; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome; IgAN, immunoglobulin A nephropathy; AAV, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; NA, not applicable.[a]Two cases had insufficient tissue remaining for IgM and C1q staining. The first case had AAV, and the second case had aHUS.

*IgA nephropathy and ANCA-associated vasculitis*

One patient had TMA associated with IgA nephropathy. C4d deposits were only present in glomeruli and stained in a diffuse pattern; C1q deposits were present in the glomeruli and arterioles, whereas MBL and C5b-9 deposits were absent. Another patient had TMA associated with granulomatosis with polyangiitis. Complement deposits were not observed in this patient.

**Transplanted kidneys**

Twelve of the 42 renal TMA samples were obtained from transplanted kidneys with TMA (Table 3). C4d deposits were present in all 12 transplanted kidney samples; specifically, C4d deposits were present in the glomeruli, peritubular capillaries, and arterioles of 8 (66.7%), 3 (25.0%), and 10 (83.3%) of these samples, respectively. C5b-9 deposits were present in 83.3% of the transplanted kidneys.

*Recurrence of aHUS*

We found that aHUS recurred in six renal allograft samples of three patients. Complement deposition patterns resembled that of the native aHUS cases. Both C4d and C5b-9 deposits were predominantly present in glomeruli (83.3%) and arterioles (83.3%).

*Drug toxicity and rejection*

TMA was attributed to drug toxicity of cyclosporine and tacrolimus in two cases. In three cases (four biopsy samples) TMA was attributed to a rejection episode. Clinical overlap was observed in both groups. Two cases with rejection received concomitant high dose-immunosuppressive therapy, but changing the therapeutic regimen did not improve renal function. Similarly, one case attributed to drug toxicity had diffuse staining of C4d in peritubular capillaries, suggesting a humoral rejection episode. Both groups were characterized by C4d deposits in all scored renal vascular structures. C5b-9 deposits were present in all samples of drug toxicity cases and in three of four cases with rejection. Examples of these stains are shown in Figure 3, Q-T.

### Controls

C4d deposits were significantly more prevalent in cases than in controls ($p<0.001$); this difference remained significant when the deposits were subcategorized for glomeruli ($p<0.001$), peritubular capillaries ($p<0.05$) and arterioles ($p<0.001$). All samples obtained from healthy controls, Alport syndrome controls and kidney transplant controls were C4d negative, but C4d deposits were present in three control biopsy samples with focal segmental glomerulosclerosis (FSGS) (5.6% of all control samples). C5b-9 deposits were also significantly more prevalent in cases than in controls ($p<0.001$), and this difference remained significant when the deposits were subcategorized for glomeruli ($p<0.001$) and arterioles ($p<0.05$). C5b-9 deposits were not observed in the peritubular capillaries of any sample. C5b-9 deposits were present in 21 control samples (39.6%), including 7 healthy control samples, 5 FSGS control samples, and 9 transplant control samples; all 5 Alport syndrome controls were negative for C5b-9 deposits. In the control samples, C5b-9 deposits were almost exclusively localized in arterioles; only 1 sample had glomerular C5b-9 deposits, which were focal.

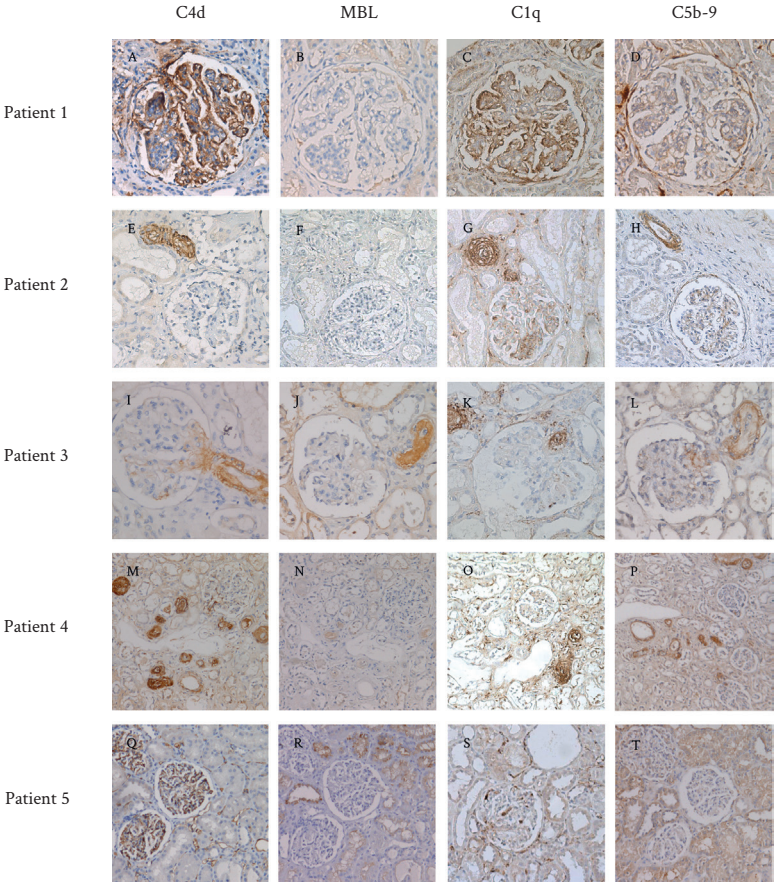


**Figure 3. Typical examples of complement deposits in TMA patients with different underlying clinical conditions.** The sections were stained for C4d (A, E, I, M, Q), MBL (B, F, J, N, R), C1q (C, G, K, O, S) or C5b-9 (D, H, L, P, T). Patient 1 developed TMA after systemic lupus erythematosus and had C4d (A) and C1q (C) deposits in the glomeruli without MBL deposition (B) or colocalization with C5b-9 deposits (D). Original magnification, x100 in A-D. Patient 2 had aHUS and had C4d (E) and C1q (G) deposits in the arterioles without MBL deposits (F) but with colocalization of C5b-9 deposits (H). Original magnification, x60 in E-H. Patients 3 and 4 developed TMA following hematopoietic stem cell transplantation. Patient 3 had C4d deposits in an arteriole at the vascular pool of a glomerulus (I) with colocalization of MBL (J), C1q (K), and C5b-9 (L). Patient 4 had C4d (M) and C1q (O) deposits in the arterioles without MBL deposits (N) but with colocalization of C5b-9 deposits (P). Original magnification, x100 in I-L; x40 in M-P. Patient 5 developed TMA following kidney transplantation and had C4d (Q) and C1q (S) deposits in the peritubular capillaries without colocalization of MBL (R) or C5b-9 deposits (T). Original magnification, x60 in Q-T.

**Table 4. Complement pathways in TMA kidney sections from patients with various underlying clinical conditions**

| Complement pathway | Kidney allografts (n=12) | | | Native kidneys (n=30) | | | | | | All TMA (n=42) |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Recurrent aHUS (n=6)* | *Drug toxicity (n=2)* | *Rejection (n=4)* | *aHUS (n=11)* | *STEC-HUS (n=1)* | *SLE/APS (n=10)* | *HSCT-TMA (n=6)* | *IgAN (n=1)* | *AAV (n=1)* | |
| Complement negative | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (0.0) | 1 (2.4) |
| Classical | 1 (16.7) | 2 (100.0) | 3 (75.0) | 6 (54.5) | 1 (100.0) | 7 (70.0) | 5 (83.3) | 1 (100.0) | 0 (0.0) | 26 (61.9) |
| Both classical and lectin | 5 (83.3) | 0 (0.0) | 1 (25.0) | 3 (27.3) | 0 (0.0) | 2 (20.0) | 1 (16.7) | 0 (0.0) | 0 (0.0) | 12 (28.6) |
| Unknown | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (18.2) | 0 (0.0) | 1 (10.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 3 (7.1) |

Values are expressed as number (%). TMA, thrombotic microangiopathy; HUS, hemolytic uremic syndrome; aHUS, atypical HUS; STEC-HUS, Shiga toxin-producing *Escherichia coli*-associated HUS; Drug toxicity refers to cases with TMA in the renal allograft caused by toxicity of immunosuppressive therapeutics; Rejection refers to cases with TMA in the renal allograft during a rejection episode; HSCT-TMA, TMA associated with hematopoietic stem cell transplantation; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome; IgAN, immunoglobulin A nephropathy; AAV, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis.

## DISCUSSION

Our study demonstrates that complement activation in the kidney is a common denominator of TMA in a heterogeneous group of patients. Our findings provide insight into the etiology of TMA and may have therapeutic consequences. The relatively widespread presence of C4d deposits indicates that complement is activated—and may be causally involved—in most histologically confirmed cases of TMA. Moreover, our observation that terminal complement complex C5b-9 deposits were present and were colocalized with C4d deposits suggests that at least 75% of all TMA cases—both with and without confirmed mutations in complement regulatory genes—might benefit from complement-inhibiting therapeutics. This is consistent with results from recent clinical trials and case reports describing the effects of eculizumab, a terminal complement inhibitor.[12-18]

Different C4d staining patterns were observed. Cases with SLE and IgA nephropathy were characterized by diffuse glomerular C4d staining. Kidney transplant cases attributed to drug toxicity or a rejection episode had varied staining patterns. Native cases with aHUS and transplant cases in which aHUS recurred were characterized by arteriolar C4d staining. Interestingly, cases with HSCT-TMA also showed arteriolar C4d staining. Arteriolar C4d staining has been described previously in native kidneys, transplanted kidneys and other transplanted organs.[19-22] The etiology of arteriolar C4d deposition is unknown. In light of our data, arteriolar C4d deposition in biopsy samples of patients with TMA may reflect a deficit in complement regulation (such as in aHUS), whereas C4d staining in glomeruli and peritubular capillaries occurs mainly in association with antibody-mediated complement activation (such as in SLE or antiphospholipid syndrome).

C4d is a widely used biomarker for complement activation that remains covalently bound, long after the complement pathway-initiating factors have dissociated.[23] To distinguish between the lectin and classical pathways, we stained samples for MBL and C1q. Lectin pathway activation was observed in patients with aHUS, either in native kidney biopsy specimens or renal transplant biopsy specimens in which aHUS recurred. The origin of a microthrombotic lesion as a result of complement activation in the lectin pathway is unknown.

The classical pathway was activated in most patients. A large subgroup had underlying clinical conditions in which antibodies could have mediated the

renal endothelial injury. Classical pathway activation was observed in 90.0% of patients with SLE with or without antiphospholipid syndrome, either solely (70.0% of cases) or in combination with the lectin pathway (20.0%). These findings are consistent with previous data reporting an association between glomerular C4d staining and microthrombi in SLE and antiphospholipid syndrome.[9, 24] In these patients, accumulation of antibodies in the glomeruli probably leads to classical pathway activation, endothelial injury, and the subsequent formation of microthrombi. In addition, all samples obtained from six patients who developed TMA after hematopoietic stem cell transplantation contained C4d deposits, predominantly in glomeruli but also in arterioles. Four patients had graft-versus-host disease, and three of these patients had both C4d and C5b-9 deposits in arterioles. C4d represented the classical pathway in all cases, suggesting that the classical complement pathway led to activation of the terminal complement complex in these patients with graft-versus-host disease. Similar findings were reported by others, suggesting that an antibody-mediated component of graft-versus-host disease may cause TMA as a result of severe endothelial damage, possibly in combination with drug toxicity.[22, 25]

Classical pathway activation was also observed in cases without apparent antibody-mediated injury such as cases with drug toxicity-associated TMA, native aHUS, recurrent aHUS and STEC-HUS. Here, the presence of complement deposits along the renal vasculature may reflect a consequence of renal damage, rather than—or perhaps in addition to—reflecting an underlying cause of the damage. Two hypotheses may explain the presence of C4d as a consequence of damage. First, chronic endothelial cell injury may cause the endothelial cells to produce components of the glomerular basement membrane. This can result in the formation of a duplicate glomerular basement membrane, entrapment of aspecific IgM and C3 and thus mimicry of immune complex deposition.[26] In our cohort, the presence of glomerular C4d and IgM were associated with each other. Furthermore, all eight TMA cases with a duplicated glomerular basement membrane had classical pathway activation, and four had both glomerular IgM and C4d deposits. Second, complement activation may be induced by intravascular cellular debris and hypoxic or injured endothelium. Experimental studies in humans, C3 and C4 knock-out mice, and mice treated with C5a receptor antagonists show that complement activation is involved in ischemia-reperfusion injury in the context of transplantation or stroke.[27-30] In TMA, ischemia-reperfusion injury could lead

to complement activation, possibly amplifying complement-mediated injury.

Although C4d deposits were present in 88.1% of the TMA samples, C4d deposits were colocalized with C5b-9 deposits in only 59.5%. It would be interesting to investigate whether terminal complement inhibitors would have therapeutic benefits in particular in patients showing a combination of C4d and C5b-9 deposits. For future studies evaluating the effect of complement-inhibiting therapeutics in various clinical settings of TMA, we would like to make the following recommendations: the main benefit of C4d as a biomarker lies in identifying cases in which tissue samples may not reveal the TMA lesion due to sampling error, as our previous study showed.[9] C5b-9 might seem the most interesting biomarker to predict an effect of its direct agent in the form of a terminal complement inhibitor, but our data show that C5b-9 deposits are present in a considerable percentage of controls, in particular in arterioles, and are sometimes co-localized with hyalinosis (data not shown). Therefore, the combination of C4d and C5b-9 may eventually be the most useful to evaluate a likely benefit of complement-inhibiting therapeutics for patients with TMA.

Our study has several limitations. Because of technical limitations, we were unable to use the paraffin-embedded tissue samples to retrospectively test cases for the most prevalent mutations in genes that encode complement regulatory proteins. Future studies should investigate whether distinct complement-staining patterns in the kidney reflect specific genotypes in the background of TMA, as well as possible differences in the expression of complement regulatory proteins in the kidneys of patients with TMA. Furthermore, our sample size is relatively small and selection bias may have occurred because patients with straightforward clinical presentation or contraindications such as severe bleeding risk often do not undergo biopsy. Because TMA is a rare complication of many diseases, a large multi-center and prospective study would be necessary to overcome these issues.

In conclusion, our study shows that C4d is a common denominator in TMA, regardless of the underlying clinical condition. That C5b-9 is present in >75% of renal biopsy specimens from patients with TMA, suggests that terminal complement inhibitors may have a beneficial effect in these patients. Finally, C4d and C5b-9 should be investigated as possible diagnostic biomarkers in the clinical work-up of patients suspected of having complement-mediated TMA.

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**Supplemental Table S1. C4d deposits in TMA kidney sections from patients with various underlying clinical conditions**

| Immuno-histochemical stain | Kidney allografts (n=12) | | | Native kidneys (n=30) | | | | | | All TMA (n=42) |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Recurrent aHUS (n=6)* | *Drug toxicity (n=2)* | *Rejection (n=4)* | *aHUS (n=11)* | *STEC-HUS (n=1)* | *SLE/APS (n=10)* | *HSCT-TMA (n=6)* | *IgAN (n=1)* | *AAV (n=1)* | |
| **C4d in glomeruli** | | | | | | | | | | |
| Absent | 1 (16.7) | 0 (0.0) | 2 (50.0) | 5 (45.5) | 0 (0.0) | 1 (10.0) | 0 (0.0) | 0 (0.0) | 1 (100.0) | 10 (23.8) |
| Focal | 5 (83.3) | 1 (50.0) | 2 (50.0) | 4 (36.4) | 1 (100.0) | 4 (40.0) | 4 (66.7) | 0 (0.0) | 0 (0.0) | 21 (50.0) |
| Diffuse | 0 (0.0) | 1 (50.0) | 0 (0.0) | 2 (18.1) | 0 (0.0) | 5 (50.0) | 2 (33.3) | 1 (100.0) | 0 (0.0) | 11 (26.2) |
| **C4d in ptc** | | | | | | | | | | |
| Absent | 5 (83.3) | 1 (50.0) | 3 (75.0) | 10 (90.9) | 1 (100.0) | 10 (100.0) | 6 (100.0) | 1 (100.0) | 1 (100.0) | 38 (90.5) |
| Focal | 1 (16.7) | 0 (0.0) | 1 (25.0) | 1 (9.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 3 (7.1) |
| Diffuse | 0 (0.0) | 1 (50.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (2.4) |
| **C4d in arterioles** | | | | | | | | | | |
| Absent | 1 (16.7) | 1 (50.0) | 0 (0.0) | 5 (45.5) | 1 (100.0) | 5 (50.0) | 2 (33.3) | 1 (100.0) | 1 (100.0) | 17 (40.5) |
| Focal | 4 (66.6) | 1 (50.0) | 3 (75.0) | 2 (18.1) | 0 (0.0) | 3 (30.0) | 1 (16.7) | 0 (0.0) | 0 (0.0) | 14 (33.3) |
| Diffuse | 1 (16.7) | 0 (0.0) | 1 (25.0) | 4 (36.4) | 0 (0.0) | 2 (20.0) | 3 (50.0) | 0 (0.0) | 0 (0.0) | 11 (26.2) |

Values are expressed as number (%).TMA, thrombotic microangiopathy; HUS, hemolytic uremic syndrome; aHUS, atypical HUS; STEC-HUS, Shiga toxin-producing *Escherichia coli*-associated HUS; Drug toxicity refers to cases with TMA in the renal allograft caused by toxicity of immunosuppressive therapeutics; Rejection refers to cases with TMA in the renal allograft during a rejection episode; HSCT-TMA, TMA associated with hematopoietic stem cell transplantation; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome; IgAN, immunoglobulin A nephropathy; AAV, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; ptc, peritubular capillaries.

**Supplemental Table S2. C5b-9 deposits in TMA kidney sections from patients with various underlying clinical conditions**

| Immuno-histochemical stain | Kidney allografts (n=12) | | | Native kidneys (n=30) | | | | | | All TMA (n=42) |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Recurrent aHUS (n=6)* | *Drug toxicity (n=2)* | *Rejection (n=4)* | *aHUS (n=11)* | *STEC-HUS (n=1)* | *SLE/APS (n=10)* | *HSCT-TMA (n=6)* | *IgAN (n=1)* | *AAV (n=1)* | |
| **C5b-9 in glomeruli** | | | | | | | | | | |
| Absent | 1 (16.7) | 2 (100.0) | 3 (75.0) | 5 (45.5) | 1 (100.0) | 4 (40.0) | 3 (50.0) | 1 (100.0) | 1 (100.0) | 21 (50.0) |
| Focal | 5 (83.3) | 0 (0.0) | 1 (25.0) | 6 (54.5) | 0 (0.0) | 4 (40.0) | 3 (50.0) | 0 (0.0) | 0 (0.0) | 19 (45.2) |
| Diffuse | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (20.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (4.8) |
| **C5b-9 in ptc** | | | | | | | | | | |
| Absent | 6 (100.0) | 2 (100.0) | 4 (100.0) | 11 (100.0) | 1 (100.0) | 10 (100.0) | 6 (100.0) | 1 (100.0) | 1 (100.0) | 42 (100.0) |
| Focal | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Diffuse | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| **C5b-9 in arterioles** | | | | | | | | | | |
| Absent | 1 (16.7) | 0 (0.0) | 1 (25.0) | 4 (36.4) | 1 (100.0) | 4 (40.0) | 0 (0.0) | 1 (100.0) | 1 (100.0) | 13 (31.0) |
| Focal | 3 (50.0) | 2 (100.0) | 2 (50.0) | 6 (54.5) | 0 (0.0) | 5 (50.0) | 2 (33.3) | 0 (0.0) | 0 (0.0) | 20 (47.6) |
| Diffuse | 2 (33.3) | 0 (0.0) | 1 (25.0) | 1 (9.1) | 0 (0.0) | 1 (10.0) | 4 (66.7) | 0 (0.0) | 0 (0.0) | 9 (21.4) |

Values are expressed as number (%).TMA, thrombotic microangiopathy; HUS, hemolytic uremic syndrome; aHUS, atypical HUS; STEC-HUS, Shiga toxin-producing *Escherichia coli*-associated HUS; Drug toxicity refers to cases with TMA in the renal allograft caused by toxicity of immunosuppressive therapeutics; Rejection refers to cases with TMA in the renal allograft during a rejection episode; HSCT-TMA, TMA associated with hematopoietic stem cell transplantation; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome; IgAN, immunoglobulin A nephropathy; AAV, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; ptc, peritubular capillaries.

**Supplemental Table S3. Complement pathways in different renal vascular structures of patients with TMA**

| Complement pathway | Kidney allografts (n=12) | | | Native kidneys (n=30) | | | | | | All TMA (n=42) |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Recurrent aHUS (n=6)* | *Drug toxicity (n=2)* | *Rejection (n=4)* | *aHUS (n=11)* | *STEC-HUS (n=1)* | *SLE/APS (n=10)* | *HSCT-TMA (n=6)* | *IgAN (n=1)* | *AAV (n=1)* | |
| **Glomeruli** | | | | | | | | | | |
| No complement | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (18.2) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (100.0) | 3 (7.1) |
| Classical only | 1 (16.7) | 2 (100.0) | 3 (75.0) | 6 (54.5) | 1 (100.0) | 7 (70.0) | 5 (83.3) | 1 (100.0) | 0 (0.0) | 26 (61.9) |
| Classical & Lectin | 5 (83.3) | 0 (0.0) | 1 (25.0) | 3 (27.3) | 0 (0.0) | 2 (20.0) | 1 (16.7) | 0 (0.0) | 0 (0.0) | 12 (28.6) |
| Unknown | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (10.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (2.4) |
| **Ptc** | | | | | | | | | | |
| No complement | 3 (50.0) | 1 (50.0) | 2 (50.0) | 9 (81.8) | 1 (100.0) | 9 (90.0) | 5 (83.3) | 1 (100.0) | 1 (100.0) | 32 (76.2) |
| Classical only | 2 (33.3) | 0 (0.0) | 1 (25.0) | 1 (9.1) | 0 (0.0) | 1 (10.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 5 (11.9) |
| Lectin only | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (16.7) | 0 (0.0) | 0 (0.0) | 1 (2.4) |
| Unknown | 1 (16.7) | 1 (50.0) | 1 (25.0) | 1 (9.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 4 (9.5) |
| **Arterioles** | | | | | | | | | | |
| No complement | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 3 (30.0) | 0 (0.0) | 0 (0.0) | 1 (100.0) | 4 (9.5) |
| Classical only | 3 (50.0) | 1 (50.0) | 3 (75.0) | 7 (63.6) | 1 (100.0) | 6 (60.0) | 4 (66.7) | 1 (100.0) | 0 (0.0) | 26 (61.9) |
| Lectin only | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (9.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (2.4) |
| Classical & Lectin | 2 (33.3) | 0 (0.0) | 1 (25.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 3 (7.1) |
| Unknown | 1 (16.7) | 1 (50.0) | 0 (0.0) | 3 (27.3) | 0 (0.0) | 1 (10.0) | 2 (33.3) | 0 (0.0) | 0 (0.0) | 8 (19.0) |

Values are expressed as number (%).TMA, thrombotic microangiopathy; HUS, hemolytic uremic syndrome; aHUS, atypical HUS; STEC-HUS, Shiga toxin-producing *Escherichia coli*-associated HUS; Drug toxicity refers to cases with TMA in the renal allograft caused by toxicity of immunosuppressive therapeutics; Rejection refers to cases with TMA in the renal allograft during a rejection episode; HSCT-TMA, TMA associated with hematopoietic stem cell transplantation; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome; IgAN, immunoglobulin A nephropathy; AAV, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; ptc, peritubular capillaries.

# Chapter 3

## Complement-mediated microangiopathy in IgA nephropathy and IgA vasculitis with nephritis

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# ABSTRACT

Complement factor C4d was recently observed in renal biopsies from patients who had IgA nephropathy and a poor prognosis. We previously reported that C4d is a common denominator in microangiopathies. In this retrospective cohort study, we investigated whether C4d is a marker of microangiopathy in both IgA nephropathy and IgA vasculitis with nephritis, and whether patients with C4d and microangiopathy have poor renal outcome. We examined 128 renal biopsies from adult and pediatric patients, including normotensive and hypertensive patients, who presented with IgA nephropathy or IgA vasculitis with nephritis. Biopsies were re-evaluated in accordance with the Oxford classification, scored for additional lesions, and stained for complement proteins using immunohistochemistry, including C4d and C5b-9. Clinical data were collected with a mean (±SD) follow-up period of 51 ± 39 months. Changes in estimated glomerular filtration rate over time were compared using linear mixed-effects models. Renal survival was analyzed using multivariable Cox regression. Microangiopathic lesions were present in 20% of all biopsies (23% and 9% of patients with IgA nephropathy and IgA vasculitis with nephritis, respectively). Microangiopathy was associated with C4d and C5b-9 deposits, a higher number of chronic lesions, and hypertension (all $p<0.05$). Patients with C4d and microangiopathic lesions had significantly poorer renal survival than patients without these findings, corrected for hypertension ($p<0.01$). In conclusion, patients with IgA nephropathy or IgA vasculitis with nephritis with a combination of C4d positivity and microangiopathy comprise a clinical subgroup with an increased number of chronic lesions, lower estimated glomerular filtration rate, and poorer renal survival, even when corrected for hypertension. These data suggest that complement activation is involved in the development of microangiopathy in patients with IgA nephropathy and IgA vasculitis with nephritis, and that complement-mediated microangiopathy contributes to disease progression.

# INTRODUCTION

Worldwide, immunoglobulin A (IgA) nephropathy is the most common primary glomerulonephritis.[1] The clinical features of IgA nephropathy include microscopic hematuria with or without macroscopic hematuria, varying amounts of proteinuria, and hypertension. The diagnostic hallmark is a predominance of IgA deposits in the glomerular mesangium on renal biopsy. Light microscopic features vary widely and can be evaluated by the Oxford classification for IgA nephropathy.[2,3] Similar to glomerular lesions, renal arterial and arteriolar lesions can vary widely, ranging from arteriolar wall thickening and hyaline changes to microangiopathy with or without thrombosis.[4-6]

Complement activation is considered to play an important role in the pathogenesis of IgA nephropathy, and various complement proteins have been correlated with disease progression in IgA nephropathy, including differences in the patterns of renal complement protein deposits and/or complement levels in the serum and urine.[7,8] Furthermore, some patients with IgA nephropathy have been reported to have genetic deficiencies in complement regulatory proteins.[7,9-14] Moreover, recent case reports suggest that some patients with IgA nephropathy may benefit from complement-inhibiting therapy.[15-18]

Two recent studies involving patients with IgA nephropathy reported that complement activation and microangiopathic lesions, respectively, are important in determining renal outcome.[5,19] Espinosa et al.[19] found that C4d-positive staining in glomeruli was present in 20% of biopsies obtained from patients with IgA nephropathy; in addition, they found that glomerular C4d staining was an independent risk factor for the development of end-stage renal disease. El Karoui et al.[5] found that more than half of renal biopsies obtained from patients with IgA nephropathy revealed renal microangiopathy with thrombosis (acute thrombotic microangiopathy) or renal microangiopathy without thrombosis (organized thrombotic microangiopathy without platelet thrombi). Although this prevalence of renal microangiopathy may have been an overestimation relative to the general population—patients were selected at an active hypertensive clinic—the findings are clinically relevant, as microangiopathy in biopsies with IgA nephropathy was associated with severe hypertension and poor renal outcome.[5] Combining the results from these two studies with our own recent finding that complement factor C4d is

a common denominator in patients with renal thrombotic microangiopathy,[20] we hypothesized that an intricate relationship may exist between complement activation and microangiopathic lesions in IgA nephropathy and IgA vasculitis with nephritis (also known as Henoch-Schönlein purpura nephritis), possibly indicating a subgroup of patients with relatively poor clinical outcome.

# METHODS

### Patients and clinical data

We retrospectively searched the archives in the Department of Pathology at Leiden University Medical Center for patients who underwent a renal biopsy from January 2003 through May 2013. The search terms included "IgA nephropathy" and "Henoch-Schönlein". We excluded biopsies from transplanted kidneys, inadequate or missing biopsies, and biopsies from patients with a concomitant kidney disease. Cases were reviewed by an experienced nephropathologist. A diagnosis of IgA nephropathy was based on the predominance of IgA deposits in the glomerular mesangium.[2] A diagnosis of IgA vasculitis with nephritis was based on the concurrent presence of palpable purpura.[21] The clinical and laboratory data were obtained retrospectively from the medical records and included patient demographics, blood pressure, the number and type of antihypertensive agents used, serum creatinine, proteinuria, clinical thrombotic microangiopathy, and requirement of renal replacement therapy. For adults, hypertension was defined as systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, or the need for antihypertensive medication to maintain blood pressure below these values.[22] For children and adolescents, hypertension was defined as blood pressure higher than the 95th percentile for the patient's gender, age, and height, which was based on the fourth report on the diagnosis, evaluation, and treatment of high blood pressure in children and adolescents.[23] Malignant hypertension was defined as hypertension with grade 3 or grade 4 hypertensive retinopathy. Clinical thrombotic microangiopathy was defined as the presence of microangiopathic hemolytic anemia (anemia, low haptoglobin levels, schistocytosis, and/or elevated lactate dehydrogenase levels), renal dysfunction, and thrombocytopenia. Mutations in complement regulatory genes were not tested in this historic cohort. Estimated glomerular filtration rate was determined in adults using the simplified Modification of Diet in Renal

Disease (MDRD) formula; estimated glomerular filtration rate was determined in children and adolescents using the bedside Schwartz formula.[24, 25] For each patient, the date of renal biopsy was used as the patient's baseline data point.

**Renal biopsy evaluation**

The renal tissue was fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Paraffin-embedded sections were stained with Masson's trichrome, hematoxylin and eosin, periodic acid-Schiff, and silver stain using standard protocols. The biopsies were re-evaluated in accordance with the Oxford classification for IgA nephropathy using the MEST-C criteria (Mesangial hypercellularity, Endocapillary hypercellularity, Segmental glomerulosclerosis, Tubular atrophy/interstitial fibrosis, and Crescents).[2, 26] Microangiopathic lesions were scored as microangiopathy with or without thrombosis.[5, 27] Active microangiopathy was defined as follows: the presence of fibrin, endothelial swelling or denudation, mesangiolysis, or microaneurysms in the glomeruli; thrombi, endothelial swelling or denudation, intramural fibrin or intimal swelling in the arterioles; thrombi or myxoid intimal swelling in the arteries. Chronic microangiopathy was defined as follows: the presence of fibrous intimal thickening with concentric lamination and/or recanalization in the arterioles or arteries; these lesions may or may not be accompanied by double contours in glomerular peripheral capillary walls. Arterial intimal sclerosis was scored semi-quantitatively on a scale from 0 to 4 (0, absent; 1, arterial intimal sclerosis without luminal occlusion; 2, arterial intimal sclerosis with 1-25% luminal occlusion; 3, arterial intimal sclerosis with 26–50% luminal occlusion; 4, arterial intimal sclerosis with >50% luminal occlusion). Arteriolar hyalinosis was scored semi-quantitatively on a scale from 0 to 2 (0, absent; 1 mild, non-occlusive hyalinosis; 2, severe, extensive, and/or occlusive hyalinosis).

**Immunostaining**

To measure human renal complement activation and co-localization, we performed immunostaining for various complement proteins on adjacent kidney sections as previously described.[20] In brief, 4-μm-thick paraffin-embedded sections were prepared, deparaffinized, and subjected to antigen retrieval. After blocking endogenous peroxidases, the sections were incubated in the relevant primary antibody for 1 h. Binding of the primary antibody was

visualized using the appropriate horseradish peroxidase–labeled secondary antibodies and diaminobenzidine as the chromogen. Finally, the sections were counterstained with hematoxylin. For immunohistochemistry, we used primary antibodies against the following proteins: C4d (BI-RC4d; Biomedica Gruppe, Vienna, Austria; 1:50), a cleavage product of C4 that binds covalently to the target tissue and can arise from the classical and lectin pathways; C1q (A0136; Dako, Glostrup, Denmark; 1:800), which reflects activation of the classical complement pathway; mannose-binding lectin (MBL) (HPA002027; Sigma-Aldrich, St. Louis, MO; 1:500) which reflects activation of the lectin pathway; and sC5b-9 (A239; Quidel, San Diego, CA; 1:150) which reflects the terminal complement pathway and can be formed after activation of any of the three complement pathways. Twelve biopsies lacked sufficient paraffin-embedded tissue for immunostaining; these cases were excluded from the subanalyses. For factor B and MBL, frozen sections were acetone-fixed, then incubated for 1 h with antibodies against factor B (a227; Quidel, San Diego, CA; 1:400) and MBL (mAb 3E7, Hycult biotech, Uden, the Netherlands; 1:200). Fifty-one biopsies had insufficient frozen tissue for factor B and MBL analyses; these cases were excluded from the subanalyses. For each staining protocol, the optimum antibody dilution and incubation time were determined empirically for each antibody by performing a titration experiment using positive control sections.

**Quantification of immunostaining**

For complement proteins, two independent observers who were blinded with respect to the subjects' clinical data scored immunostained renal sections. We scored both the glomeruli and arterioles in each section. In non-sclerotic segments of glomeruli, immunostaining was scored as absent (representing either an absence or trace levels of staining) or present in the mesangium, peripheral glomerular capillary walls, or both. If immunostaining was present, each biopsy was further classified as having either focal (≤50% of glomeruli) or diffuse (>50% of glomeruli) deposits, the staining in the majority of positive glomeruli was classified as either segmental or global glomerular staining. In the arterioles, immunostaining was scored as either absent or present, with present defined as circumferential staining along the vessel lumina; positivity only present along the elastic lamina was excluded. Finally, for each stain, an overall score was given to a biopsy sample, combining the scores obtained for

the glomeruli and arterioles; biopsy samples could be "negative" (no staining in glomeruli or arterioles) or "positive" (staining in glomeruli, arterioles, or both). In addition, IgA, IgG, and IgM staining scores were obtained from the pathology reports and were re-scored as absent (for a score of 0 or trace staining) or present (for a score of 1, 2, or 3+).

### Statistical analysis

Proportions were analyzed using the chi-squared test or Fisher's exact test, where appropriate. Continuous variables were analyzed using the unpaired Student's t-test. Changes in estimated glomerular filtration rate over time were compared using a linear mixed-effects model with random intercept and random slopes, to account for the repeated measurements of variables obtained from the same individual. For this analysis we tested interactions, including group-by-time interactions, and excluded non-significant interactions from the model. Renal survival was analyzed using Cox regression and the log-rank test. Renal survival is presented as a Kaplan-Meier curve without adjustment for baseline covariates. The predictive values of C4d and interstitial fibrosis/tubular atrophy in Cox regression models was assessed using Harrell's C.[28] All analyses were performed using the SPSS statistical software package (version 20.0; IBM Corp). Differences with $p<0.05$ were considered to be statistically significant.

### Ethics

The study was conducted in accordance with the Declaration of Helsinki, and all biopsies were coded and then handled and analyzed anonymously in accordance with the Dutch National Ethics Guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies).

# RESULTS

## Biopsy search

Our search strategy yielded 220 renal biopsies of which tissue was available. We excluded 70 transplant biopsies, 14 cases with inadequate biopsy samples, 5 cases with renal comorbidity, and 3 follow-up biopsies. Thus, we included a total of 128 native renal biopsies from 128 patients who were diagnosed with IgA nephropathy or IgA vasculitis with nephritis.

## Total cohort

The patient characteristics of the study cohort are summarized in Table 1. The mean (±SD) follow-up period was 51 ± 39 months. Microangiopathy was present in 26 of the 128 biopsies (20.3%) with IgA nephropathy or IgA vasculitis with nephritis. When present, microangiopathy was focal, and localized in glomeruli (in 4 biopsies; 15%), in arterioles (in 21 biopsies; 81%), or in both (in 1 biopsy; 4%). Active microangiopathy was present in 9 biopsies (35%), and chronic microangiopathy was present in 17 biopsies (65%) (Fig. 1). In the group of 22 pediatric patients, microangiopathy was observed in 1 of 15 cases with IgA nephropathy, but not in the 7 patients with IgA vasculitis with nephritis.

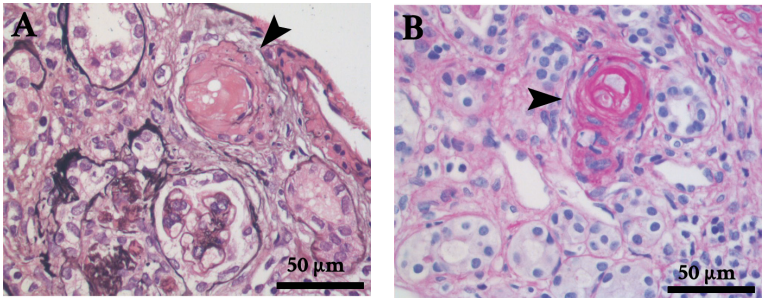


**Figure 1. Typical examples of active and chronic microangiopathy.** Typical example of a renal biopsy with active microangiopathy with thrombosis (A), showing a microthrombus (arrowhead) in an arteriole in the absence of inflammatory changes. Typical example of a renal biopsy with chronic microangiopathy (B), showing an organized thrombus (arrowhead) in the vessel wall with recanalization and obliteration of the vessel lumen due to intimal hyperplasia. A: silver stain, original magnification x400. B: Periodic acid–Schiff stain, original magnification x400.

**Table 1. Clinical characteristics at the time of biopsy**

| | Microangiopathy absent (n=102) | Microangiopathy present (n=26) | Total (n=128) | *P* value |
|---|---|---|---|---|
| Diagnosis | | | | 0.126 |
| *IgAN* | 81 (79) | 24 (92) | 105 (82) | |
| *IgAVN* | 21 (21) | 2 (8) | 23 (18) | |
| Female patients | 32 (31) | 7 (27) | 39 (31) | 0.660 |
| Pediatric patients | 21 (21) | 1 (4) | 22 (17) | 0.043 |
| Age, years | 41.1 ± 22.1 | 45.9 ± 15.6 | 42.1 ± 21.0 | 0.203 |
| Clinical thrombotic microangiopathy | 0 (0) | 1 (4) | 1 (1) | 0.203 |
| Malignant hypertension | 2 (2) | 2 (8) | 4 (3) | 0.183 |
| Hypertension | 50 (49) | 20 (77) | 70 (55) | 0.011 |
| Systolic BP (mmHg) | 136.2 ± 25.4 | 153.0 ± 34.1 | 139.7 ± 28.1 | 0.006 |
| Diastolic BP (mmHg) | 79.6 ± 15.7 | 93.5 ± 20.7 | 82.5 ± 17.7 | <0.001 |
| Number of antihypertensive drugs | 0.9 ± 1.1 | 1.4 ± 1.1 | 1.0 ± 1.1 | 0.042 |
| Type of antihypertensive drugs[a] | | | | 0.033 |
| *None* | 52 (52) | 7 (27) | 59 (47) | |
| *ACE-I and/or ARB* | 34 (34) | 16 (62) | 50 (40) | |
| *Other* | 14 (14) | 3 (11) | 17 (13) | |
| Use of corticosteroids | 42 (41) | 10 (39) | 52 (41) | 0.801 |
| Proteinuria (g/day) | 1.93 ± 2.37 | 3.25 ± 2.31 | 2.29 ± 2.41 | 0.035 |
| SCr in adults (mg/dL) | 2.2 ± 2.7 | 2.8 ± 2.4 | 2.4 ± 2.7 | 0.328 |
| SCr in children (mg/dL) | 0.7 ± 0.3 | 0.4 | 0.7 ± 0.3 | 0.423 |
| eGFR (mL/min/1.73m$^2$) | 63.9 ± 41.3 | 37.7 ± 25.3 | 58.6 ± 39.9 | <0.001 |
| IgA (g/L) | 3.5 ± 1.6 | 3.5 ± 1.4 | 3.5 ± 1.5 | 0.950 |
| Serum IgA : C3 | 3.1 ± 2.0 | 2.8 ± 1.2 | 3.1 ± 1.9 | 0.702 |
| Serum C3 (g/L) | 1.2 ± 0.3 | 1.1 ± 0.2 | 1.2 ± 0.3 | 0.425 |
| Serum C4 (mg/L) | 265.3 ± 113.7 | 232.3 ± 74.6 | 260.3 ± 108.8 | 0.434 |

Values are expressed as the mean ± SD or as number of cases (%). IgAN, IgA nephropathy; IgAVN, IgA vasculitis with nephritis; BP, blood pressure; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; eGFR, estimated glomerular filtration rate; SCr, serum creatinine.

[a] Data on antihypertensive treatment was not available for two patients.

## Clinical differences at time of biopsy

The patients with microangiopathy had significantly lower estimated glomerular filtration rate values at the time of biopsy than the patients without microangiopathy (the mean values were 37.7 and 63.9 mL/min/1.73m$^2$, respectively; $p<0.001$) (Table 1). Although the pediatric patients had a significantly lower prevalence of microangiopathy in their biopsy samples than the adults ($p<0.05$), no significant difference was found with respect to mean age between the patients with microangiopathy and the patients without microangiopathy ($p=0.203$). The prevalence of hypertension was higher among patients with microangiopathy (77%) than among patients without microangiopathy (49%; $p<0.05$). Notably, 6 of the 26 patients with microangiopathy (23%) were normotensive; these patients either used no medication (n=3) or used only angiotensin-converting enzyme inhibitors (n=1) or angiotensin-receptor blockers (n=2) for persistent proteinuria. One of 26 patients with microangiopathy (4%) had clinical evidence for systemic thrombotic microangiopathy. Two other patients with microangiopathy had malignant hypertension, and one additional patient had preeclampsia and HELLP (hemolysis, elevated liver enzyme levels, and low platelet levels) syndrome noted in the medical history but absent at the time of biopsy. No other potential causes of systemic thrombotic microangiopathy (such as antiphospholipid syndrome, a history of drug-induced thrombotic microangiopathy, mutations in complement regulatory genes, or infection with Shiga toxin–producing bacteria) were documented in our cohort. Eleven patients had evidence of liver disease prior to the diagnosis of IgA nephropathy (n=9) or IgA vasculitis with nephritis (n=2), these are described in Supplemental Table S1.

## Renal biopsy findings

In the renal biopsies, chronic lesions such as global glomerulosclerosis or tubular atrophy and interstitial fibrosis were more prevalent in biopsies with microangiopathy than in biopsies without microangiopathy ($p<0.01$) (Table 2). The prevalence and severity of arterial intimal sclerosis and arteriolar hyalinosis were significantly higher among the patients with microangiopathy than among patients without microangiopathy (p values $<0.01$). No significant histopathological differences were observed between cases with respect to active microangiopathy versus chronic microangiopathy. Crescents were observed in 6 of 23 cases with IgA vasculitis with nephritis (26%) and in 11 of 105 cases

**Table 2. Renal biopsy characteristics**

| | Microangiopathy absent (n=102) | Microangiopathy present (n=26) | Total (n=128) | *P* value |
|---|---|---|---|---|
| Percentage of glomeruli with global sclerosis, mean ± SD | 13 ± 18 | 33 ± 26 | 17 ± 22 | 0.001 |
| Mesangial hypercellularity (M1) | 49 (48) | 18 (69) | 67 (52) | 0.053 |
| Endocapillary proliferation (E1) | 26 (25) | 11 (42) | 37 (29) | 0.091 |
| Segmental glomerulosclerosis (S1) | 41 (40) | 16 (62) | 57 (45) | 0.051 |
| Tubular atrophy and interstitial fibrosis (T) | | | | <0.001 |
| *≤25 % (T0)* | 63 (62) | 8 (31) | 71 (56) | |
| *26-50 % (T1)* | 28 (27) | 3 (11) | 31 (24) | |
| *>50 % (T2)* | 11 (11) | 15 (58) | 26 (20) | |
| Crescents (C) | | | | 0.550 |
| *absent (C0)* | 87 (85) | 24 (92) | 111 (87) | |
| *1-24 % of glomeruli (C1)* | 12 (12) | 2 (8) | 14 (11) | |
| *≥ 25 % of glomeruli (C2)* | 3 (3) | 0 (0) | 3 (2) | |
| Glomerular necrosis | 4 (4) | 0 (0) | 4 (3) | 0.582 |
| Arteriolar hyalinosis | 29 (28) | 15 (58) | 44 (34) | 0.010 |
| Arterial intimal sclerosis[a] | 23 (24) | 21 (81) | 44 (36) | <0.001 |

Values are expressed as number of cases (%), unless specified otherwise. [a] Arterial sclerosis was not scored in seven biopsies that lacked arterial branches.

with IgA nephropathy (10%). This difference was statistically significant (p=0.046). In the renal biopsies from the two IgA vasculitis with nephritis cases with microangiopathy, one had crescents and the other did not. Both cases progressed to end-stage renal disease and required renal replacement therapy. In addition to the predominance of IgA deposits, glomerular deposits of IgG and IgM were observed in 8% and 30% of all renal biopsies, respectively; no significant differences were observed between cases with microangiopathy and cases without microangiopathy. Among the 26 cases with microangiopathy, only 2 patients had weak (1+) staining intensity of IgA by immunofluorescence;

both cases had mesangial hypercellularity. Deposits of C4d, C5b-9, and C1q in the glomeruli and/or arterioles were more prevalent in the patients with microangiopathy than in the patients without microangiopathy (Table 3). Typical examples of complement staining in IgA nephropathy are shown in Fig. 2 and Supplemental Fig. S1. Complement proteins were co-localized with microthrombi (Figure 2C, D). Glomerular C4d deposition was associated with C1q and IgM deposits ($p<0.001$ and $p<0.05$, respectively), but not with IgG or MBL deposits.

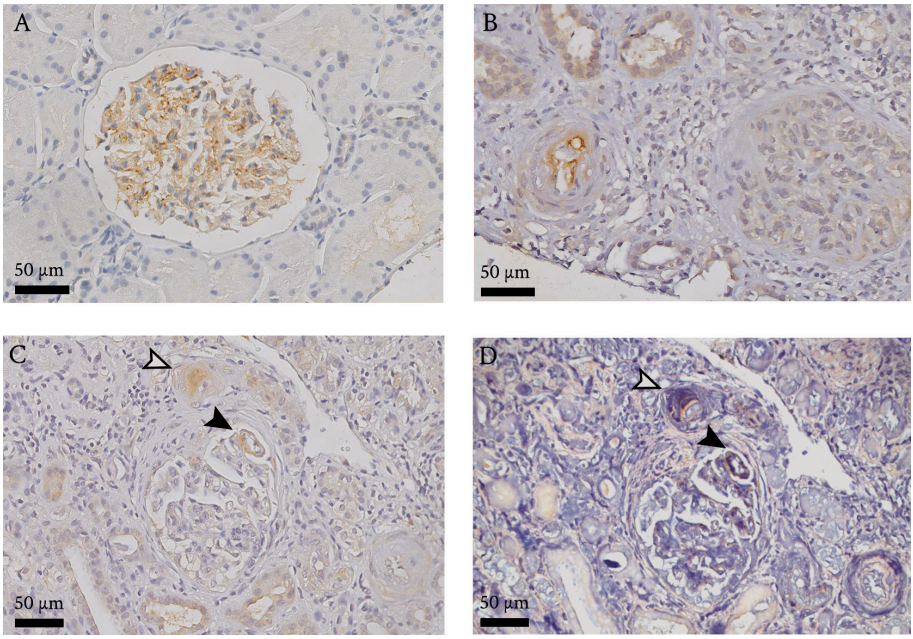


**Figure 2. Typical examples of complement staining and co-localization with microangiopathy.** Typical examples of complement staining in glomeruli (A) and arterioles (B). C4d staining (C) was co-localized with microangiopathy (D) in the same glomerular capillary (black arrowhead) and arteriole (white arrowhead) of a patient with microangiopathy in sequentially sectioned tissue. A-C show C4d staining; D shows phosphotungstic acid hematoxylin (PTAH) staining, with fibrin staining as deep blue.

**Table 3. Complement proteins in cases with or without microangiopathy**

| Immunohistochemical stain | Microangiopathy absent (n=94) | Microangiopathy present (n=22) | Total (n=116) | *P* value |
|---|---|---|---|---|
| C4d positive | 16 (17) | 17 (77) | 33 (28) | <0.001 |
| in glomeruli | 14 (15) | 12 (55) | 26 (22) | <0.001 |
| in arterioles | 5 (5) | 11 (50) | 16 (14) | <0.001 |
| C5b-9 positive | 44 (47) | 16 (73) | 60 (52) | 0.029 |
| in glomeruli | 11 (12) | 6 (27) | 17 (15) | 0.063 |
| in arterioles | 43 (46) | 15 (68) | 58 (50) | 0.058 |
| C1q positive | 34 (36) | 13 (59) | 47 (41) | 0.049 |
| in glomeruli | 30 (32) | 10 (46) | 40 (35) | 0.229 |
| in arterioles | 8 (9) | 6 (27) | 14 (12) | 0.015 |
| MBL positive | 2 (2) | 0 (0) | 2 (2) | 0.490 |
| in glomeruli | 1 (1) | 0 (0) | 1 (1) | 0.627 |
| in arterioles | 1 (1) | 0 (0) | 1 (1) | 0.627 |
| Factor B positive[a] | 14/57 (25) | 5/20 (25) | 19/77 (25) | 0.969 |
| in glomeruli | 14/57 (25) | 5/20 (25) | 19/77 (25) | 0.969 |
| in arterioles | 0 (0) | 0 (0) | 0 (0) | NA |

Values are expressed as number of cases (%). MBL, mannose-binding lectin; NA, not applicable.

[a] Factor B was performed on fresh frozen tissue, which was available for 77 biopsy samples.

## Clinical differences at follow-up

The follow-up duration was similar between patients with microangiopathy and patients without microangiopathy (48.4 ± 36.2 and 51.5 ± 39.2 months, respectively; p=0.717). Patients with a combination of C4d and microangiopathy had significantly poorer renal survival than patients who lacked these findings (p<0.01) (Fig. 3). Cox proportional hazard regression analysis revealed that this difference in renal survival remained significant after we corrected for hypertension (Table 4), estimated glomerular filtration rate, and interstitial fibrosis with tubular atrophy, respectively (Supplemental Tables S2 and S3). C4d deposition was not a better predictor than the Oxford T-score or the presence of interstitial fibrosis with tubular atrophy (Supplemental Table S4).

Linear mixed-effects model analysis revealed that hypertensive patients who have a combination of microangiopathy and C4d had a mean estimated glomerular filtration rate of 23.4 mL/min/1.73m$^2$ at the time of biopsy (Table 5).

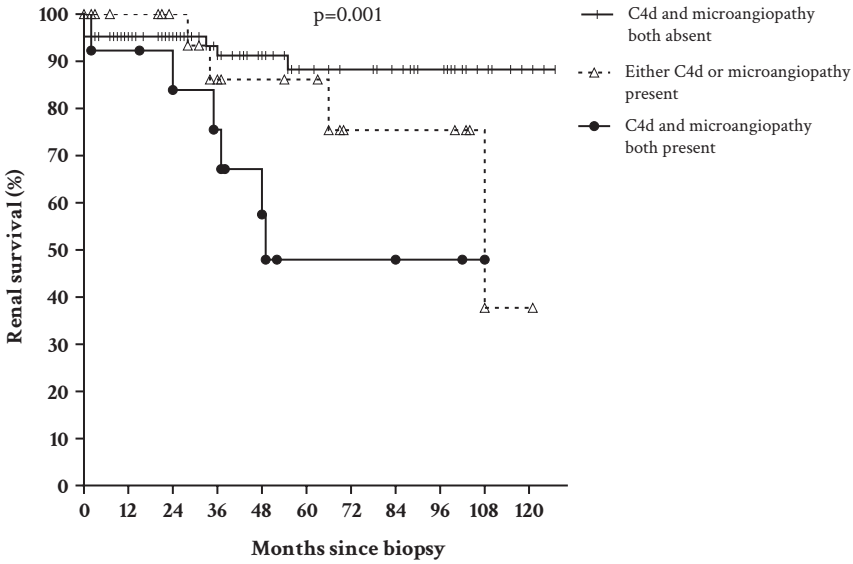


**Figure 3. Renal survival of patients with microangiopathy and C4d.** All patients with IgA nephropathy or IgA vasculitis with nephritis were divided into three groups based on the presence or absence of microangiopathy and C4d. Patients in which microangiopathy and C4d were both present had significantly lower renal survival than patients in which C4d and microangiopathy were both absent (p=0.001).

After we corrected for hypertension, these patients with both microangiopathy and C4d still had significantly lower estimated glomerular filtration rate values at the time of biopsy compared to patients who lacked both of these findings, leading to a mean estimated glomerular filtration rate of 46.8 mL/min/1.73m$^2$ for the latter group. The rate of estimated glomerular filtration rate decline was not significantly different between the groups. Although overall renal survival differed between cases with and without microangiopathy (Supplemental Table S5 and Supplemental Fig. S2), the difference in renal survival did not differ significantly between the cases with active microangiopathy and the cases with chronic microangiopathy (Supplemental Fig. S3).

**Table 4. Risk factors for renal replacement therapy**

| Variable | Hazard Ratio | 95% C.I. | *P* value |
|---|---|---|---|
| Both microangiopathy and C4d absent | Reference (1.000) | NA | 0.028 |
| Either microangiopathy or C4d present | 2.007 | 0.600 – 7.193 | 0.249 |
| Both microangiopathy and C4d present | 4.439 | 1.492 – 13.207 | 0.007 |
| Hypertension present | 2.779 | 0.746 – 10.504 | 0.127 |

Multivariable Cox proportional hazard regression analyses. The hazard ratios for requiring renal replacement therapy are shown for microangiopathy and C4d staining, corrected for hypertension. C.I., confidence interval; NA, not applicable.

**Table 5. Change in estimated glomerular filtration rate over time**

| Variable | eGFR (mL/min/1.73m$^2$) | 95% C.I. | *P* value |
|---|---|---|---|
| Intercept | 23.4 | 7.8 – 39.1 | 0.004 |
| Both C4d and microangiopathy absent | + 23.4 | 5.2 – 41.6 | 0.012 |
| Either C4d or microangiopathy present | + 9.3 | -12.0 to 30.7 | 0.388 |
| Both C4d and microangiopathy present | Reference | NA | NA |
| Hypertension absent | + 42.8 | 30.4 – 55.1 | <0.001 |
| Hypertension present | Reference | NA | NA |
| Time, years | 0.04 | -0.06 to 0.2 | 0.414 |

Linear mixed-effects model using random intercepts and random slopes. The intercept reflects the mean estimated glomerular filtration rate at baseline for hypertensive patients with microangiopathy and C4d. The table shows adjusted differences to this mean estimated glomerular filtration rate at baseline given the absence of microangiopathy, C4d, or hypertension. C.I., confidence interval; NA, not applicable; eGFR, estimated glomerular filtration rate.

## DISCUSSION

Here, we report that the presence of microangiopathic lesions in IgA nephropathy is strongly associated with complement activation in general and with C4d deposits in particular. Patients whose renal biopsies show both C4d and microangiopathy represent a clinical subgroup with poorer renal outcome compared to patients who lack these findings. Our results underscore the important role that microangiopathy plays in IgA nephropathy and adds a nuanced perspective to previous reports. For example, Chang et al.[4] reviewed the renal pathology reports of 435 cases with IgA nephropathy and found that 2% of cases had microangiopathy either with or without thrombosis. Nasri et al.[29] and Oruc et al.[30] reported a similar prevalence. In contrast, El Karoui et al.[5] re-examined 128 renal biopsies from patients with IgA nephropathy and found a prevalence of 53%. This higher prevalence may be explained—at least in part—by the fact that El Karoui et al. re-examined the renal biopsies with the specific aim of identifying microangiopathic lesions, as well as the fact that patients were selected from an active hypertensive clinic. Although the patients in our cohort did not come from a hypertension clinic, we re-examined at least four different biopsy sections from each patient and found that 23% of biopsies with IgA nephropathy had microangiopathy with or without thrombosis, which is higher than we had expected based on other studies.[4, 29-31] Thus, our findings indicate that microangiopathy may be underdiagnosed in clinical practice, which is an important point given our finding that microangiopathy is clinically relevant—specifically, we found that patients with microangiopathy had a higher prevalence of hypertension, a higher number of chronic lesions, and poorer renal outcome compared to patients without microangiopathy.

The precise pathogenic mechanism underlying microangiopathy in the setting of IgA nephropathy and IgA vasculitis with nephritis remains to be determined. However, case reports indicate that various factors may increase the risk of developing of microangiopathy in IgA nephropathy; these factors may include drug toxicity, HELLP syndrome, and the presence of antiphospholipid antibodies.[5, 32-37] Multiple factors may be required to cause microangiopathy in general, and in IgA nephropathy in particular; however, our data provide evidence that complement activation plays an important role in the development of microangiopathy in IgA nephropathy, irrespective of other

factors. In this respect, hypertension deserves specific attention here, given that high levels of shear stress induced by hypertension can cause microangiopathic changes.[38] However, in our study, several patients with microangiopathy were normotensive; moreover, only a fraction of patients with malignant hypertension had microangiopathy, and the clinical outcome of patients with both microangiopathy and complement deposits remained significantly worse even after we corrected for hypertension. Therefore, our results suggest that hypertension may not be a primary cause of the microangiopathic lesions. A linking factor in this discussion may be the association between complement regulatory deficits and hypertension-related microangiopathy reported by Timmermans et al.[39] They recently described a cohort of patients with biopsy-proven microangiopathy that was attributed clinically to severe hypertension; however, they found that 67% of patients had a mutation in the genes that encode complement factor C3 or the complement regulators CFH, CFI, or CD46, with concomitant evidence of complement activation *in vivo*, and poor renal outcome.[39] It would therefore be interesting to investigate whether IgA nephropathy patients with hypertension-associated microangiopathy share a similar genetic predisposition with the patients reported by Timmermans et al.,[39] particularly given the evidence that disease progression is more rapid in patients who have IgA nephropathy and a deficiency in complement regulation, as well as case reports describing atypical hemolytic uremic syndrome as a co-morbidity in IgA nephropathy.[18, 40-43]

In the pathogenesis of IgA nephropathy, complement activation is an important trigger of inflammation and progression, acting predominantly via the lectin and alternative pathways.[7, 8] The presence of the complement cleavage product C4d has been shown to predict the progression of renal damage in patients with IgA nephropathy;[19, 44-47] however, these results did not take into account vascular lesions. In IgA nephropathy, C4d deposition is generally considered a consequence of lectin pathway activation.[47] Roos et al.[46] demonstrated that C4d deposition was associated with the deposition of various lectin proteins. In our study, we found that microangiopathy was associated with C4d, C1q, and C5b-9 deposits; in contrast, MBL deposits were not detected in most patients, even after we repeated the experiment using different anti-MBL antibodies or using fresh-frozen tissue samples (data not shown). Proteomics-based analyses of laser-captured micro-dissected glomeruli revealed

that the classical pathway components C1q, C1r, and C1s were significantly higher in patients with progressive IgA nephropathy compared to patients with non-progressive IgA nephropathy.[48] These data suggest that C4d may reflect activation of both the lectin and classical pathways in IgA nephropathy. In our study, factor B deposition was observed in 25% of cases, suggesting activation of the alternative pathway. Evidence of alternative pathway activation is commonly observed in IgA nephropathy.[7] Genome-wide association studies in IgA nephropathy point to a role of deletions of CFHR1, CFHR3, and rare CFHR5 variants for IgA nephropathy susceptibility.[7] These genes code for the Factor H-Related proteins (FHR), which may function as competitive antagonists of factor H, reducing complement regulation. Elevated circulating levels of FHR proteins in patients with IgA nephropathy were shown to predict the progression of renal disease.[42] A possible relationship with the development of microangiopathic lesions remains to be investigated. The mechanism by which the complement pathway is activated in microangiopathy could also be distinct from the mechanism that involves IgA1-containing immune complexes. Severe vascular lesions and shear stress-induced endothelial injury have been shown to activate the classical pathway, and several proteins in the coagulation cascade have bi-directional interactions with the complement system, causing a vicious cycle that can be particularly harmful in patients who lack adequate complement regulation.[49, 50] This is particularly interesting given the recent case reports showing that complement-inhibiting therapeutics—which are known to benefit patients with systemic microangiopathy—are also beneficial to at least some patients with IgA nephropathy, including patients with complement-mediated microangiopathy.[15-18, 51]

In our study, there were 11 patients with liver disease prior to the diagnosis of IgA nephropathy or IgA vasculitis with nephritis. Because it was uncertain whether these cases were to be considered as "secondary IgA nephropathy", we did not exclude them from the study group. We found no important differences between cases with or without liver disease and all relevant reported associations remained significant in subanalyses in which these cases were excluded (data not shown). Importantly, C4d deposition and microangiopathy remained a subgroup with more chronicity and inferior outcome.

Our study has limitations that warrant discussion. First, serum and DNA samples from our patients were unavailable, limiting our ability to investigate

possible risk factors in the development of microangiopathy other than the factors examined during the original clinical work-up. Furthermore, although C4d is conducted at a number of institutions worldwide, there is considerable variability in C4d staining across institutions.[52] Therefore, our observations on complement deposition need to be validated in other centers using local staining procedures. In addition, evaluation of prognosis was limited by the sample size of 26 patients with microangiopathy of which only a proportion required renal replacement therapy. Although our data suggests that interstitial fibrosis and tubular atrophy is a better predictor for renal outcome than C4d, this study lacked sufficient power to examine the incremental effect of microangiopathy and C4d deposition in addition to all other known markers of poor prognosis, including clinical data, parameters of the Oxford classification, different treatment modalities, and other biomarkers.[3, 53] Multi-center prospective studies on IgA nephropathy should determine whether C4d and microangiopathy have an additional prognostic value to these variables. Moreover, we did not take into account relatively subtle cases with only ultrastructural microangiopathic lesions, as electron microscopy data were not available for most cases. Our study's strengths include the relatively high number of biopsy samples examined specifically for C4d and microangiopathy, the long follow-up period, and a study cohort that was not biased with respect to recruiting hypertensive patients. Moreover, we describe the prevalence and clinical significance of microangiopathy in pediatric patients with IgA nephropathy and patients with IgA vasculitis with nephritis, both of which had a low prevalence of microangiopathy.

In our cohort of 128 patients with IgA nephropathy or IgA vasculitis with nephritis, microangiopathic lesions were present in 20% of biopsies, and these lesions were primarily combined with C4d deposition, based on a thorough histopathological examination involving multiple levels of the renal biopsy. Patients with IgA nephropathy or IgA vasculitis with nephritis together with C4d positivity and microangiopathy comprise a clinical subgroup with a higher number of chronic lesions, lower estimated glomerular filtration rate, and poorer renal survival compared to patients without microangiopathy or C4d deposits, even after we corrected for hypertension. These data suggest that complement activation plays an important role in the development of microangiopathy in patients with IgA nephropathy and IgA vasculitis with nephritis, and that complement-mediated microangiopathy contributes to disease progression.

**Disclosures:** The authors have nothing to disclose.

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# SUPPLEMENTAL DATA

**Supplemental Table S1. Patients with or without liver disease**

| Variable | Patients without liver disease (n=117) | Patients with liver disease (n=11) | Total (n=128) | *P* value |
|---|---|---|---|---|
| Liver disease | 0 (0) | 11 (100) | 11 (9) | NA |
| Alcoholic liver cirrhosis | NA | 5 (45) | 5 (4) | NA |
| Hepatitis B | NA | 4 (36) | 4 (3) | NA |
| Klebsiella Pneumoniae in liver abscess | NA | 1 (9) | 1 (1) | NA |
| Granulomatous hepatitis of unknown cause | NA | 1 (9) | 1 (1) | NA |
| Female patients | 39 (33) | 0 (0) | 39 (30) | 0.022 |
| Age, years | 41.5 ± 21.4 | 47.9 ± 14.7 | 42.1 ± 21.0 | 0.336 |
| Hypertension | 63 (54) | 7 (64) | 70 (55) | 0.553 |
| Microangiopathy | 23 (20) | 3 (27) | 26 (20) | 0.548 |
| M1 | 61 (52) | 6 (55) | 67 (52) | 0.878 |
| E1 | 35 (30) | 2 (18) | 37 (29) | 0.412 |
| S1 | 51 (44) | 6 (55) | 57 (45) | 0.485 |
| T | | | | 0.063 |
| T0 | 68 (58) | 3 (27) | 71 (56) | |
| T1 | 28 (24) | 3 (27) | 31 (24) | |
| T2 | 21 (18) | 5 (46) | 26 (20) | |
| C | | | | 0.159 |
| C0 | 101 (86) | 10 (91) | 111 (87) | |
| C1 | 14 (12) | 0 (0) | 14 (11) | |
| C2 | 2 (2) | 1 (9) | 3 (2) | |
| C4d positivity[a] | 29 (27) | 4 (40) | 33 (28) | 0.397 |
| C1q positivity[a] | 42 (40) | 5 (50) | 47 (41) | 0.523 |
| MBL positivity[a] | 2 (2) | 0 (0) | 2 (2) | 0.654 |
| C5b-9 positivity[a] | 54 (51) | 6 (60) | 60 (52) | 0.584 |
| Factor B positivity[b] | 18 (25) | 1 (20) | 19 (25) | 0.802 |

Positivity was defined as the presence in glomeruli, arterioles, or both. MBL, mannose-binding lectin; NA: not applicable. Values are expressed as the mean ± SD or number (%). [a] Tissue samples for immunohistochemistry were available for 116 patients. [b] Fresh-frozen tissue was available for factor B in 77 cases.

**Supplemental Table S2. Subanalysis: risk factors for RRT, correcting for eGFR**

| Variable | Hazard Ratio | 95% Confidence Interval | *P* value |
|---|---|---|---|
| Both microangiopathy and C4d absent | Reference | | 0.015 |
| Either microangiopathy or C4d present | 3.020 | 0.770 – 11.855 | 0.113 |
| Both microangiopathy and C4d present | 4.673 | 1.639 – 13.326 | 0.004 |
| Estimated glomerular filtration rate | 0.921 | 0.888 – 0.955 | <0.001 |

Multivariable Cox proportional hazard regression analysis. The hazard ratios for requiring renal replacement therapy (RRT) are shown for microangiopathy and C4d staining, corrected for estimated glomerular filtration rate (eGFR).

**Supplemental Table S3. Subanalysis: risk factors for RRT, correcting for IF/TA**

| Variable | Hazard Ratio | 95% Confidence Interval | *P* value |
|---|---|---|---|
| Both microangiopathy and C4d absent | Reference | | 0.032 |
| Either microangiopathy or C4d present | 1.347 | 0.384 – 4.725 | 0.641 |
| Both microangiopathy and C4d present | 3.748 | 1.331 – 10.551 | 0.012 |
| Interstitial fibrosis / tubular atrophy present | 11.034 | 2.405 – 50.630 | 0.002 |

Multivariable Cox proportional hazard regression analysis. The hazard ratios for requiring renal replacement therapy (RRT) are shown for microangiopathy and C4d staining, corrected for the presence of interstitial fibrosis and tubular atrophy (IF/TA).

**Supplemental Table S4. Harrell's C score for IF/TA and C4d**

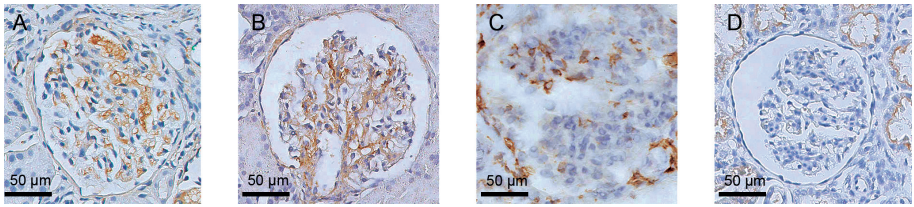
| Variable | Harrell's C |
|---|---|
| Oxford T-score | 0.85 |
| Presence of interstitial fibrosis / tubular atrophy | 0.76 |
| Presence of C4d in glomeruli or arterioles | 0.66 |

Harrell's C was used to assess the predictive performance of the COX regression models. It measures the correlation between the prediction and the outcome on a scale from 0-1; a higher value of C indicates a better prediction.

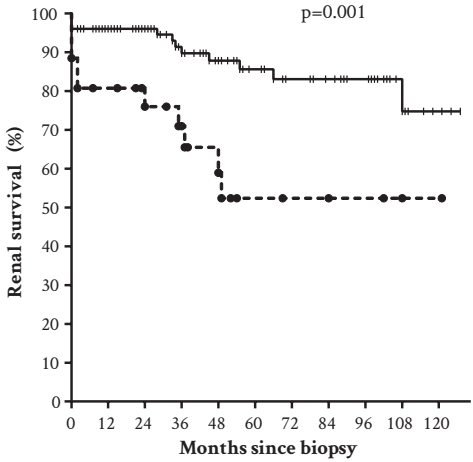
**Supplemental Table S5. Subanalysis: microangiopathy as risk factor for RRT, correcting for hypertension**

| Variable | Hazard Ratio | 95% Confidence Interval | *P* value |
|---|---|---|---|
| Microangiopathy | 2.739 | 1.152 – 6.513 | 0.023 |
| Hypertension | 3.052 | 1.002 – 9.296 | 0.049 |

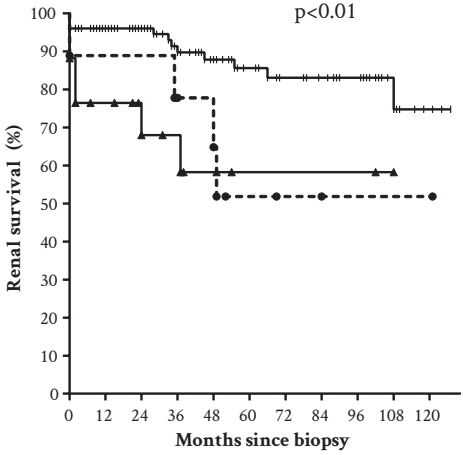
Multivariable Cox proportional hazard regression analysis. The hazard ratios for requiring renal replacement therapy (RRT) are shown for microangiopathy (corrected for hypertension) and for hypertension (corrected for microangiopathy).




**Supplemental Figure S1. Complement staining of C1q, C5b-9, Factor B, and MBL.** Representative examples of glomerular staining of C1q (A), C5b-9 (B), and factor B (C). In contrast, glomerular mannose-binding lectin (MBL) deposition (D) was typically absent.




**Supplemental Figure S2. Kaplan-Meier plot of renal survival in patients with or without microangiopathy.** All patients with IgA nephropathy or IgA vasculitis with nephritis were divided into two groups based on the presence or absence of microangiopathy. Patients with microangiopathy had significantly lower renal survival than patients without microangiopathy (p=0.001).




**Supplemental Figure S3. Kaplan-Meier curve for renal survival in patients with active, chronic, or no microangiopathy.** Patients with IgA nephropathy or IgA vasculitis with nephritis were divided into three groups: no microangiopathy, active microangiopathy, and chronic microangiopathy. Although there was an overall difference (p<0.01), there was no significant difference in renal survival between patients with active microangiopathy and patients with chronic microangiopathy.

# Chapter 4

## Glomerular C4d deposits can mark structural capillary wall remodelling in thrombotic microangiopathy and transplant glomerulopathy: *C4d beyond active antibody-mediated injury: a retrospective study*

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# ABSTRACT

Peritubular capillary C4d (ptc-C4d) usually marks active antibody-mediated rejection, while pseudolinear glomerular capillary C4d (GBM-C4d) is of undetermined diagnostic significance, especially when seen in isolation without concurrent ptc-C4d. We correlated GBM-C4d with structural GBM abnormalities and antibody-mediated rejection in 319 renal transplant and 35 control native kidney biopsies. In kidney transplants, ptc-C4d was associated with GBM-C4d in 97% by immunofluorescence microscopy (IF) and 61% by immunohistochemistry (IHC; $p<0.001$). Transplant glomerulopathy was correlated with GBM-C4d ($p<0.001$) and presented with isolated GBM-C4d lacking ptc-C4d in 69% by IF and 40% by IHC. Strong isolated GBM-C4d was found post year-1 in repeat biopsies with transplant glomerulopathy. GBM-C4d staining intensity correlated with Banff cg scores ($rs = 0.45$, $p<0.001$). Stepwise exclusion and multivariate logistic regression corrected for active antibody-mediated rejection showed significant correlations between GBM duplication and GBM-C4d ($p=0.001$). Native control biopsies with thrombotic microangiopathies demonstrated GBM-C4d in 92% (IF, $p < 0.001$), and 35% (IHC). In conclusion, pseudolinear GBM-C4d staining can reflect two phenomena: (i) structural GBM changes with duplication in native and transplant kidneys or (ii) active antibody-mediated rejection typically accompanied by ptc-C4d. While ptc-C4d is a dynamic 'etiologic' marker for active antibody-mediated rejection, isolated strong GBM-C4d can highlight architectural glomerular remodelling.

# INTRODUCTION

Pioneering work by H. Feucht and colleagues on the complement degradation product C4d began a new era of research on antibody-induced graft injury.[1-5] Over the past 15 years, C4d has attracted much interest, and has become an important biomarker for the diagnosis and classification of active antibody-mediated rejection in solid organ transplant biopsies.[6-15]

In ABO compatible kidney allografts, C4d deposits along peritubular capillaries (ptc-C4d) follow a dynamic expression pattern.[9] Ptc-C4d staining is found during active antibody-mediated graft rejection (AMR) with acute tissue injury, and vanishes following therapeutic intervention with reduction in circulating donor-specific antibodies. The ptc-C4d staining pattern is scored according to Banff guidelines on the interpretation of allograft pathology,[11] and incorporated into diagnoses. However, C4d deposits in other anatomic compartments of the kidney, such as in glomeruli, are currently incompletely characterized and are not used to diagnose active rejection.[9-11, 15] For example, mesangial C4d staining is interpreted to be 'normal'.[16] Distinct granular intra-glomerular C4d staining in native and transplanted kidneys typically marks the presence of immune complexes and glomerulonephritides.[17, 18] In renal allografts, linear or pseudolinear glomerular basement membrane C4d deposits (GBM-C4d) are often,[19-27] but not always,[28] associated with signs of chronic active AMR, including transplant glomerulitis, ptc-C4d and transplant glomerulopathy. Pseudolinear GBM-C4d in native kidney biopsies was reported in preeclampsia and some but not all renal biopsies with thrombotic microangioapthy.[29-31] Thus, C4d deposition along GBM segments has been described in different forms of glomerular injury.

Previously, we hypothesized that in contrast to ptc-C4d, isolated pseudolinear GBM-C4d does not mark active ongoing AMR but might rather indicate structural GBM remodelling. Subsequently, this hypothesis got support from Chua et al.[29] The aim of the current analysis was to evaluate the prevalence and significance of pseudolinear GBM-C4d in a large cohort of renal biopsies. Special emphasis was placed on GBM-C4d and architectural glomerular capillary wall remodelling with subendothelial new lamina densa formation and duplication: can GBM-C4d mark structural changes rather than active and ongoing AMR as highlighted by ptc-C4d? The answer to this question furthers our understanding of the pathophysiological mechanisms of GBM remodelling and the role of complement in structural damage and repair.

# METHODS

## Study cohorts

We reviewed 319 consecutively obtained diagnostic kidney allograft biopsies collected during a 64-month study period between February 2008 and February 2013 from 219 patients. The majority of allograft recipients were transplanted at the University of North Carolina in Chapel Hill between December 1991 and December 2012. A total of 140 biopsies were taken within the first year post-transplantation (44%); 39 between the first and the second year (12%); 133 after 2 years (42%) and 7 without local knowledge of the specific transplantation date (2%). The study biopsy group contained 219/319 'index' biopsies, that is first biopsy obtained during study period, and 100/319 additional follow-up biopsies from 61/219 patients (1-5 follow-up biopsies per patient during study period). Implantation biopsies, cases of glomerulonephritides or polyomavirus nephropathy, and specimens from ABO incompatible transplants were not included into the study cohort. A comparative control group consisted of selected native renal biopsies from nine patients with minimal change disease and 26 patients with thrombotic microangiopathy of various underlying etiologies and GBM remodelling (n=2 scleroderma renal crisis, n=2 infection including typical HUS, n=4 malignant neoplasms, therapeutic intervention including radiation, bone marrow transplantation, n=1 systemic lupus erythematosus, n=9 severe hypertension, n=2 atypical HUS, n=1 preeclampsia, n=5 undetermined). This study was approved by the Institutional Review Board of UNC (10-1353).

## Clinical data

The following clinical data were collected from the UNC hospital database: patient demographics, year of transplantation, cross-match positivity, panel reactive antibody positivity, donor-specific antibody positivity at time of grafting and during follow-up until end of study period 3/2013, evidence of a hepatitis C infection (see Appendix S1 for further details on antibody testing). Post-transplantation patients with clinical suspicion of AMR were tested for donor-specific antibodies. In this study 'antibody-positive cases' were defined as patients having detectable donor-specific antibodies for HLA-class I and/or class-II at least once during the entire post-transplantation time period starting at time of grafting and ending 1 March 2013, and 'antibody-negative cases' as patients without detectable alloantibodies at any time during follow-up.

## Histological analysis

All biopsies sufficed for rendering a diagnosis according to Banff criteria.[32] Sections were prepared for light microscopy (LM) and immunofluorescence microscopy (IF) according to standard protocols. Tissue for electron microscopy (EM) analysis was collected from all transplants older than 1-year post-grafting and from all cases during year-1 with proteinuria and/or haematuria.

*C4d staining*

For IF, 3-μm cryostat sections were incubated with a monoclonal anti-human C4d antibody (Quidel A213, diluted 1:50) following routine procedures. For immunohistochemistry (IHC), performed in a randomly selected representative subgroup of study cases, formalin-fixed and paraffin-embedded tissue sections were pressure cooked at 125 and 90 °C for antigen retrieval and subsequently incubated with a polyclonal rabbit anti-human C4d antibody (ALPCO Diagnostics® Cat.# 004-BIRC4d, diluted 1:20 and incubated for 30 min at 37 °C).

## Scoring of histology

Two experienced renal pathologists (VN, AG) who were blinded to the patients' clinical data and C4d staining results scored all renal biopsies according to Banff '97 and '09 criteria.[32, 33] In allograft biopsies, transplant glomerulopathy (TG) was defined according to Banff '97 criteria based on LM (cg scores 0-3). The same approach was arbitrarily used for scoring native control kidney biopsies including GBM duplications in cases of chronic thrombotic microangiopathy.

## Scoring of immunostaining

Along peritubular capillaries, C4d staining by IF and IHC was scored according to Banff criteria as absent (0), minimal (1; <10% of peritubular capillaries staining), focal (2; 10-50%) or diffuse positivity (3; >50%).[11] For study purposes, ptc-C4d was also categorized as either positive (for IHC at Banff scores ≥1 and for IF at Banff scores ≥2), or as negative. GBM-C4d staining along peripheral glomerular capillaries with linear and/or dense granular deposits, termed 'pseudolinear GBM-C4d' for current study purposes, was assessed separately; mesangial C4d staining was not analysed. Cases with staining along the entire capillary wall circumference of at least one perfused glomerular capillary in at least one glomerulus by IF or IHC were arbitrarily classified as 'GBM-C4d

positive' (minimum positive cut-off). GBM scoring was performed by VN and AG and recorded as consensus result. The GBM-C4d staining intensity was semi-quantitatively scored for IF and IHC on a scale from 0-3+ as 0 (absent or trace), weak (1+), moderate (2+) or strong (3+). The GBM staining pattern was scored as either focal (<50% of the total number of glomeruli) or diffuse (≥50% of the total number of glomeruli) and as either segmental (<50% of the glomerular tuft) or global (≥50% of the glomerular tuft). The same case of acute AMR with C4d staining along ptc served as comparative positive staining control.

**Scoring of ultrastructural GBM remodelling**

Glomeruli (at least two per case) were studied by EM to investigate ultrastructural evidence of GBM remodelling and duplication. Three experienced renal pathologists (VN, AG, HKS), who were blinded to the patients' clinical data and C4d staining status, evaluated low- and high-power digital EM images from cases with at least eight inflated glomerular capillary loops available for review. Ultrastructural GBM changes were grouped based on 'round-table' consensus into four categories: (i) circumferential GBM duplication with widening of the lamina rara interna and subendothelial new densa formation (one or more layers) involving at least one entire circumference of at least one glomerular capillary loop; other loops could show different changes including widening of the rara interna; (ii) noncircumferential GBM duplication with widening of the lamina rara interna and subendothelial new densa formation, involving less than an entire circumference in at least one capillary loop, circumferential GBM duplications per definition absent; (iii) lamina rara interna widening, activation of endothelial cells including loss of fenestration and cytoplasmic thickening but no subendothelial new densa deposition (following descriptions given by Wavamunno et al.[34]); and (iv) normal GBM.

**Statistical analyses**

The statistical analysis was performed using SPSS 20.0 software (SPSS, Chicago, IL, USA) and the SAS (University Edition) statistical software package (SAS Institute Inc., Cary, NC) for multivariate exact logistic regression. $P<0.05$ was considered statistically significant. Continuous variables were expressed as mean ± SD. For comparison of numerical and categorical clinical and histological data, the Student's t-test, one-way ANOVA, chi-squared and Fisher's exact tests

were utilized. Relationships between different categories of GBM duplication and GBM-C4d staining intensities were expressed using Spearman's rank correlation, supplemented by testing of group differences with ANOVA F-test. Multivariate logistic regression and multivariate exact logistic regression were used to determine independent factors associated with GBM-C4d. Successively eliminating variables with nonsignificant p values starting with the highest p value, did not indicate the presence of multicollinearity in the full multivariate model.

**Clinical data – Testing of circulating donor-specific antibodies**
(See Supplemental Methods for details)

**Immune electron microscopy**
(See Supplemental Methods for details)

# RESULTS

## Renal allografts

*Glomerular basement membrane changes*

Transplant glomerulopathy (TG) by LM was observed in 52/319 biopsies (16.3%; Banff '97 score cg-1: 9/52, cg-2: 2/52, cg-3: 41/52), collected from 35/219 patients (15.9%). EM, performed on 198/319 biopsies, showed GBM duplication in 61/198 cases (40 circumferential, 21 noncircumferential); 31/61 biopsies had concurrent signs of TG by LM and 30/61 only revealed ultrastructural GBM duplication (10 circumferential, 20 noncircumferential). Eleven of 52 cases of TG seen by LM were not accompanied by EM changes as a reflection of lesion sampling. In total, 82/319 transplant biopsies (25.7%) demonstrated GBM duplications by LM and/or EM. By ultrastructural examination, 15/198 cases revealed widening of the lamina rara interna without GBM duplication and 111/198 biopsies were normal both by LM and EM.

## C4d staining

*Immunofluorescence staining*

C4d along peritubular capillaries (ptc-C4d) by IF was noted in 37/319 biopsies; in 12/37 with a focal distribution pattern (concomitant GBM-C4d deposits in all cases), and in 25/37 with a diffuse ptc-C4d pattern (concomitant GBM-C4d in 24/25 biopsies). Overall, pseudolinear GBM-C4d by IF was present in 196/319 transplant biopsies (61%, Table 1), 36/196 with concomitant ptc-C4d staining and 160/196 in isolation. The GBM-C4d staining intensity was strong in 30/319 (9%) and weak to moderate in 166/319 cases (52%). The staining pattern of biopsies with strong GBM-C4d staining was most frequently diffuse and global (24/30, 80%), whereas the staining of cases with weak-to-moderate staining was often focal and segmental (83/166, 50%) (Fig. 1).

*Immunohistochemical staining*

Ptc-C4d staining by IHC was noted in 31/116 cases; in 9/31 with a focal distribution pattern (concomitant GBM-C4d in 3/9 biopsies), and in 22/31 with diffuse staining (concomitant GBM-C4d in 16/22 biopsies). Overall, pseudolinear GBM-C4d by IHC was present in 37/116 transplant biopsies (32%, Table 1); 19/37 with concomitant ptc-C4d and 18/37 in isolation. The GBM-

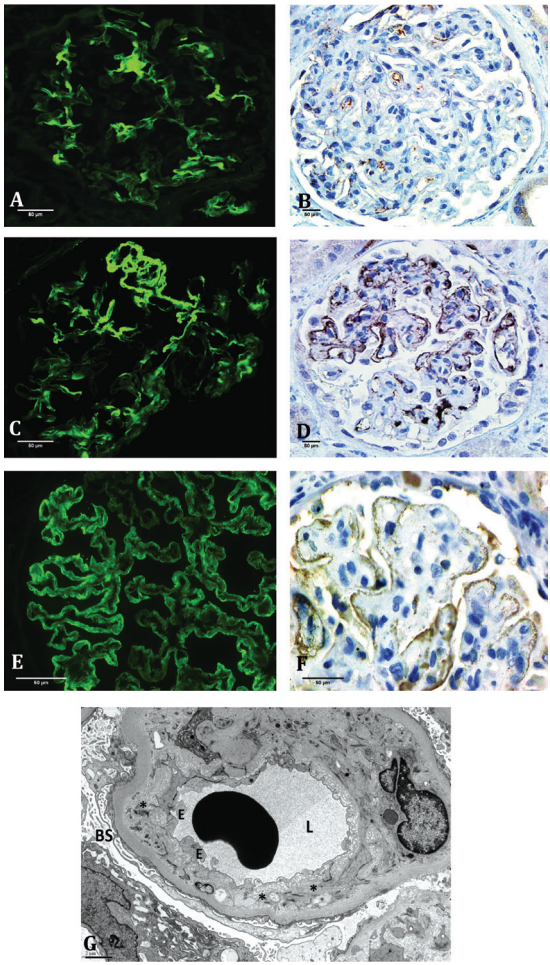


**Figure 1 (A-F).** Normal glomeruli illustrated in (A,B) do not show distinct GBM-C4d by IF (A) or IHC (B). However, by IF they often reveal C4d deposits in mesangial/ paramesangial zones (A); this mesangial staining pattern does not carry any diagnostic significance. Mesangial C4d staining is not seen by IHC showing only nondiagnostic C4d staining in intracapillary protein precipitations ('fixation artifact' in B). Segmental and global GBM-C4d staining are illustrated in C-F. The deposits are dense, granular or segmentally linear (termed here 'pseudolinear') and located along endothelial and epithelial aspects of thickened glomerular capillary walls; intramembranous GBM-C4d staining can be less pronounced. Indirect immunofluorescence microscopy with a monoclonal antibody directed against C4d, x400 (A,C) and x600 oil (E); immunohistochemistry on formalin-fixed and parrafin-embeded tissue sections with a polyclonal rabbit anti human C4d antibody, x400 original magnification (B,D) and x600 oil (F).

C4d staining intensity was strong in 12/116 (10%) and weak to moderate in 25/116 cases (22%). The staining pattern of biopsies with strong GBM-C4d was most frequently diffuse and global (n=11/12, 92%), whereas the staining pattern of cases with weak to moderate staining was often focal and segmental (n=10/25, 40%) (Fig. 1).

Both by IF and IHC, GBM-C4d staining was most pronounced under endothelial cells and podocytes; staining within thickened GBM segments was often less intense. The staining pattern was dense granular or linear, often detected side by side in the same glomerulus (termed "pseudolinear"; Fig. 1).

## Glomerular basement membrane duplications and GBM-C4d

*Light Microscopy*

TG was significantly associated with GBM-C4d staining, both by IF (94%, 49/52 TG) and IHC (67%, 20/30 TG), p<0.001 (Table 1). In particular, strong GBM-C4d expression corresponded with TG (24/30 biopsies (80%) with strong GBM-C4d by IF had TG and 9/12 (75%) biopsies with strong GBM-C4d by IHC had TG; p<0.001). The GBM-C4d staining intensity by IF and IHC increased as Banff cg scores increased; this correlation was statistically significant (IF: rs=0.453, p<0.001; IHC: rs=0.478, p<0.001; Table 1). GBM-C4d in cases with TG was seen in isolation, that is without corresponding ptc-C4d, in 73% (36/49) of biopsies by IF and in 60% (12/20) by IHC.

*Electron Microscopy*

Ultrastructural evidence of GBM duplication was significantly associated with GBM-C4d positivity (IF: 53 biopsies GBM-C4d positive/61 biopsies with GBM duplication by EM, 87%, p<0.001; IHC: 22/40 cases, 55%, p<0.01, Table 1). The C4d staining intensity correlated with the degree of ultrastructural GBM changes (IF: rs=0.474, p<0.001 and IHC: rs=0.433; p<0.001; Fig. 2). Cases with an unremarkable GBM by both LM and EM (n = 111) had no GBM-C4d staining by IF in 52% (85% by IHC), and weak-to-moderate GBM-C4d by IF in 47% (10% by IHC); strong GBM-C4d staining with normal GBM was exceptionally rare (1% by IF and 5% by IHC).

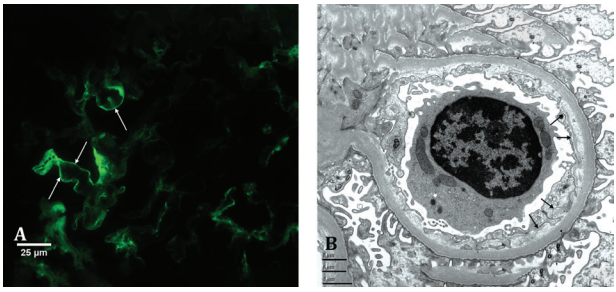


**Figure 2 (A and B).** GBM-C4d deposits associated with structural capillary wall remodelling: Minor GBM duplication, often limited to few glomerular capillaries and only noted by electron microscopy, can be reflected by segmental pseudolinear GBM-C4d staining. (A) GBM-C4d staining by IF is seen along few peripheral glomerular capillary walls (arrows); mesangial C4d deposits are considered to be nondiagnostic. (B) Corresponding EM illustrates minor structural capillary wall changes with thin layers of rudimentary new densa (arrows) under activated glomerular endothelial cells. The newly formed subendothelial lamina densa is considered to be the morphologic correlate for the GBM-C4d deposits. The illustrated changes can be seen in native kidney biopsies with TMA or in early transplant glomerulopathy. Indirect immunofluorescence microscopy with a monoclonal antibody directed against C4d, x600 oil (A); transmission electron microscopy, uranyl acetate staining, x6000 (B).

**Table 1. GBM remodelling by light microscopy or electron microscopy and corresponding GBM-C4d staining intensities by immunofluorescence microscopy or immunohistochemistry**

| | | IF GBM-C4d | | | | | IHC GBM-C4d | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Absent (0+) | Weak (1+) | Moderate (2+) | Strong (3+) | Total | Absent (0+) | Weak (1+) | Moderate (2+) | Strong (3+) | Total |
| Degree of GBM duplication by LM (TG) | TG absent; cg0 | 120 (97) | 114 (90) | 27 (67) | 6[a] (20) | 267 (83) | 69 (87) | 7 (70) | 7 (47) | 3[b](25) | 86 (74) |
| | TG present; cg1 | 1 (1) | 5 (4) | 1 (3) | 2 (7) | 9 (3) | 2 (3) | 1 (10) | 2 (13) | 0 (0) | 5 (4) |
| | TG present; cg2 | 1 (1) | 1 (1) | 0 (0) | 0 (0) | 2 (1) | 1 (1) | 0 (0) | 0 (0) | 0 (0) | 1 (1) |
| | TG present; cg3 | 1 (1) | 6 (5) | 12 (30) | 22 (73) | 41 (13) | 7 (9) | 2 (20) | 6 (40) | 9 (75) | 24 (21) |
| | Total | 123 (100) | 126 (100) | 40 (100) | 30 (100) | 319 (100) | 79 (100) | 10 (100) | 15 (100) | 12 (100) | 116 (100) |
| Degree of GBM remodelling by EM | Normal GBM | 58 (77) | 47 (71) | 13 (43) | 2 (7) | 120 (60) | 37 (62) | 4 (50) | 1 (7) | 3 (27) | 45 (48) |
| | Lamina rara interna widening | 9 (12) | 4 (6) | 4 (13) | 0 (0) | 17 (9) | 5 (8) | 0 (0) | 3 (21) | 0 (0) | 8 (9) |
| | Non-circumferential new lamina densa formation | 5 (7) | 8 (12) | 7 (23) | 1 (4) | 21 (11) | 9 (15) | 1 (13) | 1 (7) | 0 (0) | 11 (12) |
| | Circumferential new lamina densa formation | 3 (4) | 7 (11) | 6 (20) | 24 (89) | 40 (20) | 9 (15) | 3 (37) | 9 (64) | 8 (73) | 29 (31) |
| | Total | 75 (100) | 66 (100) | 30 (100) | 27 (100) | 198 (100) | 60 (100) | 8 (100) | 14 (100) | 11 (100) | 93 (100) |

Values are presented as number of biopsies (%). GBM, glomerular basement membrane; LM, light microscopy; EM, electron microscopy; TG, transplant glomerulopathy by LM evaluated according to Banff '97 cg scoring criteria (cg-0 = no or only minimal TG by LM up to cg-3 = global, marked TG); IF, immunofluorescence microscopy; IHC, immunohistochemistry. [a] Four of six cases with strong GBM-C4d by IF and no TG had GBM duplication by EM. [b] Three of three cases with strong GBM-C4d by IHC and no TG had concomitant ptc-C4d staining.

Also, a combined cohort of 82 cases with GBM duplication either by LM (classical TG), and/or by EM demonstrated a significant association between GBM remodelling and GBM-C4d deposits ($p<0.001$). In this group, GBM-C4d was seen in isolation without corresponding ptc-C4d in 62% (51/82) by IF and 31% (15/48) by IHC. By IF, the sensitivity and specificity of GBM-C4d for GBM duplication was 89% (73/82) and 48% (114/237), respectively. In comparison, isolated, strong GBM-C4d was less sensitive (22%, 18/82) but more specific (99%, 235/237). IHC, performed in 48/82 with GBM duplication and 68/237 cases without GBM duplication, had a sensitivity of 54% and a specificity of 84%. By IHC, isolated, strong GBM-C4d was most specific 100% (68/68) and least sensitive (8%, 4/48). Of note: in Table 1, four of six cases with strong GBM-C4d by IF had circumferential GBM duplication by EM only.

Isolated strong GBM-C4d occurred in temporal association with the development of GBM duplication and typical TG by LM. In early biopsies taken <1 year post-transplantation, TG was present in 7/140 (5%) biopsies; isolated strong GBM-C4d was not found. In late biopsies, taken ≥1 year post-transplantation, TG by LM was present in 45/172 (26%) biopsies. Isolated strong GBM-C4d by IF was seen in 20/172 (12%) cases (in 16/45, 36%, cases with TG) and by IHC in 4/28 (14%) cases with TG.

In the cohort of 61 patients with available repeat biopsies, 3/61 had strong isolated GBM-C4d by IF and circumferential GBM duplication by LM and/or EM in their index biopsy. From these three patients, between one and three follow-up biopsies were obtained over 7-16 months. All repeat biopsies had continuous isolated GBM-C4d staining of various intensity; there was no evidence of circulating donor-specific antibodies in 2/2 patients tested.

**GBM-C4d staining and chronic active presumably AMR: a step-wise exclusion analysis**

To determine whether GBM-C4d staining was associated with GBM remodelling in cases lacking diagnostic evidence of antibody-mediated injury, we performed a stepwise exclusion analysis. In steps 1 and 2, we excluded cases with morphologic signs commonly associated with active antibody-mediated tissue injury and in step 3 additionally cases based on their donor-specific antibody status (Table 2).

*Step 1) Exclusion of cases with peritubular capillary C4d staining.*

All biopsies with ptc-C4d deposits either by IF (37/319) or IHC (31/116) were excluded. GBM-C4d staining was seen in 36/39 (92%) remaining TG cases by IF and in 12/18 (67%) by IHC. Correlations in this subgroup closely reflected findings described above: GBM-C4d remained significantly associated with the presence of TG ($p<0.001$), and typically, strong GBM-C4d staining indicated the underlying presence of GBM duplication.

*Step 2) Additional exclusion of cases with transplant glomerulitis.*

Glomerulitis is often associated with active antibody-mediated rejection. All biopsies with transplant glomerulitis and/or peritubular capillary C4d deposits (as outlined in step 1) were excluded. By IF, GBM-C4d staining was seen in 22/24 (92%) remaining TG cases and in 6/10 (60%) by IHC. GBM-C4d staining remained significantly associated with TG ($p<0.001$).

*Step 3) Additional exclusion of cases with suspected donor-specific antibodies.*

We chose a conservative approach and excluded all biopsies from patients who were positive for donor-specific antibodies at least once post-transplantation or who had an undetermined donor-specific antibody status (i.e. no data on donor-specific antibodies available). By IF, GBM-C4d staining was seen in 8/8 remaining TG cases and by IHC in 1/1.

**Table 2. Correlations between GBM-C4d staining and TG: *stepwise exclusion of parameters associated with AMR***

| | | IF GBM-C4d | | | | | | IHC GBM-C4d | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Step** | **TG** | **Absent (0+)** | **Weak (1+)** | **Moderate (2+)** | **Strong (3+)** | **Total** | ***P* value** | **Absent (0+)** | **Weak (1+)** | **Moderate (2+)** | **Strong (3+)** | **Total** | ***P* value** |
| Step 0 *all cases* | Absent | 120 (98) | 114 (91) | 27 (68) | 6 (20) | 267 (84) | <0.001 | 69 (87) | 7 (70) | 7 (47) | 3 (25) | 86 (74) | <0.001 |
| | Present | 3 (2) | 12 (9) | 13 (33) | 24 (80) | 52 (16) | | 10 (13) | 3 (30) | 8 (53) | 9 (75) | 30 (26) | |
| | Total | 123 (100) | 126 (100) | 40 (100) | 30 (100) | 319 (100) | | 79 (100) | 10 (100) | 15 (100) | 12 (100) | 116 (100) | |
| Step 1 | Absent | 119 (98) | 98 (89) | 22 (71) | 4 (20) | 243 (86) | <0.001 | 61 (91) | 4 (80) | 2 (22) | 0 (0) | 67 (79) | <0.001 |
| | Present | 3 (2) | 11 (11) | 9 (29) | 16 (80) | 39 (14) | | 6 (9) | 1 (20) | 7 (78) | 4 (100) | 18 (21) | |
| | Total | 122 (100) | 109 (100) | 31 (100) | 20 (100) | 282 (100) | | 67 (100) | 15 (100) | 9 (100) | 4 (100) | 85 (100) | |
| Step 2 | Absent | 116 (98) | 96 (92) | 22 (76) | 4 (36) | 238 (91) | <0.001 | 61 (94) | 4 (100) | 2 (29) | 0 (0) | 67 (87) | <0.001 |
| | Present | 2 (2) | 8 (8) | 7 (24) | 7 (64) | 24 (9) | | 4 (6) | 0 (0) | 5 (71) | 1 (100) | 10 (13) | |
| | Total | 118 (100) | 104 (100) | 29 (100) | 11 (100) | 262 (100) | | 65 (100) | 4 (100) | 7 (100) | 1 (100) | 77 (100) | |
| Step 3* | Absent | 69 (100) | 45 (95) | 11 (79) | 1 (25) | 126 (94) | <0.001 | 33 (100) | 1 (100) | 0 (0) | 0 (0) | 34 (97) | 0.057 |
| | Present | 0 (0) | 2 (4) | 3 (21) | 3 (75) | 8 (6) | | 0 (0) | 0 (0) | 1 (100) | 0 (0) | 1 (3) | |
| | Total | 69 (100) | 47 (100) | 14 (100) | 4 (100) | 134 (100) | | 33 (100) | 1 (100) | 1 (100) | 0 (100) | 35 (100) | |

Step 0, all cases included; Step 1, excluding cases with C4d staining along peritubular capillaries (ptc-C4d); Step 2, excluding cases with ptc-C4d and/or transplant glomerulitis; Step 3, excluding cases with ptc-C4d, transplant glomerulitis, and/or DSA.* In this conservative approach 'erroring on the side of DSA positivity' a patient/case was defined as 'DSA positive' if either donor-specific antibodies were detected at any time during follow-up, or if the DSA status was undetermined/DSA data lacking. Values are presented as number of biopsies (%). GBM-C4d, pseudolinear C4d staining along the glomerular basement membrane; TG, transplant glomerulopathy by LM evaluated according to Banff '97 criteria; IF, immunofluorescence microscopy; IHC, immunohistochemistry; DSA, donor-specific antibodies.

**Uni- and Multivariate analysis of GBM-C4d staining.**

By univariate analysis, the presence of GBM-C4d staining was significantly correlated with the presence of a duplicated GBM by LM and/or EM (combined group of n=82 biopsies) and with signs of antibody-mediated rejection, that is presence of donor-specific antibodies, transplant glomerulitis and ptc-C4d (Table 3). By multivariate logistic regression, only the presence of a duplicated GBM by LM and/or EM and the presence of ptc-C4d independently correlated with GBM-C4d (variables with $p<0.25$ in the univariate analysis were included as independent variables; Table 4).

**Table 3. Univariate analysis correlating GBM-C4d with various morphologic and clinical parameters**

| Variable | IF GBM-C4d absent (n=123) | IF GBM-C4d present (n=196) | *P* value |
|---|---|---|---|
| GBM duplications by LM or EM | 9 (7) | 73 (37) | <0.001 |
| DSA positivity | 32 (31) | 66 (46) | 0.013 |
| Positive cross-match at time of transplantation | 6 (6) | 9 (6) | 0.969 |
| PRA > 30% | 9 (9) | 19 (12) | 0.368 |
| Transplant glomerulitis | 4 (3) | 29 (15) | 0.001 |
| IF ptc-C4d present | 1 (1) | 36 (18) | <0.001 |
| Female sex | 49 (40) | 94 (48) | 0.156 |
| Race | | | 0.557 |
| Caucasian | 47 (38) | 86 (44) | |
| African American | 64 (52) | 95 (49) | |
| Other | 12 (10) | 15 (7) | |
| Hepatitis C Virus infection | 8 (7) | 20 (12) | 0.187 |

Values are presented as number of biopsies (%). IF GBM-C4d = pseudolinear C4d staining along the glomerular basement membrane observed by immunofluorescence microscopy. IF ptc-C4d = C4d staining along peritubular capillaries observed by immunofluorescence microscopy. GBM, glomerular basement membrane; LM, light microscopy; EM, electron microscopy; DSA, donor-specific antibodies; PRA, panel reactive antibodies.

**Table 4. Multivariable regression analysis of parameters associated with GBM-C4d deposits**

| Variable | O.R. | 95% Confidence Interval | *P* value |
|---|---|---|---|
| GBM duplication by LM or EM | 4.712 | 1.853 - 11.980 | 0.001 |
| Transplant glomerulitis | 1.306 | 0.349 - 4.887 | 0.692 |
| DSA positivity | 1.163 | 0.641 - 2.110 | 0.619 |
| IF ptc-C4d present* | 17.656 | 3.616 - infinity | <0.001 |
| Female sex | 0.851 | 0.486 - 1.490 | 0.573 |
| Hepatitis C Virus infection | 1.480 | 0.567 - 3.861 | 0.423 |

GBM, glomerular basement membrane; LM, light microscopy; EM, electron microscopy; DSA, donor-specific antibodies. IF ptc-C4d = C4d staining along peritubular capillaries observed by immunofluorescence microscopy. Variables with p<0.25 in the univariate analysis (see Table 3) were included into the multivariable logistic regression analysis. * Exact conditional logistic regression was performed.

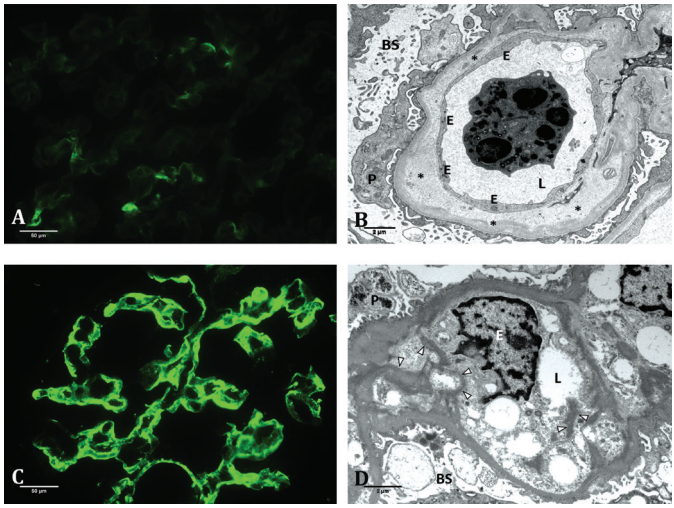


**Figure 3 (A-D)**. Native kidneys with thrombotic microangiopathy. TMA without GBM-C4d (A, corresponding EM in B): EM illustrates activated glomerular endothelial cells and widened subendothelial zones with watery/flocculent material (asterisks in B) but no GBM duplication/ no new subendothelial densa formation, explaining the lack of GBM-C4d deposits. TMA with GBM-C4d staining: in contrast, global, marked GBM-C4d can be associated with intracapillary remodelling and ill-formed matrix accumulation (white arrow heads in D). Illustrated in C and D is a case of TMA in the setting of preeclampsia. L, glomerular capillary lumen; BS, Bowman's space; P, podocyte; E, glomerular capillary endothelial cells. Indirect immunofluorescence microscopy with a monoclonal antibody directed against C4d, x400 (A), x600 oil (C); transmission electron microscopy, uranyl acetate staining, x6000 (B) and x7000 (D).

### Native kidneys

To investigate the association between GBM duplication and GBM-C4d staining in a setting without donor-specific antibodies, 35 native control biopsies were studied (n=26 chronic TMA with GBM duplication, and n=9 minimal change disease without GBM duplication). In native controls, GBM-C4d was significantly associated with GBM duplication, both by IF and IHC (IF: $p<0.001$; IHC: $p<0.05$; Fig. 3). In biopsies with minimal change disease, GBM-C4d was not observed. In biopsies with TMA, GBM-C4d was observed in 24/26 (92%) cases by IF, 14/24 with weak to moderate and 10/24 with strong GBM-C4d staining intensity. GBM-C4d staining intensity by IF was significantly correlated with the Banff cg GBM duplication scores (IF: $rs=0.764$, $p<0.001$; strong C4d expression was associated with a cg-3 Banff glomerular remodelling score in 10/10 cases). By IHC, GBM-C4d was present in 9/26 (35%) TMA controls. Ptc-C4d staining was not observed in any native kidney biopsy.

### Morphologic correlate for GBM-C4d staining intensity in native and transplant kidneys

The degree of GBM-C4d staining was significantly associated with the degree of GBM duplication and the degree of subendothelial new densa multilamination. No GBM-C4d was noted in cases with only 'watery' widening of the lamina rara interna lacking new subendothelial densa formation/duplication (Fig 3A, B). Such ultrastructural changes are seen in very early stages of microangiopathy-like glomerular injury. In comparison, strongest staining was found in glomeruli with multiple subendothelial layers of rudimentary new densa, often seen post recurrent glomerular endothelial injury. Also accumulation of intracapillary matrix-like proteinaceous material, as seen in a case of preeclampsia, showed marked GBM-C4d staining (Fig. 3C, D).

### Immunogold labelled glomerular C4d deposits

Immunogold staining signals were noted in subendothelial glomerular zones with GBM duplication and to a lesser degree also along visceral epithelial cells (Fig. S1).

## DISCUSSION

Our study sheds new light on the significance of C4d deposits along glomerular capillary walls. In a heterogeneous group of transplant and native kidney biopsies, we demonstrated for the first time that pseudolinear C4d accumulation along glomerular capillary walls was tightly associated with subendothelial new lamina densa formation and GBM duplication. GBM-C4d as a reflection of structural glomerular capillary wall remodelling was observed in native and transplant kidneys, independent of the underlying etiology (Fig. 4). The GBM-C4d staining pattern mirrored the degree of glomerular capillary wall remodelling with no staining in cases showing only widening of the lamina rara interna but lacking subendothelial new lamina densa formation/duplication, focal and segmental staining in cases with minor GBM duplication, and global staining in biopsies with widespread capillary wall restructuring. The strongest staining intensities were seen in cases with marked subendothelial new densa multilamination or intracapillary matrix accumulation as seen with repetitive and severe endothelial injury, including TMA in the setting of preeclampsia. GBM-C4d was also noted under podocytes, likely reflecting subepithelial capillary wall turnover and restructuring originating from visceral epithelial cells. By immunogold labelling, C4d deposits were mainly detected in architecturally altered subendothelial zones. Structural glomerular changes were reflected with the highest sensitivity by IF on frozen tissue samples, whereas IHC revealed highest specificity; overall, our C4d staining characteristics (IF versus IHC) were in agreement with previously published observations.[35]

Glomerular C4d deposits can be seen in various settings and must be interpreted in the appropriate context. For example, weak linear GBM and more pronounced paramesangial C4d staining is found in normal glomeruli by IF as a reflection of physiologic complement and GBM turnover, lacking specific diagnostic significance but providing positive internal IF staining controls.[16, 36] In our study, physiologic complement turnover might explain the high prevalence of linear GBM-C4d in normal glomeruli noted by IF. Such a glomerular C4d pattern is less apparent in formalin-fixed biopsies examined by IHC, likely due to differences in assay sensitivities and antibody affinities.[35, 37] In the setting of glomerulonephritides, distinct, finely-granular C4d staining can be found marking the presence of immune complex deposits along the GBM

and in mesangial zones.[17, 18, 36] Post kidney transplantation, antibody-mediated rejection with complement activation commonly shows C4d along peritubular capillaries and often concomitant pseudolinear GBM-C4d staining, as reported here and elsewhere (Fig. 4).[17, 21-23, 27] Ptc-C4d accumulation can occur and vanish within days,[9, 33] and serves as dynamic 'etiologic' marker for antibody-induced graft injury. In contrast, pseudolinear GBM-C4d, in particular when seen in isolation without concurrent ptc-C4d, can be of different significance. We noted isolated GBM-C4d only in older grafts post year-1 when TG developed. Isolated GBM-C4d did not vanish but rather remained a constant finding over months based on the evaluation of repeat biopsies. The late occurrence and tight link to glomerular capillary wall remodelling, suggest structural GBM alterations with duplication rather than active antibody-mediated rejection as a cause for complement accumulation along glomerular capillary walls. This notion is further supported by several of our findings. First, 60-70% of transplant biopsies with TG demonstrated isolated GBM-C4d, rendering it a rather common finding. Second, GBM-C4d was significantly associated with GBM duplication by multivariate analysis, post-exclusion of antibody-mediated graft injury, and importantly, in native kidney biopsies with thrombotic microangiopathies of various etiologies. Third, the degree of GBM-C4d staining was correlated with the degree of architectural changes. We, therefore, conclude that distinct, especially isolated global or segmental GBM-C4d can indicate glomerular basement membrane remodelling with new lamina densa formation. Its detection should stimulate a targeted search for thrombotic microangiopathy-like glomerular injury that might be in an early stage of development. As stains to detect C4d are standard practice, the approach outlined here is simple and can facilitate an early diagnosis when glomerular duplication is limited and progression under new therapies potentially preventable.[38, 39] Whether ptc-C4d with concomitant GBM-C4d in cases of acute antibody-mediated rejection might represent – at least in some cases – a very early form of GBM remodelling is undetermined and requires future studies.

If GBM duplication is reflected by GBM-C4d, then can architectural changes along peritubular capillaries be reflected by ptc-C4d because multilamination of peritubular capillary basement membranes is a frequent finding in thrombotic microangiopathies and chronic rejection?[40] Although not specifically studied here, we think there is no data to support such speculation.

For example, we found that native kidney specimens with chronic TMA revealed isolated GBM-C4d in 46% of cases; ptc-C4d was not seen. Potential differences in the response to injury in the microvasculature of peritubular capillaries versus glomerular capillaries are currently not defined.

Complement is known to play a role in diseases affecting glomerular capillaries. Complement activation is associated with the atypical haemolytic uremic syndrome, a form of TMA caused by dysregulation of the alternative complement pathway (reviewed by George et al.[41]). Also the complex lectin pathway of complement activation, including serine proteases that have activating effects on endothelial cells and the complement cascade via C4, might be of pathophysiologic significance during glomerular capillary wall remodelling and duplication.[42-45] The capacity of glomerular endothelial cells to syntheize complement factor C4,[46] adds to the role that the complement system might play in this scenario. Recently, complement C4d and C5b-9 deposits were described in TMA and interpreted as possible diagnostic biomarkers in the clinical work-up of patients.[29]

In the current study, we identify subendothelial and subepithelial zones as 'construction sites' with GBM-C4d accumulation. We provide, together with Chua et al.,[29] strong evidence that complement is an integral building block during GBM remodelling in various settings, independent of a specific underlying etiology. However, it is beyond the scope of the current biopsy-based study to specifically address many aspects of pathophysiology or therapeutic intervention.

In conclusion, we show that pseudolinear C4d deposits along peripheral glomerular basement membranes can be seen in different settings: (i) as etiologic markers for active ongoing antibody-induced renal graft rejection, especially when seen with concurrent ptc-C4d, or (ii) as structural markers for microangiopathy-like GBM duplication in transplant and native kidneys, independent of the underlying remote or recent triggering event. In renal transplants, GBM-C4d as a structural marker becomes most apparent if detected with strong staining intensity and in isolation without concurrent ptc-C4d. Thus, GBM-C4d does not necessarily indicate a specific underlying disease etiology, and its presence should not be in-and-by itself interpreted as a reliable diagnostic marker for active ongoing antibody-mediated rejection (Fig. 4).

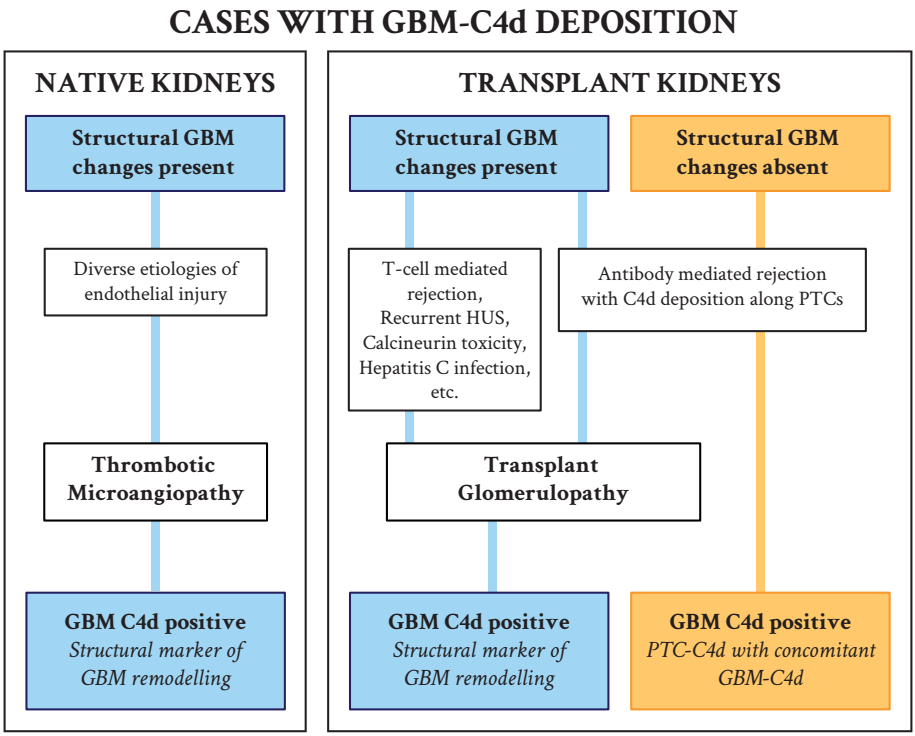


**Figure 4.** Flowchart highlighting two pathways associated with pseudolinear complement factor C4d deposits along glomerular capillary walls. (i) Both in native and transplant kidneys, various injuries can lead to structural, microangiopathy-like GBM remodelling with capillary wall duplication and newly formed subendothelial lamina densa as the morphologic correlate for GBM-C4d. Thus, in these cases, GBM-C4d deposits mark 'architectural' glomerular changes (in the flowchart: outlined in light blue). Of note: in particular strong and isolated GBM-C4d deposits not accompanied by C4d staining along peritubular capillaries mark structural glomerular capillary wall remodelling independent of the underlying etiology. (ii) In comparison, in renal allografts with active antibody-mediated rejection, including C4d staining along peritubular capillaries (ptc-C4d), GBM-C4d can be observed in normal glomeruli lacking capillary wall remodelling and GBM duplication (in the flowchart: outlined in yellow). These latter GBM-C4d deposits represent a concomitant 'spill over' phenomenon in the setting of ptc-C4d.

**Disclosures:** The authors have nothing to disclose.

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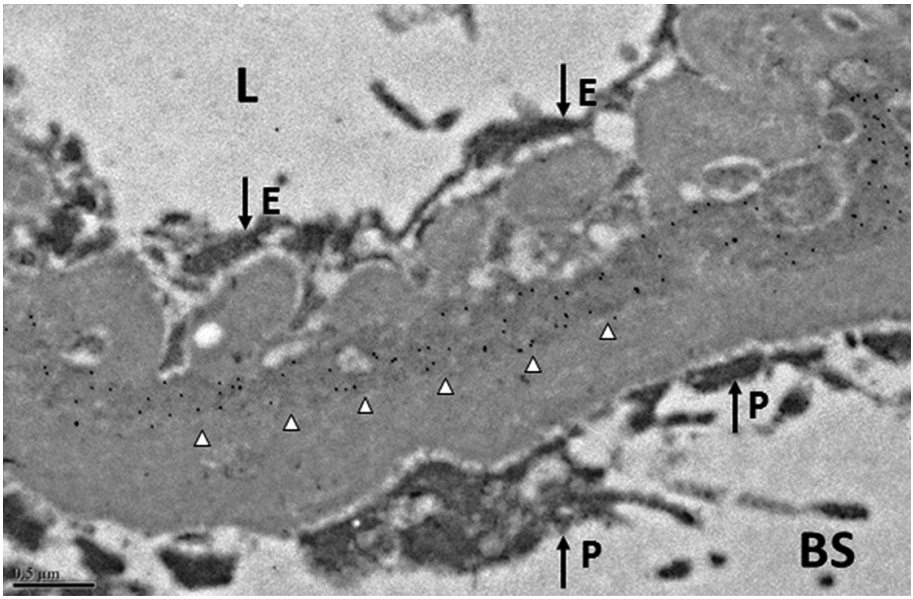
# SUPPLEMENTAL DATA




**Supplemental Figure S1. Immunogold labelling, EM image of transplant glomerulopathy.** The glomerular capillary wall is duplicated with new subendothelial densa deposition and GBM-C4d deposits in remodelled subendothelial zones (marked by black dots representing gold particles, white arrow heads). Only few scattered C4d deposits/black dots are noted within the GBM proper and along podocytes. Immunogold labeling EM with a rabbit polyconal anti-human C4d antibody, 5-nm gold particles. Frozen tissue originally collected for diagnostic purposes was subsequently processed for EM/immunogold studies, x14000. L, glomerular capillary lumen; BS, Bowman's space; P, podocyte; E, glomerular capillary endothelial cells.

## Supplemental Methods

*Clinical data – Testing of antibodies*

Cross-match data (T-cell and B-cell flow cytometry) and donor-specific antibody data were available from 158 and 156 patients, respectively. Following standard laboratory testing, alloantibody profiles were assessed by solid phase analysis with flow cytometry or multiplex bead arrays. Sera were screened for class I and II human leukocyte antigen antibodies with FlowPRA Class I and II beads (Thermofisher); positive cases were further analysed using Class I and/or II single antigen bead arrays (Thermofisher). At UNC, a 'positive' donor-specific antibody status was defined as a mean fluorescence intensity (MFI) of 1000 or greater. A positive cross-match was based upon a 'mean channel fluorescence shift' when test serum was compared to negative control serum.

*Immune electron microscopy*

To investigate the ultrastructural location of GBM-C4d, biopsy specimens obtained from one patient with a TMA in the native kidney and one additional patient with TG (both with cg-3 scores and diffuse, global and strong C4d staining by IF and IHC) were prepared for immunogold studies using a modified pre-embedding staining protocol.[47] Thin slices (<0.5mm) of frozen biopsy tissue were thawed and fixed in 4% paraformaldehyde/0.15M sodium phosphate buffer overnight at 4°C. After buffer washes and blocking with Aurion goat blocking solution (Electron Microscopy Sciences), sections were incubated with mouse monoclonal (Quidel) or rabbit polyclonal (ALPCO) anti-human C4d antibodies (diluted 1:50) for 24 hours at 4°C. Negative controls were incubated concurrently in dilution buffer only. Following buffer washes, samples were incubated with Aurion Ultrasmall GAM or GAR 0.8nm immunogold (diluted 1:100) for 16 hours at 4°C, embedded in LR White resin followed by ultrathin sectioning and grid preparation. GOLDENHANCE™-EM Formulation (Nanoprobes) was used for 3 or 10 minutes to facilitate signal detection. Sections were observed and photographed without further contrast enhancement using a LEO EM910 transmission electron microscope operating at 80kV (Carl Zeiss Microscopy, LLC, Peabody, MA). Digital images were acquired using a Gatan Orius SC1000 CCD Digital Camera and Digital Micrograph 3.11.0 (Gatan, Inc., Pleasanton, CA).

# Chapter 5

## Classical complement pathway activation in the kidneys of women with preeclampsia

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## ABSTRACT

A growing body of evidence suggests that complement dysregulation plays a role in the pathogenesis of preeclampsia. The kidney is one of the major organs affected in preeclampsia. Because the kidney is highly susceptible to complement activation, we hypothesized that preeclampsia is associated with renal complement activation. We performed a nationwide search for renal autopsy material in the Netherlands using a computerized database (PALGA). Renal tissue was obtained from 11 women with preeclampsia, 25 pregnant controls, and 14 nonpregnant controls with hypertension. The samples were immunostained for C4d, C1q, mannose-binding lectin, properdin, C3d, C5b-9, IgA, IgG, and IgM. Preeclampsia was significantly associated with renal C4d—a stable marker of complement activation—and the classical pathway marker C1q. In addition, the prevalence of IgM was significantly higher in the kidneys of the preeclamptic women. No other complement markers studied differed between the groups. Our findings in human samples were validated using a soluble fms-like tyrosine kinase 1 mouse model of preeclampsia. The kidneys in the soluble fms-like tyrosine kinase 1–injected mice had significantly more C4 deposits than the control mice. The association between preeclampsia and renal C4d, C1q, and IgM levels suggests that the classical complement pathway is involved in the renal injury in preeclampsia. Moreover, our finding that a soluble fms-like tyrosine kinase 1–injected mice develop excess C4 deposits indicates that angiogenic dysregulation may play a role in complement activation within the kidney. We suggest that inhibiting complement activation may be beneficial for preventing the renal manifestations of preeclampsia.

# INTRODUCTION

Preeclampsia is a severe multisystem pregnancy-related complication that causes high maternal and perinatal morbidity and mortality rates worldwide.[1] Preeclampsia complicates 2% to 8% of pregnancies and is characterized by endothelial damage, resulting in maternal hypertension and proteinuria after gestational week 20.[2]

Although the precise pathogenesis of preeclampsia is unknown, a growing body of evidence suggests that complement dysregulation plays a role in the development of preeclampsia.[3] In support of this notion, women with preeclampsia have complement dysregulation in the placenta and elevated circulating levels of complement degradation products.[4, 5] In addition, individuals with mutations in genes that encode complement regulatory proteins are predisposed to developing preeclampsia.[6] Finally, in a case report, a terminal complement inhibitor was used successfully to reduce preeclampsia-associated conditions, thereby prolonging pregnancy in a patient with preeclampsia.[7]

In preeclampsia, the kidney is a target organ that develops severe damage leading to renal dysfunction, proteinuria, and abnormal renal histology.[8] These symptoms are believed to reflect endothelial damage because of a dysregulation of proangiogenic and antiangiogenic factors.[8, 9] For example, an increase in the antiangiogenic factor soluble fms-like tyrosine kinase 1 (sFlt-1) can prevent vascular endothelial growth factor from maintaining the renal endothelium, thereby leading to endothelial damage.[9, 10] Damage to the fenestrated glomerular endothelium can activate the complement system.[11-13] A recent study showed that patients with severe preeclampsia have a higher prevalence of urinary excretion of the terminal complement complex compared with controls, suggesting that the complement system may be involved in generating or mediating renal damage in preeclampsia.[14] In addition, treating preeclamptic mice with complement inhibitors can reverse proteinuria and histopathological lesions.[15] Interestingly, a case report showed glomerular C4d deposits in a patient with preeclampsia.[16] We previously demonstrated that preeclampsia is associated with activation of the classical complement pathway in the placenta.[4] Here, we investigated whether preeclampsia is associated with classical complement activation in the kidney. To address this question, we measured the presence of complement components in a unique cohort of renal autopsy tissue samples collected from preeclamptic patients. To validate our findings, we studied complement components in an sFlt-1–induced mouse model of preeclampsia.

# METHODS

**Patient selection and nationwide PALGA search for renal autopsy tissue**

To study the role of the complement system in the renal pathology of preeclampsia, we performed a nationwide search for renal autopsy tissues in the Netherlands using the Dutch Pathology Registry (PALGA), a histopathology and cytopathology network and registry that includes all pathology laboratories within the Netherlands.[17] The search parameters were autopsy, women, age between 18 and 45 years, and since 1990. We included all patients who were pregnant and were confirmed cases of preeclampsia.[18] In addition, we included 2 control groups: (1) pregnant women without a hypertensive disorder either before or during their pregnancy; this group was included to investigate the effect of pregnancy alone; and (2) young nonpregnant women with a medical history of chronic hypertension; this group was included to investigate the effect of hypertension alone. The search yielded paraffin-embedded kidney samples from 11 patients with preeclampsia, 25 pregnant controls, and 14 nonpregnant chronic hypertensive controls. If available, clinical characteristics were obtained from the autopsy reports. The records of the National Maternal Mortality Committee of the Dutch Society of Obstetrics and Gynecology were used to confirm the cause of death of each pregnant case.[19] All tissue samples were coded and treated anonymously in accordance with Dutch national ethics guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies). This study was approved by the Medical Ethics Committee of the Leiden University Medical Center (P12.107).

**sFlt-1 mouse model of preeclampsia**

All animal experiments were performed at the Beth Israel Deaconess Medical Center in accordance with International Animal Care and Use Committee guidelines. Preeclampsia is a multifactorial disease in which immunological factors, genetic factors, oxidative stress, and antiangiogenic factors such as sFlt-1 are involved.[20] We used the sFlt-1–induced mouse model of preeclampsia, which overexpresses the sFlt-1 protein and develops high blood pressure, proteinuria, and endotheliosis.[21] In brief, on gestational day 9.5, pregnant female CD1 mice (Charles River, Wilmington, MA) received a tail vein injection of 2 x $10^9$ pfu of either an adenovirus encoding sFlt-1 (Ad-sFlt-1) or an equivalent dose of

the empty adenovirus vector CMV null (Vector Laboratories, Philadelphia, PA). This model has been well characterized in both rats and mice and leads to hypertension, proteinuria, and glomerular endothelial damage 7 to 10 days after adenoviral injection of sFlt-1.[9, 21-24] For our studies, mice were euthanized on gestational day 17.5, and one kidney from each mouse was frozen for immunofluorescence; the other kidney was formalin-fixed and embedded in paraffin. Paraffin-embedded kidney sections were stained with Periodic Acid Schiff or silver using standard protocols.

**Histology, immunohistochemistry, and immunofluorescence**

Sections of human kidney samples were stained with Periodic Acid Schiff and silver using standard protocols. To measure human renal complement activation, immunohistochemistry was performed on adjacent kidney sections. We used primary antibodies against the following proteins: C4d (1:50; Biomedica Gruppe, Vienna, Austria), a cleavage product of C4 that binds covalently to the target tissue and can arise from the classical and lectin pathways; C1q (1:800; DakoCytomation, Glostrup, Denmark), which reflects activation of the classical complement pathway; mannose-binding lectin (MBL) (1:500; Sigma-Aldrich Biotechnology, St. Louis, MO), which reflects activation of the lectin pathway; properdin (1:200; kindly provided by the Department of Nephrology, LUMC, Leiden, Netherlands), which reflects activation of the alternative complement pathway; and C3d (1:800; Abcam, Cambridge, UK) and SC5b-9 (1:150; Quidel, San Diego, CA), both of which are formed by activation of any of the three aforementioned pathways.

To identify immunoglobulin deposits in the human glomeruli, immunofluorescence was performed for IgA, IgG, and IgM. First, the sections were treated with protease XXIV (Sigma-Aldrich) at 37°C for one hour. The sections were then incubated for one hour with fluorescein isothiocyanate-labeled rabbit antihuman IgA (1:20; DakoCytomation), fluorescein isothiocyanate-labeled goat antihuman IgG (1:25; Protos Immuno Research, Burlingame, CA), or fluorescein isothiocyanate- labeled rabbit antihuman IgM (1:20; DakoCytomation).

To identify apoptotic cells, the samples were immunostained for caspase-3 (1:300; Cell Signaling Technology, Inc, Danvers, MA).

Immunohistochemistry was performed after the sections were deparaffinized and treated for antigen retrieval. Staining was visualized using the appropriate horseradish peroxidase-labeled secondary antibodies with diaminobenzidine as the chromogen. Finally, the sections were counterstained with hematoxylin. In addition, the TdT-mediated dUTP nick-end labeling (TUNEL) technique was used in accordance with the manufacturer's instructions (In Situ Cell Detection Kit; Roche, Basel, Switzerland).

To study colocalization of complement and endothelial cells in the mouse kidneys, frozen sections were immunostained using a rat monoclonal anti-C4 antibody (1:200; Cedarlane Laboratories, Burlington, ON, Canada), which binds to murine C4, C4b, and C4d, and a goat polyclonal antibody against von Willebrand factor (1:250; Affinity Biologicals Inc, Ancaster, ON, Canada), an endothelial marker. Staining was visualized using a fluorescein isothiocyanate-conjugated rabbit antirat antibody and a TRITC-conjugated rabbit antigoat antibody.

To measure IgM deposits in mouse glomeruli, frozen sections were fixed with acetone for 5 minutes, then incubated for 1 hour with Alexa 488-conjugated goat antimouse IgM (1:200; Invitrogen, Carlsbad, CA).

**Complement activation**

Mouse C3 fragments (C3b/C3c/iC3b) were measured by sandwich ELISA using a specific rat-anti-mouse mAb for capture (clone 2/11, HM1065; Hycult Biotechnology) and biotinylated rabbit antimouse C3 pAb (HP8012; Hycult Biotechnology) for detection.[25] Zymosan-activated CD1 mouse serum (IMSCD1-COMPL, Innovative Research) was used as a standard and set to 100 AU/mL as described previously.[26]

**Quantification of immunohistochemistry and immunofluorescence**

The human kidney sections were scored histologically by an experienced renal pathologist who was blinded with respect to the subjects' clinical data. Each immunostained sample was evaluated and scored by 2 independent observers. Because the renal pathological manifestations of preeclampsia are present in the glomerulus, we scored the staining of the various markers in the glomerulus only, scoring ≥50 glomeruli per section. The immunostained complement components were scored semi-quantitatively as follows: 0 represents an absence

of—or traces of—glomerular staining; 1 represents segmental glomerular staining; and 2 represents global staining of the glomeruli. If positive (score ≥1), the kidney sections were further classified as having either focal (10% to 50% of the glomeruli) or diffuse (>50% of the glomeruli) deposits. Caspase-3 staining was analyzed by counting the number of caspase-3–positive cells in 50 glomeruli and comparing the number of positive cells between the study groups. TUNEL staining was scored as absent or present. For immunofluorescence, the slides were analyzed for either the absence or presence of immune deposits in the glomeruli using both a fluorescence microscope (DM5500B; Leica Instruments) and a confocal laser-scanning microscope (LSM 700; Zeiss).

**Statistical analysis**

Continuous variables and the frequencies of categorical variables were analyzed using the Student's t-test or the $\chi^2$ test as appropriate. Differences in quantitative parameters between groups were analyzed using a 1-way ANOVA (for normally distributed data) or the non-parametric Kruskal-Wallis test (for non-normally distributed data). Correlations between ordinal data and numeric data were calculated using the Spearman or Pearson coefficient, respectively. All analyses were performed using the SPSS statistical software package (version 20.0; IBM Corp.). Differences with $p<0.05$ were considered to be statistically significant.

# RESULTS

## Clinical data

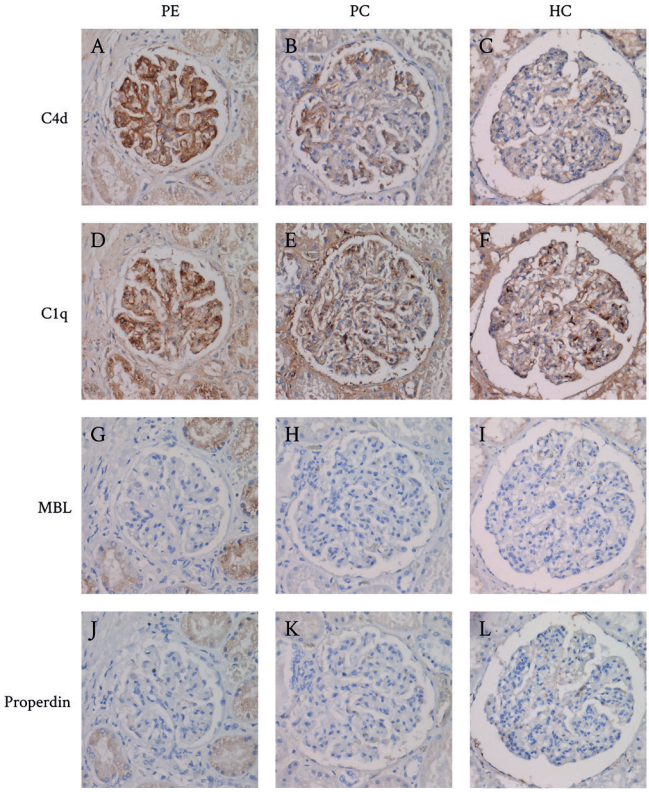
The clinical characteristics of the human subjects included in the study were previously described.[27] In brief, for the preeclamptic women, the median age was 32.5 years (interquartile range, 29-36), median gestational age was 35.7 weeks (interquartile range, 34-39), mean parity was 0.6 children, and median proteinuria was 0.36 g/24 hours (interquartile range, 0.3-6.1). The hypertensive control group was significantly older than the other 2 study groups ($p<0.05$), and the preeclamptic women had significantly higher systolic and diastolic blood pressure ($p<0.05$) than pregnant controls; no other significant differences were observed with respect to the remaining clinical characteristics. Median death-autopsy interval in the preeclamptic women was 18.0 hours (interquartile range, 6.0-32.3), which did not differ significantly from the control groups (24.0 hours; interquartile range, 16.5-24.0). The preeclamptic women and control subjects had no previously reported renal disease. The cause of death was preeclampsia-associated complications in all preeclamptic cases; the cause of death in the majority of controls included thromboembolism, aortic dissection, or infection.

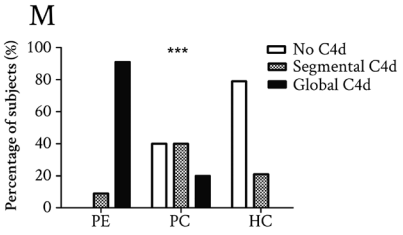
## Histology

The renal histology of this cohort was previously published.[27] In brief, the majority (82%) of preeclamptic women had prominent glomerular lesions, including various degrees of endotheliosis, podocyte swelling, and tram tracking (i.e., double contours of the glomerular basement membrane). The prevalence of endotheliosis in the preeclamptic women (55%) was higher than in the pregnant (12%; $p<0.05$) and hypertensive controls (15%; $p<0.05$). Tram tracking (36%) and podocyte swelling (18%) were present exclusively in the preeclamptic women ($p<0.05$ versus both control groups).

## Immunohistochemistry

Figure 1 shows typical examples of immunostained adjacent kidney sections from a patient with preeclampsia, a pregnant control, and a hypertensive control. The glomeruli in all 11 preeclamptic women (100%) were positive for C4d; in contrast, only 15/25 (60%) pregnant controls and 3/14 (21%) nonpregnant hypertensive controls had C4d staining in the glomeruli. Positive C4d staining



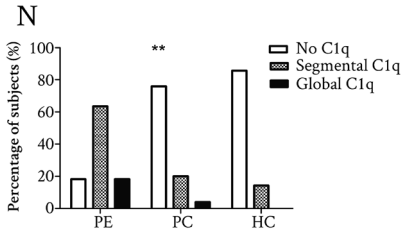




**Figure 1. Immunohistochemistry of human kidney sections.** Adjacent sections of glomeruli were immunostained for C4d (A–C), C1q (D–F), mannose-binding lectin (MBL; G–I), or properdin (J–L). Each column contains adjacent sections and shows a single glomerulus. The left column shows a glomerulus from a patient with preeclampsia (PE), with global C4d staining. The middle column shows a glomerulus from a pregnant control (PC), with segmental C4d staining. The right column shows a C4d-negative glomerulus from a hypertensive control (HC). C1q staining was present in C4d-positive glomeruli (D) but also in C4d-negative glomeruli. In C4d-positive glomeruli, colocalization of C1q and C4d was observed (A and D). MBL was rarely observed (G-I) and properdin was never observed (J-L). Summary of the prevalence of each C4d (M) and C1q (N) staining pattern in the 3 groups. Kidney sections from all preeclamptic patients were positive for C4d, with global staining in the majority of the kidney sections. In contrast, the majority of the pregnant and hypertensive controls showed a segmental or negative C4d staining pattern. Overall comparison revealed that C4d was significantly increased in preeclampsia ($p<0.001$). N, The staining patterns for C1q; C1q was significantly increased in preeclampsia ($p<0.01$). **$p<0.01$, ***$p<0.001$.

was strongly associated with preeclampsia ($p<0.001$), and global C4d staining was more prevalent in the preeclamptic women (91%) than in the controls (13%; $p<0.001$). The presence of C4d was correlated significantly with endotheliosis and tram tracking ($p<0.05$). C1q staining was observed in the glomeruli of 82% of the preeclamptic women; in contrast, C1q was detected in only 24% of the pregnant controls and 14% of the nonpregnant hypertensive controls. Positive C1q staining was significantly associated with both preeclampsia ($p<0.01$) and C4d staining ($p<0.001$). MBL was present in 1 preeclamptic woman and one pregnant control; in both samples, the staining pattern was segmental; no significant differences were found between cases and controls with respect to MBL staining ($p=0.515$). Properdin was not detected in any of the kidney samples. C3d staining was typically observed in a segmental staining pattern and was present in the glomeruli of 45% of preeclamptic women, 8% of the pregnant controls, and 14% of the nonpregnant hypertensive controls. The prevalence of glomerular C3d was significantly higher in the preeclamptic women than the controls ($p<0.05$); however, no significant correlation was found between C4d and C3d staining ($p=0.184$). The most abundant C5b-9 staining was detected in sclerotic glomeruli; C5b-9 was only present segmentally in functioning glomeruli of three autopsy samples (one from each group). Supplemental Figure S1 shows typical staining patterns of C3d and C5b-9. All positive immunostained sections had a diffuse staining pattern; none of the samples had a focal staining pattern.

No significant difference was found between the patient and control groups with respect to caspase-3 staining ($p=0.529$). Specifically, the samples from the preeclamptic women had an average of 0.05 caspase-3–positive cells/glomerulus, and the samples from the pregnant controls and hypertensive controls had an average of 0.02 and 0.12 caspase-3–positive cells/glomerulus, respectively. TUNEL staining revealed similar results (data not shown).

**Immunofluorescence**

IgA was not detected in any of the samples. However, the preeclamptic patients, pregnant controls, and nonpregnant hypertensive controls had weak mesangial IgG staining (in 27%, 8%, and 21% of the subjects, respectively; $p=0.265$ between the groups). IgM (Figure 2) was detected in 36%, 4%, and 21% of the preeclamptic patients, pregnant controls, and nonpregnant hypertensive controls, respectively ($p<0.05$ between the groups). We also analyzed the prevalence of IgM staining

based on whether the sections were C4d-positive or -negative; 14% (3/21) C4d-negative kidney sections contained IgM deposits; 7% (1/14) kidney sections with segmental C4d staining contained IgM; and 27% (4/15) kidney sections with global C4d staining contained IgM. Although IgM deposits were more prevalent in the kidney sections with global C4d, this correlation was not statistically significant. In contrast, the presence of IgM was correlated significantly with tram tracking ($p<0.001$).

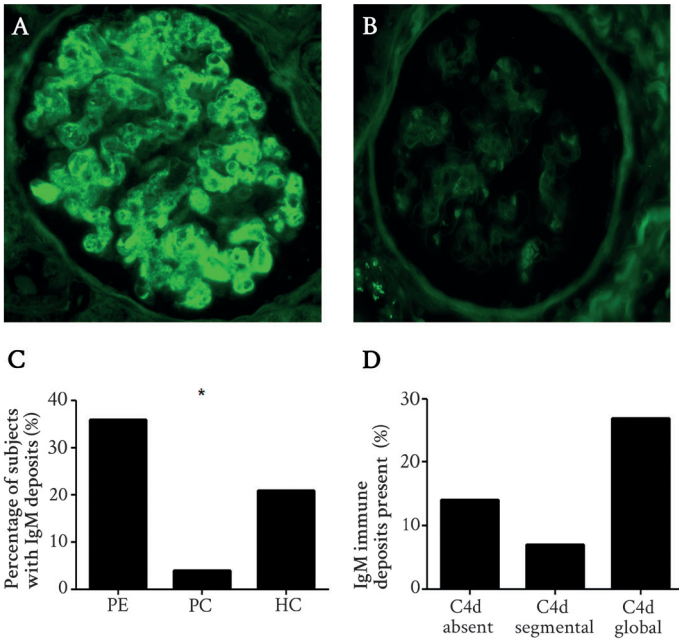



**Figure 2. Immunofluorescent staining of IgM.** Representative images of an IgM-positive glomerulus (A) and an IgM-negative glomerulus (B). C, IgM deposits were significantly more prevalent in the kidney sections from the preeclamptic women compared with the 2 control groups. D, Distribution of the percentage of IgM-positive sections based on C4d staining pattern ($p>0.05$). *$p<0.05$. HC indicates hypertensive control; PC, pregnant control; and PE, preeclampsia.

## Correlation between clinical characteristics and C4d

Among the 36 samples obtained from the preeclamptic patients and pregnant controls, 10 samples were negative for C4d, 11 samples were C4d-positive with segmental staining, and 15 samples were C4d-positive with global staining. Global C4d deposits were significantly correlated with increased gestational age ($p<0.05$), whereas C4d-negative staining was not correlated significantly with gestational age. Neither the level of proteinuria nor peak blood pressure was correlated with the pattern of C4d staining.

## sFlt-1 mouse model of preeclampsia

Next, we validated our findings in an sFlt-1 mouse model that develops endothelial dysfunction and manifests preeclampsia-like signs and symptoms.[9, 21-23] As reported previously, injecting sFlt-1 into the tail vein caused a preeclampsia-like phenotype, with significantly elevated blood pressure, urinary albumin secretion, and endotheliosis (measured using open capillary volume).[9, 28, 29] The sFlt-1–injected mice (n=6) had significantly more C4-positive glomeruli ($p<0.05$) and significantly higher levels of circulating activated C3 fragment in serum ($p<0.01$) compared with control-treated mice (n=5), indicating increased complement activation (Figure 3). IgM was present in the glomeruli of 100% and 60% of sFlt-1–injected and control-treated mice, respectively ($p=0.151$). C4 deposits were present on the endothelial cells, as demonstrated by the colocalization of C4 and von Willebrand factor in double-stained sections (Figure 4).

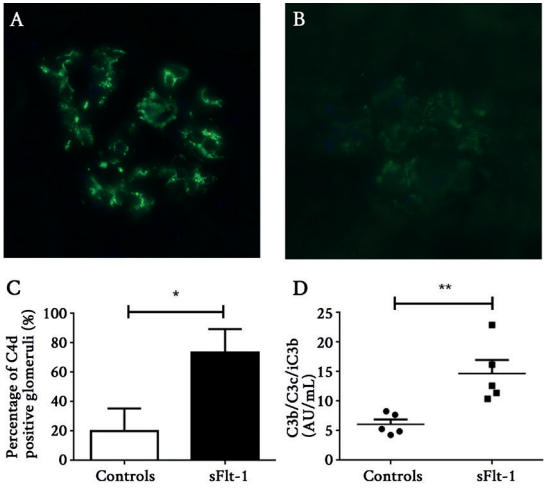



**Figure 3. Complement activation in the kidneys of soluble fms-like tyrosine kinase 1 (sFlt-1)–injected mice as a model of preeclampsia.** Representative images of C4 deposits in a glomerulus from an sFlt-1–injected (A) and a control-treated (B) mouse. The average (±SD) percentage of C4-positive glomeruli (C) is significantly higher in the kidneys of sFlt-1–injected mice (n=6 mice) than control-treated mice (n=5 mice). sFlt-1–injected mice had significantly higher levels of activated C3 fragments in the serum compared with control-treated mice (D). *$p<0.05$, **$p<0.01$. AU = arbitrary units.

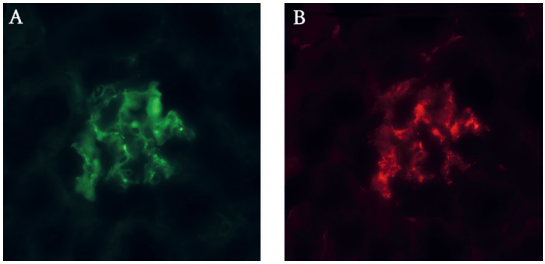



**Figure 4. Double-staining for C4 and von Willebrand factor.** Mice injected with soluble fms-like tyrosine kinase 1 (sFlt-1) have colocalization of complement factor C4 (A) and von Willebrand factor (B).

# DISCUSSION

The mechanisms that underlie the renal pathology in preeclampsia are poorly understood. Here, we report that the glomeruli of all preeclamptic women in our study were positive for C4d deposits, with a predominantly global staining pattern. In contrast, C4d deposits were significantly less prevalent in the 2 control groups, which were comprising nonhypertensive pregnant women and nonpregnant women with chronic hypertension. Importantly, C4d was correlated with C1q, whereas C4d was not correlated with MBL, and properdin was not observed; thus, the complement system seems to be activated via the classical pathway but not via the lectin or alternative pathways. In sFlt-1-injected mice, an established model of preeclampsia, the prevalence of C4-positive glomeruli and the levels of activated C3 fragment in the serum were significantly higher than in control-treated mice. Taken together, these findings suggest that preeclampsia is associated with activation of the classical complement pathway in the kidney.

We previously described a similar relationship between preeclampsia and activation of the classical complement pathway in the placenta.[4] Both the previous study and our current study raise the question of what drives activation of the classical complement pathway in the setting of preeclampsia.

In general, complement imbalance can be caused by excessive activation, inadequate regulation of the complement system, or both. Excessive complement activation could result from endothelial damage caused by angiogenic dysregulation. Angiogenic dysregulation is believed to cause the initial preeclampsia-related renal injury because of increased sFlt-1 levels preventing vascular endothelial growth factor from maintaining the renal endothelium.[9, 10] In our study, the presence of the IgM isotype was significantly associated with preeclampsia. Although glomerular IgM deposits have been observed in a wide range of renal diseases, the role of these deposits has remained elusive, suggesting that these pentameric IgM deposits might not be involved in the pathogenesis of these diseases, but rather represent nonspecific IgM entrapment.[30, 31] However, the presence of IgM deposits might have other explanations.

First, the presence of IgM antibodies and the activation of the classical complement pathway in the kidneys of preeclamptic women could have resulted from autoantibodies, such as angiotensin II type 1 receptor agonistic

antibodies ($AT_1$-AA),[32, 33] antiphospholipid auto-antibodies,[34] and antilaminin autoantibodies.[35] In the context of preeclampsia, complement activation could result from these autoantibodies binding to glomerular structures or by the deposition of circulating antibody–antigen immune complexes and their subsequent entrapment in renal tissue. In our study, although we observed glomerular immunoglobulins in preeclamptic patients and in some controls, IgM was the only immunoglobulin isotype that was significantly more prevalent in the patients with preeclampsia. If immunoglobulin deposits had resulted from auto-antibodies, we would have expected to find increased IgG deposits in the kidneys of these women. Therefore, based on our observations, it is unlikely that the glomerular complement deposits in the kidneys of the preeclamptic women were caused by autoantibodies.

Second, the presence of IgM deposits could reflect the binding of IgM antibodies to damaged endothelium. Natural IgM antibodies play a major role in the clearance of damaged cells,[36, 37] and they can bind to both hypoxic,[38] and apoptotic cells,[30, 39] through intracellular antigens that become externalized under these conditions. The binding of IgM antibodies to either hypoxic or apoptotic cells activates the complement system.[30, 38, 39] Taken together, these previously reported findings suggest that the initial endothelial damage—mediated via high sFlt-1 levels in the kidneys of preeclamptic women—might trigger the binding of IgM antibodies, thereby activating the complement system. Our finding of classical complement pathway components in the glomeruli of preeclamptic women—combined with excess deposits of C4 on glomerular endothelial cells in our sFlt-1–injected mice—supports this hypothesis. However, complement activation in preeclampsia could also result from other factors, including inflammation, immune complexes, oxidative stress, and ischemia-reperfusion damage.[40, 41]

In addition, inadequate regulation of the complement system may have caused glomerular complement activation. High levels of complement regulatory proteins are expressed in the kidney,[42-44] suggesting the presence of renal complement regulation. However, in our study, we found no correlation between late complement cascade components and preeclampsia, suggesting that the complement cascade does not become activated—at least to a detectable degree—beyond the level of C3; adequate complement regulation may be responsible for our observation. Nevertheless, the association between preeclampsia and mutations in genes that encode complement regulatory proteins suggests that inadequate

complement regulation is involved in the pathogenesis of preeclampsia.[6] Mutations in factor H, a regulator of the alternative and classical complement pathways, have been observed in relation to preeclampsia, and reduced levels of factor H have been related to angiogenic imbalance within the kidney.[45, 46]

Interestingly, IgM, IgG, and C3 deposits were reported by Tribe et al. in the glomeruli of relatively few preeclamptic women and by Petrucco et al. in the glomeruli of severe cases (almost exclusively in the afferent and efferent arterioles).[47, 48] Others have reported fibrin deposits, but failed to detect antibody or complement deposits.[49, 50] In our study, C4d was observed in the glomeruli of all preeclamptic women; the pattern was predominantly global. In contrast, IgM, IgG and C3d were less prevalent than C4d deposits. The high prevalence of C4d compared with other complement factors could be explained by the ability of C4d to bind covalently, even after the pathway-initiating factors have dissociated.[51] Because C4d was present in all preeclamptic women and was primarily present in a global staining pattern, C4d was not correlated with clinical data such as proteinuria or peak blood pressure, suggesting that the presence of C4d does not necessarily reflect disease severity in preeclampsia.

Our study has several limitations that warrant discussion. First, because we studied autopsy material, we cannot exclude the possibility that post-mortem changes may have influenced our results. Furthermore, the post-mortem death-autopsy interval in our study was longer than in the cohort studied by Sheehan, who examined the renal histopathology of preeclamptic women who were autopsied within two hours of death.[52] Therefore, our observations might differ from findings in living individuals or other autopsy studies. Nevertheless, in our study, the death-autopsy interval was similar between cases and controls. In addition, we found no significant difference in death-autopsy interval with respect to the presence or absence of histological parameters, immunoglobulins, or complement deposits (data not shown). Second, this study was an association study. Complement activation may be a cause of damage in preeclampsia in some patients, which is supported by the association with mutations in complement regulatory genes, the efficacy of eculizumab during active clinical disease, and the efficacy of inhibiting complement activation in a mouse model of preeclampsia.[6, 7, 15] Complement activation may also be a result of damage, as supported by this study.

In summary, complement activation is involved in the renal damage that occurs in women with preeclampsia. Future studies that systematically manipulate complement factors will likely determine whether complement activation is a cause of renal damage in preeclampsia, a consequence of this damage, or both. Future studies should also be designed to determine whether inhibiting complement activation is a viable option for treating the renal manifestations of preeclampsia.

**Disclosures**

E.V. Khankin is supported by National Institute of Health KO8 award and S.A. Karumanchi is supported by Howard Hughes Medical Institute. S.A. Karumanchi is a coinventor on patents related to angiogenic markers in preeclampsia and is a consultant to Siemens and Aggamin. The other authors report no conflicts.

## PERSPECTIVES

Here, we report the extensive activation of the classical complement pathway in the kidneys of preeclamptic women. The presence of excess C4 deposits in our sFlt-1–induced preeclampsia mouse model strongly supports the notion that preeclampsia-related renal complement activation is initiated by endothelial damage. Our results suggest that complement activation might contribute to renal injury in preeclampsia. Moreover, our findings suggest that inhibiting the complement system might reduce both the renal and placental manifestations of preeclampsia.

## NOVELTY AND SIGNIFICANCE

### What is new?

1. The kidney sections obtained from all of the preeclamptic women in our study contained C4d deposits in the glomeruli, indicating that preeclampsia is associated with renal activation of the classical complement pathway.
2. Our hypothesis that angiogenic dysregulation plays an important role in triggering complement activation in the kidney is supported by our finding that the prevalence of C4-positive glomeruli and the serum levels of activated C3 fragments were significantly increased in soluble fms-like tyrosine kinase 1 (sFlt-1)–injected mice, an established model of preeclampsia.

### What is relevant?

1. Our study suggests that initial endothelial damage mediated via high sFlt-1 levels in the kidneys of preeclamptic women can trigger the binding of IgM antibodies, thereby activating the complement system
2. Complement activation may contribute to renal injury in preeclampsia.
3. Our findings provide compelling evidence that inhibiting the complement system could significantly reduce the renal manifestations of preeclampsia.

### Summary

The clear association between preeclampsia and renal C4d, C1q, and IgM levels suggests that the classical complement pathway plays a role in the pathogenesis of renal injury in preeclampsia. Moreover, our finding that mice injected with sFlt-1 develop excess C4 deposits and increased levels of circulating activated C3 indicates that angiogenic dysregulation may play an important role in complement activation within the kidney. Based on these findings, inhibiting complement activation may help prevent the renal manifestations of preeclampsia.

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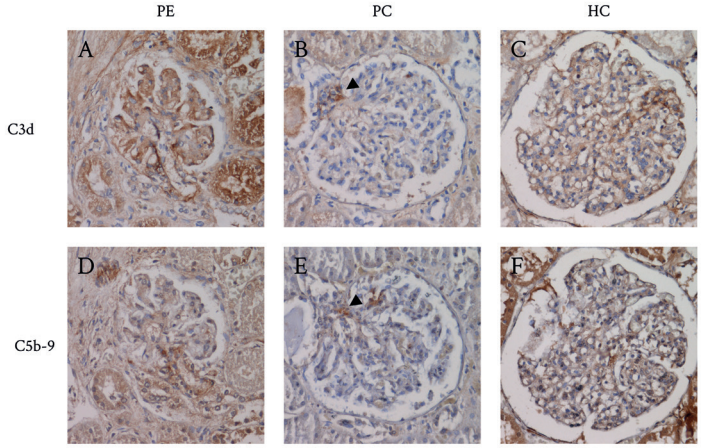
# SUPPLEMENTAL DATA




**Supplemental Figure S1. Immunohistochemical staining pattern of C3d and C5b-9 in human kidneys.** Adjacent sections were immunostained for C3d (A–C) or C5b-9 (D–F). Each column represents an individual glomerulus. The left column shows a glomerulus from a patient with preeclampsia (PE), showing global staining. The middle column shows a glomerulus from a pregnant control (PC), with a segmental staining pattern. The right column shows a glomerulus from a hypertensive control (HC). C3d deposits were observed in the glomeruli in all study groups, whereas C5b-9 deposits were relatively rare. However, these C3d deposits co-localized with C5b-9 deposits (arrowheads).

# Chapter 6

## Complement activation in patients with diabetic nephropathy

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# ABSTRACT

**Introduction**: Complement activation plays a role in various organs in patients with diabetes. However, in diabetic nephropathy (DN), the role of complement activation is poorly understood. We examined the prevalence and clinical significance of complement deposits in the renal tissue of cases with type 1 and type 2 diabetes with and without DN.

**Methods:** We measured the prevalence of glomerular C4d, C1q, mannose-binding lectin (MBL), and C5b-9 deposits in 101 autopsied diabetic cases with DN, 59 autopsied diabetic cases without DN, and 41 autopsied cases without diabetes or kidney disease. The presence of complement deposits was scored by researchers who were blinded with respect to the clinical and histological data.

**Results:** C4d deposits were more prevalent in cases with DN than in cases without DN in both the glomeruli (46% vs. 26%) and the arterioles (28% vs 12%). C1q deposits were also increased in the glomerular hili (77% vs. 55%) and arterioles (33 vs. 14%) and were correlated with DN ($p<0.01$). MBL deposits were only rarely observed. C5b-9 deposits were more prevalent in the cases with diabetes mellitus (DM) than in the cases without DM (69% vs. 32%; $p<0.001$). Finally, glomerular C4d and C5b-9 deposits were correlated with the severity of DN (rho = 0.341 and 0.259, respectively; $p<0.001$).

**Discussion:** Complement activation is correlated with both the presence and severity of DN, suggesting that the complement system is involved in the development of renal pathology in patients with diabetes and is a promising target for inhibiting and/or preventing DN in these patients.

# INTRODUCTION

Diabetic nephropathy (DN), the leading cause of end-stage renal disease worldwide, can occur in patients with either type 1 or type 2 diabetes mellitus (DM).[1-3] DN is characterized by a gradual increase in proteinuria and blood pressure, and a gradual decrease in glomerular filtration rate that may result in the need for renal replacement therapy. Prolonged hyperglycemia can lead to the development of DN via a number of pathways with complex interactions; however, the precise cellular and molecular mechanisms that underlie this process are poorly understood.[4-6]

The first evidence for a possible role of the complement system in the development of DN was provided by the finding that serum, urine, and renal samples obtained from patients with diabetes often contained activated complement proteins, and that these proteins are associated with DN.[7-14] Serum levels of mannose-binding lectin (MBL) are correlated with the severity of DN, which suggest a role for the lectin complement pathway.[15] In addition, advanced glycation end-products can directly bind C1q and activate the complement system,[16] and DN has been associated with increased renal expression of complement factors C3, C4, and C9 (at the protein level), as well as increased expression of C1q, C1s, and C1r (at the mRNA level), which also suggest a role for the classical complement pathway.[8, 17] Furthermore, hyperglycemia can cause glycation-induced dysfunction and/or inactivation of complement regulatory proteins, including CD59, which inhibits C5b-9 under physiological conditions;[11, 18] this glycation-induced complement dysregulation leads to increased C5b-9 levels in patients with DN.[11] In a rat model of type 2 diabetes, treating diabetic rats with a C3a receptor antagonist improved renal function and reduced both albuminuria and the deposition of extracellular matrix proteins.[19]

Recently, 2 groups reviewed the role of complement activation in DM.[7, 14] They concluded that the relative role of complement in the development of DM-related complications, including DN, are unknown. Moreover, it remains unclear whether these mechanisms are similar between type 1 DM and type 2 DM.[7] To address these questions, we examined whether complement activation occurred in renal autopsy samples obtained from a large cohort of cases with diabetes with and without DN. Specifically, we measured the prevalence, localization, and staining patterns of renal C4d and C5b-9 deposits. Furthermore,

because both the lectin and classical complement pathways can lead to C4d deposits, and because both pathways might be involved in the development of DN, we also determined which complement pathway was associated with the deposition of C4d in cases with DN. We validated our findings in renal biopsy samples. Finally, we correlated complement deposition with histopathology, and examined differences in complement deposition between cases with type 1 and 2 DM.

## METHODS

This study group includes a selection of a previously described cohort.[20] In brief, we retrospectively searched the database of our pathology department for native kidneys from adult cases with either type 1 or type 2 DM who were autopsied from 1984 to 2004. We initially included 184 autopsied kidneys that were prepared for light microscopy, electron microscopy, and immunohistochemistry. We subsequently excluded 25 cases due to poor tissue quality or missing tissue for immunostaining. Thus, we included a total of 159 samples from cases with diabetes for whom we confirmed the histopathological presence or absence of DN according to the classification for DN.[21] In addition, we included a control group consisting of autopsy samples obtained from 41 cases without diabetes and without renal pathology. We validated our findings with autopsy tissue by examining 12 kidney biopsies from patients with DN and 10 biopsies obtained from healthy living transplantation donors.

### Clinical data

The clinical information was obtained retrospectively via the medical records and autopsy reports available at Leiden University Medical Center, and the general practitioners of the patients. The following laboratory parameters were collected from a period starting 1 year before the patient died: serum creatinine, estimated glomerular filtration rate (eGFR) (calculated using the Modification of Diet in Renal Disease formula), microalbuminuria (defined as 30-300 mg/L), proteinuria (defined as >300 mg/L) measured via a 24-hour urine or dipstick test, systolic and diastolic blood pressures, serum hemoglobin, serum cholesterol, and serum glycosylated hemoglobin ($HbA_{1c}$).[20] The clinical data were analyzed to reflect a stable representation of the serum and/or urine levels, thereby

excluding data that were clearly affected by an unstable clinical condition (for example, patients who were clinically unstable in an intensive care unit before death). Cause of death was categorized into the following 5 general categories: cancer, cardiovascular, infection and/or sepsis, multiple pathologies, and other (e.g., high-impact trauma).

**Histopathology and transmission electron microscopy**

Renal tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections were cut and then stained with hematoxylin and eosin, periodic-acid Schiff, and silver using standard protocols. Glomerular lesions, interstitial lesions, and vascular lesions were scored according to the histopathological classification for DN.[21] Discrimination between class 0 (i.e., no DN) and class I DN was determined using transmission electron microscopy, as described previously.[20]

**Immunohistochemistry and immunofluorescence**

To measure renal complement activation, immunohistochemistry was performed on adjacent kidney sections using primary antibodies against the following proteins: C4d (1:150; Biomedica Gruppe, Vienna, Austria), which is a cleavage product of C4 that binds covalently to the target tissue and can arise from both the classical and lectin pathways; C1q (1:1200; DakoCytomation, Glostrup, Denmark), which reflects activation of the classical complement pathway; MBL (1:300; Sigma-Aldrich Biotechnology, Saint Louis, MO), which reflects activation of the lectin pathway; and sC5b-9 (1:500; Quidel, San Diego, CA), which is formed by activation of any of the aforementioned pathways. To investigate the presence of natural antibodies, immunofluorescence was performed on sections using a fluorescein isothiocyanate-labeled anti-IgM antibody (1:20; DakoCytomation).

The immunostained tissue samples were scored semi-quantitatively as follows: staining of glomeruli was scored as absent (representing either an absence of staining or trace levels of staining in <5% of glomeruli) or present (representing staining in ≥5% of glomeruli). If present, the distribution of glomerular staining was scored as focal (5-50% of the glomeruli) or diffuse (>50% of the glomeruli), and the staining pattern was scored as segmental (<50% of the glomerular tuft) or global (>50% of the glomerular tuft). In addition, if

present, glomerular staining was scored as present in the glomerular capillary walls, mesangial cells, or both. Immunohistochemical staining in the glomerular hilus, arterioles, and arterial branches, was scored as absent or present (i.e., the presence of staining in ≥1 glomerular hilus, arteriole, and/or arterial branch was scored as positive). Renal tissue specimens containing ≥100 glomeruli were scored by two investigators who were blinded with respect to the clinical data of the cases.

**Statistical analysis**

The SPSS statistical software package (version 20.0; IBM, Armonk, NY) was used for all statistical analyses. Categorical variables were compared using the chi-squared test or Fisher's exact test, where appropriate. Continuous variables were compared using the Student's t-test or the Mann-Whitney U test, where appropriate. Spearman's rank correlation coefficient was used to analyze the correlation between diabetes class and the presence of glomerular complement deposits. Differences with a p value $<0.05$ were considered statistically significant.

**Ethics statement**

All tissue samples were coded and then handled and analyzed anonymously in accordance with the Declaration of Helsinki. Approval for the study was obtained from the medical ethics committee of Leiden University Medical Center.

# RESULTS

## Clinical and histological characteristics

We included 159 diabetic cases; histologically confirmed DN was present in 101 cases (64%) and absent in 58 cases (36%). We also included an age- and sex-matched control cohort of 41 renal samples from autopsied nondiabetic cases without renal pathology. The clinical characteristics of the cases are summarized in Table 1. The duration of diabetes was significantly higher in the cases with DN than in the cases without DN (p=0.017); however, we found no difference between these 2 groups with respect to age, sex, diabetes type, presence of hypertension, serum creatinine, eGFR, or $HbA_{1c}$ levels.

The histological features of the cases with diabetes are summarized in Table 2. Among the 101 cases with DN, DN was distributed as follows: 20% with class I, 20% with class IIA, 10% with class IIB, 45% with class III, and 5% with class IV. Compared with the cases without DN, the cases with DN had a significantly higher prevalence of glomerular hyalinosis, glomerular capsular drop, arteriosclerosis, and arteriolar hyalinosis ($p<0.05$). In addition, the cases with DN had more interstitial fibrosis and tubular atrophy (IFTA) compared with the cases without DN (p=0.028).

## C4d deposition is associated with DN

C4d deposits in the glomeruli, glomerular hili, and arterioles were significantly more prevalent in cases with diabetes than in the control cases without diabetes ($p<0.05$) (Table 3), in contrast to C4d deposits in arterial branches (p=0.171) (Figure 1). In the diabetic cohort, C4d deposits were present in the glomeruli, glomerular hili, and arterioles of 38%, 48%, and 22% of the cases, respectively. In the nondiabetic control cohort, C4d deposits were rarely observed in any vascular structure.

In the cohort of cases with diabetes, cases with DN had a significantly higher prevalence of C4d deposits in the glomeruli and arterioles than cases without DN (Figure 1) (p=0.019 and p=0.022, respectively). The cases with DN had a higher prevalence of C4d in the glomerular capillary walls (45% vs. 26% of cases without DN; p=0.019) and in the mesangial cells (26% vs. 12%, respectively; p=0.041) than cases without DN. The distribution of glomerular C4d did not differ significantly between the 2 groups. However, the global staining pattern

of C4d was significantly more prevalent in cases with DN than patients without DN (15% vs 5%, p=0.044).

Within our cohort of cases with DN, eGFR was significantly lower in the cases with glomerular C4d than in the cases without glomerular C4d (39.8 ± 28.6 mL/min per 1.73m$^2$ vs. 60.3 ± 33.5 mL/min per 1.73m$^2$; respectively; p=0.004). In contrast, we found no other correlation between C4d deposits and clinical data (Table 4).

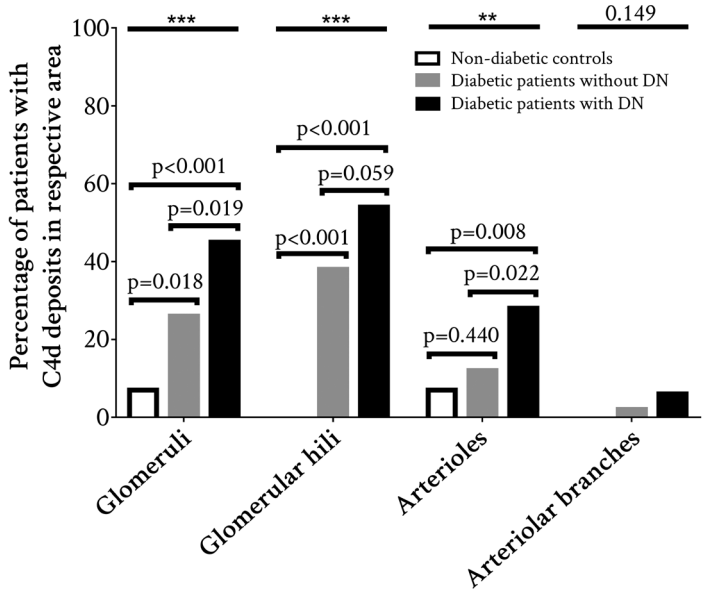


**Figure 1. Prevalence of C4d deposits in cases and controls.** The percentage of cases and controls with complement factor C4d is shown for the indicated renal structures. Asterisks represent the overall differences between the nondiabetic controls, the diabetic cases without diabetic nephropathy (DN), and the diabetic cases with DN; The p values shown between the 2 groups represent post hoc analyses. **p<0.01 and ***p<0.001.

**Table 1. Clinical characteristics of the control cases and diabetic cases**

| Clinical characteristics | Nondiabetic controls (n=41) | Diabetic cases without DN (n=58) | Diabetic cases with DN (n=101) | All diabetic cases (n=159) | *P* value |
|---|---|---|---|---|---|
| Age, years | 63.8 ± 16.8 | 69.1 ± 12.6 | 69.0 ± 12.8 | 69.1 ± 12.7 | 0.100[a] |
| Female, n (%) | 16 (39) | 24 (41) | 47 (47) | 71 (45) | 0.754[a] |
| T1DM, n (%) | NA | 5/48 (10) | 12/89 (14) | 17/137 (12) | 0.603[b] |
| Duration of diabetes, years | NA | 8.4 ± 7.3 | 15.3 ± 13.1 | 13.3 ± 10.0 | 0.003[b] |
| T1DM (median, IQR) | NA | 17.5 (2) | 28.0 (33) | 18.0 (28) | 0.215[b] |
| T2DM (median, IQR) | NA | 5.0 (5) | 10.0 (14) | 8.0 (15) | 0.004[b] |
| Serum Creatinine, mmol/L | NA | 155 ± 169 | 167 ± 112 | 163 ± 135 | 0.625[b] |
| eGFR, mL/min per 1.73m$^2$ | NA | 59 ± 36 | 50 ± 33 | 54 ± 34 | 0.184[b] |
| $HbA_{1c}$, % unit | NA | 7.5 ± 1.8 | 8.5 ± 2.4 | 8.1 ± 2.3 | 0.117[b] |
| Hypertension, n (%) | NA | 27/48 (56) | 44/85 (52) | 71/133 (53) | 0.618[b] |
| Systolic blood pressure, mmHg | NA | 134 ± 29 | 155 ± 29 | 135 ± 29 | 0.780[b] |
| Diastolic blood pressure, mmHg | NA | 75 ± 14 | 75 ± 12 | 75 ± 13 | 0.961[b] |
| Cause of death, n (%) | | | | | 0.199[a] |
| Cancer | 4 (10) | 9 (16) | 5 (5) | 14 (9) | |
| Cardiovascular | 16 (39) | 20 (34) | 49 (48) | 69 (43) | |
| Infection/Sepsis | 4 (10) | 7 (12) | 12 (12) | 19 (12) | |
| Multiple pathologies | 13 (31) | 14 (24) | 17 (17) | 31 (20) | |
| Other | 4 (10) | 8 (14) | 18 (18) | 26 (16) | |

Data are presented as the mean ± SD, unless stated otherwise. DN, diabetic nephropathy; eGFR, estimated glomerular filtration rate; $HbA_{1c}$, glycosylated hemoglobin; IQR: interquartile range; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus. [a] Between nondiabetic controls without renal disease, diabetic cases without DN and diabetic cases with DN. [b] Between diabetic cases without DN and diabetic cases with DN.

**Table 2. Histological characteristics of the diabetic cases**

| Histological characteristics | Diabetic cases without DN (n=58) | Diabetic cases with DN (n=101) | All diabetic cases (n=159) | *P* value[a] |
|---|---|---|---|---|
| DN class | | | | NA |
| 0 (no DN) | 58 (100) | 0 (0) | 58 (37) | |
| I | 0 (0) | 20 (20) | 20 (13) | |
| IIA | 0 (0) | 21 (20) | 21 (13) | |
| IIB | 0 (0) | 10 (10) | 10 (6) | |
| III | 0 (0) | 45 (45) | 45 (28) | |
| IV | 0 (0) | 5 (5) | 5 (3) | |
| Glomerular hyalinosis | 19 (33) | 85 (84) | 104 (65) | <0.001 |
| Glomerular capsular drop | 2 (3) | 15 (15) | 17 (11) | 0.025 |
| FSGS | 2 (3) | 12 (12) | 14 (9) | 0.071 |
| IFTA | | | | 0.028 |
| absent | 22 (38) | 17 (17) | 39 (25) | |
| 10-25% | 25 (43) | 56 (55) | 81 (51) | |
| 25-50% | 5 (9) | 15 (15) | 20 (13) | |
| >50% | 6 (10) | 13 (13) | 19 (12) | |
| Arteriosclerosis | 46 (79) | 92 (91) | 138 (87) | 0.035 |
| Arteriolar hyalinosis | 46 (79) | 92 (91) | 138 (87) | 0.035 |
| Cholesterol emboli | 3 (5) | 3 (3) | 6 (4) | 0.483 |

Data are presented as the number of cases (%). DN, diabetic nephropathy; FSGS, focal segmental glomerulosclerosis; IFTA, interstitial fibrosis and tubular atrophy. [a] Between diabetic cases without DN and diabetic cases with DN.

**Table 3. Summary of complement deposition in nondiabetic controls, diabetic cases without nephropathy, and diabetic cases with nephropathy**

| Location | Presence of C1q | | | | Presence of MBL | | | | Presence of C4d | | | | Presence of C5b-9 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NDC | DM+ DN- | DM+ DN+ | *P* value | NDC | DM+ DN- | DM+ DN+ | *P* value | NDC | DM+ DN- | DM+ DN+ | *P* value | NDC | DM+ DN- | DM+ DN+ | *P* value |
| **Glomerular presence** | 20 (49) | 17 (29) | 41 (41) | 0.132 | 0 (0) | 1 (2) | 8 (8) | 0.057 | 3 (7) | 15 (26) | 45 (46) | <0.001 | 13 (32) | 35 (60) | 74 (73) | <0.001 |
| Glomerular location | | | | <0.001 | | | | 0.184 | | | | <0.001 | | | | <0.001 |
| *Mesangium only* | 5 (12) | 0 (0) | 0 (0) | | 0 (0) | 0 (0) | 0 (0) | | 2 (5) | 0 (0) | 0 (0) | | 1 (2) | 2 (3) | 2 (2) | |
| *Glomerular capillary wall only* | 0 (0) | 2 (3) | 18 (18) | | 0 (0) | 1 (2) | 4 (4) | | 0 (0) | 8 (14) | 19 (19) | | 0 (0) | 0 (0) | 0 (0) | |
| *Both mesangium and capillary wall* | 15 (37) | 15 (26) | 23 (23) | | 0 (0) | 0 (0) | 4 (4) | | 1 (2) | 7 (12) | 26 (26) | | 12 (29) | 34 (59) | 72 (71) | |
| Glomerular distribution | | | | 0.023 | | | | 0.215 | | | | <0.001 | | | | <0.001 |
| *Focal* | 7 (17) | 13 (22) | 25 (25) | | 0 (0) | 1 (2) | 7 (7) | | 3 (7) | 10 (17) | 27 (27) | | 3 (7) | 8 (14) | 14 (14) | |
| *Diffuse* | 13 (32) | 4 (7) | 16 (16) | | 0 (0) | 0 (0) | 1 (1) | | 0 (0) | 5 (9) | 18 (18) | | 10 (24) | 27 (47) | 60 (59) | |
| Glomerular staining pattern | | | | 0.027 | | | | 0.197 | | | | <0.001 | | | | <0.001 |
| *Segmental* | 2 (5) | 8 (14) | 14 (14) | | 0 (0) | 1 (2) | 5 (5) | | 3 (7) | 12 (21) | 30 (30) | | 2 (5) | 11 (19) | 26 (26) | |
| *Global* | 18 (44) | 9 (16) | 27 (27) | | 0 (0) | 0 (0) | 3 (3) | | 0 (0) | 3 (5) | 15 (15) | | 11 (27) | 24 (41) | 48 (48) | |
| **Glomerular hili presence** | 4 (10) | 32 (55) | 78 (77) | <0.001 | 0 (0) | 0 (0) | 2 (2) | 0.372 | 0 (0) | 22 (38) | 54 (54) | <0.001 | 32 (78) | 55 (95) | 101 (100) | <0.001 |
| **Arteriolar presence** | 1 (2) | 8 (14) | 33 (33) | <0.001 | 0 (0) | 0 (0) | 0 (0) | NA | 3 (7) | 7 (12) | 28 (28) | 0.005 | 31 (82) | 58 (100) | 101 (100) | <0.001 |
| **Arterial branches presence** | 0 (0) | 10 (17) | 29 (29) | <0.001 | 0 (0) | 0 (0) | 0 (0) | NA | 0 (0) | 1 (2) | 6 (6) | 0.149 | 39 (98) | 58 (100) | 101 (100) | 0.136 |

All data are presented as the number of nondiabetic controls or patients (%). NDC, nondiabetic controls; DM+DN-, diabetic cases without diabetic nephropathy; DM+DN+, diabetic cases with diabetic nephropathy.

**Table 4. Correlation between clinical data and C4d deposition in autopsied cases with diabetic nephropathy**

| Clinical parameter | Glom C4d - | Glom C4d + | *P* value | Glom hilus C4d - | Glom hilus C4d + | *P* value | Art C4d - | Art C4d + | *P* value | Art branch C4d - | Art branch C4d + | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age, years | 67.6 ± 12.7 | 70.8 ± 12.7 | 0.202 | 68.9 ± 12.4 | 69.2 ± 13.1 | 0.896 | 68.3 ± 13.4 | 71.0 ± 10.9 | 0.339 | 68.9 ± 12.9 | 70.8 ± 10.6 | 0.723 |
| eGFR, mL/min/1.73m$^2$ | 60.3 ± 33.5 | 39.8 ± 28.6 | 0.004 | 58.3 ± 33.8 | 44.1 ± 30.8 | 0.053 | 53.6 ± 31.2 | 43.8 ± 35.4 | 0.211 | 51.3 ± 32.4 | 39.8 ± 37.6 | 0.414 |
| $HbA_{1c}$, % unit | 8.0 ± 2.4 | 9.0 ± 2.4 | 0.182 | 8.1 ± 3.0 | 8.7 ± 2.1 | 0.536 | 8.4 ± 2.7 | 8.7 ± 2.1 | 0.725 | 8.4 ± 2.4 | 10.3 ± 4.2 | 0.285 |
| SBP, mmHg | 129.6 ± 27.8 | 141.6 ± 29.3 | 0.092 | 139.3 ± 36.0 | 132.4 ± 23.0 | 0.381 | 133.3 ± 28.9 | 139.4 ± 29.3 | 0.425 | 133.9 ± 28.0 | 156.3 ± 40.3 | 0.135 |
| DBP, mmHg | 75.4 ± 12.9 | 74.5 ± 12.1 | 0.769 | 74.7 ± 12.9 | 75.2 ± 12.3 | 0.889 | 75.4 ± 12.6 | 74.1 ± 12.3 | 0.706 | 74.9 ± 12.6 | 76.3 ± 12.5 | 0.838 |
| Duration of DM, years | 14.1 ± 12.0 | 16.6 ± 14.3 | 0.469 | 15.8 ± 17.0 | 14.9 ± 9.8 | 0.808 | 13.0 ± 10.2 | 20.9 ± 17.5 | 0.085 | 15.5 ± 13.2 | 12.0 ± 12.5 | 0.660 |

All data are presented as the mean ± SD. Art, arterioles; Art branch, arteriolar branches; DBP, diastolic blood pressure; Glom, glomerular; $HbA_{1c}$, glycosylated hemoglobin; SBP, systolic blood pressure.

**Evidence for classical complement activation in cases with diabetes**

Next, to investigate which complement pathway(s) led to the deposition of C4d in cases with DN, we stained renal tissue for MBL (to measure the lectin pathway) and C1q (to measure the classical pathway).

MBL was only observed in 6% of the kidneys from cases with diabetes and was not observed in the control cases without diabetes. When present, the staining pattern of MBL in the glomeruli was predominantly focal and segmental; MBL was not observed in the glomerular hili, arterioles, or arterial branches. We found no significant differences between MBL deposition in the cases with diabetes and control cases without diabetes or between the cases with diabetes with DN and the cases with diabetes without DN (Figure 2A and Table 3).

Glomerular C1q was present in 36% of the cases with diabetes, and the staining pattern was predominantly focal and global. The prevalence of glomerular C1q was not significantly different between the cases with diabetes and control cases without diabetes (37% vs. 49%, respectively; $p=0.150$) (Figure 2B and Table 3). In contrast, the prevalence of C1q in the glomerular hili, arterioles, and arterial branches was significantly higher in the cases with diabetes than in the control cases without diabetes ($p\leq0.001$). Furthermore, among the cases with diabetes, the cases with DN had a significantly higher prevalence of C1q in the glomerular hili and arterioles compared with the cases without DN ($p<0.05$), whereas the prevalence of C1q in the arterial branches did not differ significantly between these 2 groups ($p=0.106$). Finally, the presence of C1q deposits was correlated with the presence of C4d deposits in the glomeruli ($p=0.006$), glomerular hili ($p=0.027$), and arterioles ($p<0.001$).

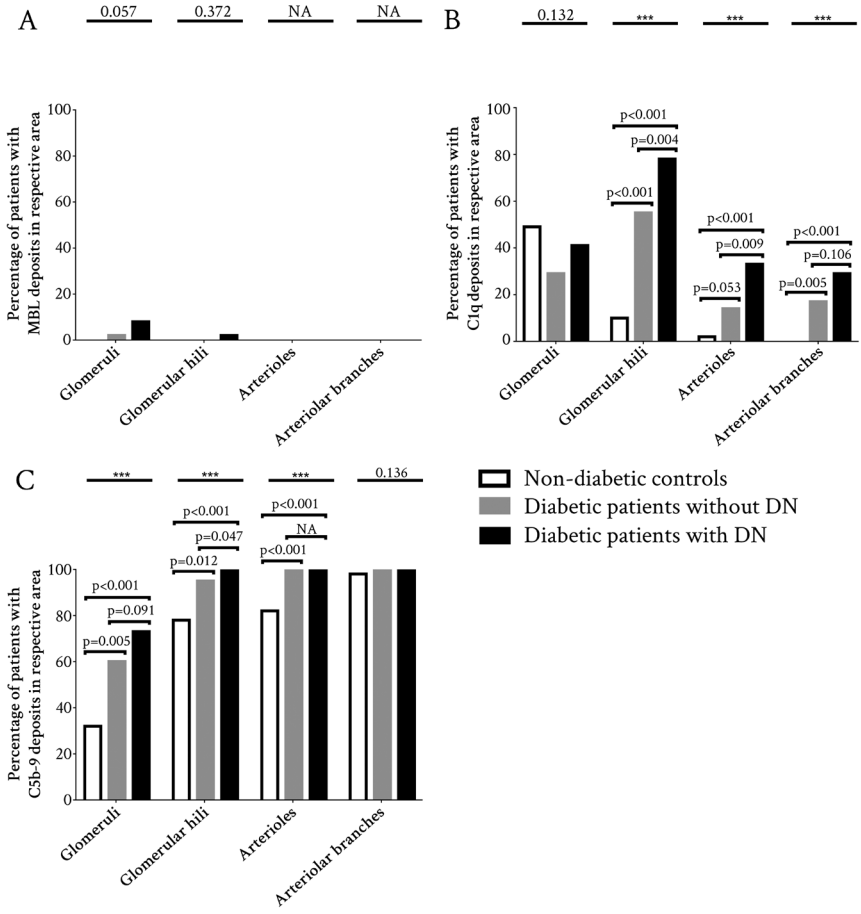


**Figure 2. Prevalence of mannose-binding lectin (MBL), C1q, and C5b-9 deposits in cases and controls.** The percentage of cases and controls with complement factor MBL (A), C1q (B), and/or C5b-9 (C) is shown for the indicated renal structures. The p values shown between the 2 groups represent post hoc analyses. ***p<0.001, $\chi^2$ test between nondiabetic controls, diabetic cases without diabetic nephropathy (DN), and diabetic cases with DN.

### C5b-9 deposits are associated with diabetes, but not with DN

In the cases with diabetes, glomerular C5b-9 staining was predominantly diffuse and global. Although the prevalence of C5b-9 deposits was significantly higher in the glomeruli, glomerular hili, and arterioles of the cases with diabetes compared with the control cases without diabetes ($p<0.001$) (Figure 2C and Table 3), the prevalence of C5b-9 was still relatively high in the control cases without diabetes. Only the prevalence of C5b-9 deposits in the glomerular hili was significantly higher in the patients with DN than in patients without DN ($p=0.047$; Figure 2C). The presence of glomerular C5b-9 deposits was correlated with the presence of glomerular C4d deposits ($p=0.002$).

The prevalence, location, distribution, and staining patterns of complement proteins in the glomeruli, glomerular hili, arterioles, and arterial branches of the cases with diabetes are listed in Table 3. Representative images of C1q, C4d, MBL, and C5b-9 staining in these structures are shown in Figure 3.

### C4d and C5b-9 deposits are correlated with histological lesions and DN class

Among the cases with diabetes, the presence of glomerular C4d was correlated with glomerular hyalinosis ($p=0.020$), IFTA ($p<0.001$), arteriosclerosis ($p=0.017$), and arteriolar hyalinosis ($p=0.017$) (Supplemental Table S1A and S1B). Moreover, the presence of C4d in the glomerular hili was correlated with glomerular hyalinosis ($p=0.002$), and the presence of C4d in the arterioles was correlated with IFTA ($p<0.001$), arteriosclerosis ($p=0.041$), and arteriolar hyalinosis ($p=0.041$). Although glomerular C1q was not correlated with histological lesions, the presence of C1q in the arterioles was correlated with the presence of glomerular capsular drop ($p=0.034$), arteriolar hyalinosis ($p=0.048$), and IFTA ($p=0.027$). The presence of MBL in the glomeruli, arterioles, and arterial branches was not correlated with any renal lesion. In addition, the presence of glomerular C5b-9 was correlated with IFTA ($p=0.008$). Finally, with respect to DN class, the prevalence of glomerular C4d and C5b-9 was correlated with more severe classes of DN ($p\leq0.001$) (Figure 4), particularly with class III and class IV DN.

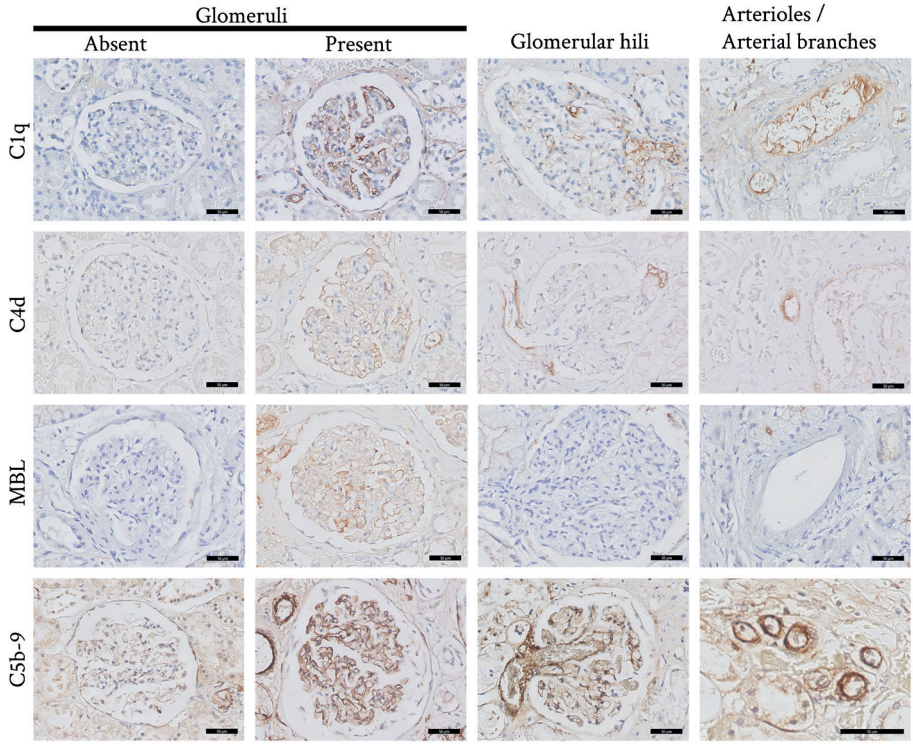


**Figure 3. Representative images of complement staining in patients.** Kidney sections were immunostained for the indicated proteins, and representative images containing the glomeruli, glomerular hili, arterioles, and arterial branches are shown. Mannose-binding lectin (MBL) staining was negative in the glomerular hilus, arterioles, and arterial branches. Bars = 50 μm.

## Differences in complement deposition between type 1 DM and type 2 DM

Data on diabetes type was available for 137 patients (86% of cases with diabetes); 17 cases (12%) had type 1 DM and 120 cases (88%) had type 2 DM. Compared to cases with type 2 DM, cases with type 1 DM had a significantly higher prevalence of C4d in glomeruli (64% vs. 33%; p=0.012), C4d in arterioles (47% vs. 18%; p=0.007), and C5b-9 in glomeruli (94% vs. 64%; p=0.013). Within the subgroup of cases with DN, cases with type 1 DM also had a significantly higher prevalence of C4d in glomeruli (75% vs. 35%; p<0.01), C4d in arterioles (50% vs. 22%; p=0.040), and C5b-9 in glomeruli (100% vs. 68%; p<0.05).

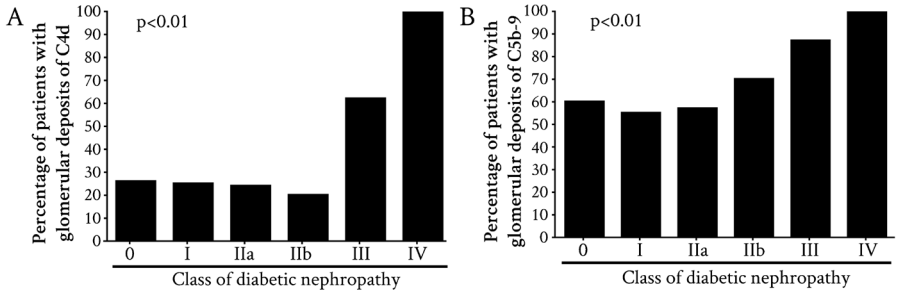


**Figure 4. Percentage of patients with glomerular C4d deposits and glomerular C5b-9 deposits plotted against diabetic nephropathy class.** The presence of glomerular C4d (A) and C5b-9 (B) was correlated with diabetic nephropathy class. For C4d and C5b-9, the Spearman rank correlation coefficient (rho) was 0.344 and 0.228, respectively (both p<0.01). Cases without diabetic nephropathy were classified as class 0.

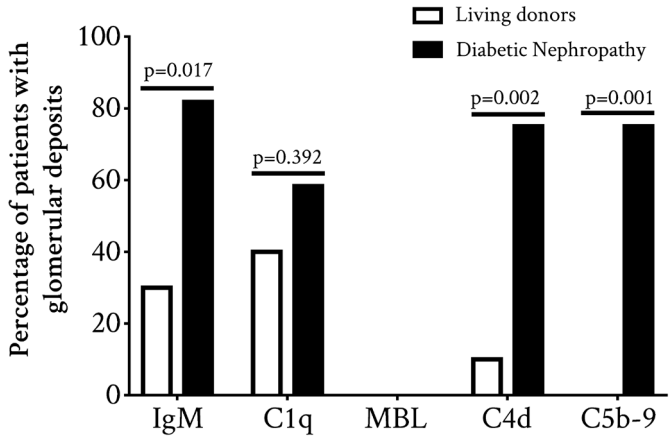


**Figure 5. Percentage of renal sections containing IgM, C1q, mannose-binding lectin (MBL), C4d, or C5b-9 deposits.** Biopsy samples were obtained from living patients with diabetic nephropathy (n=12) and healthy living renal transplantation donors (n=10) and stained for IgM, C1q, MBL, C4d, and C5b-9. P values were calculated using the $\chi^2$ test.

**Evidence for activation of the classical complement pathway in biopsies**

To support our findings with autopsy tissue, we found that the prevalence of glomerular C4d was significantly higher in biopsies from cases with DN compared with a control group of biopsies obtained from healthy living transplantation donors (75% vs. 10%, respectively; p=0.002); similar results were obtained with respect to glomerular C5b-9 deposits (75% vs. 0%, respectively; p=0.001; Figure 5). In contrast, the prevalence of glomerular C1q deposits did not differ significantly between these 2 groups (58% vs. 40%, respectively; p=0.392), and glomerular MBL deposits were not observed in either group. The prevalence of glomerular IgM deposits was significantly higher in the cases with DN compared with control cases (82% vs. 30%, respectively; p=0.017), and the prevalence of glomerular IgM deposits were significantly correlated with the prevalence of glomerular C1q (p=0.003) and C4d (p=0.001) deposits. Finally, glomerular IgM deposits co-localized with glomerular C1q and C4d deposits (Figure 6), which suggests that the classical complement pathway was activated in cases with DN.

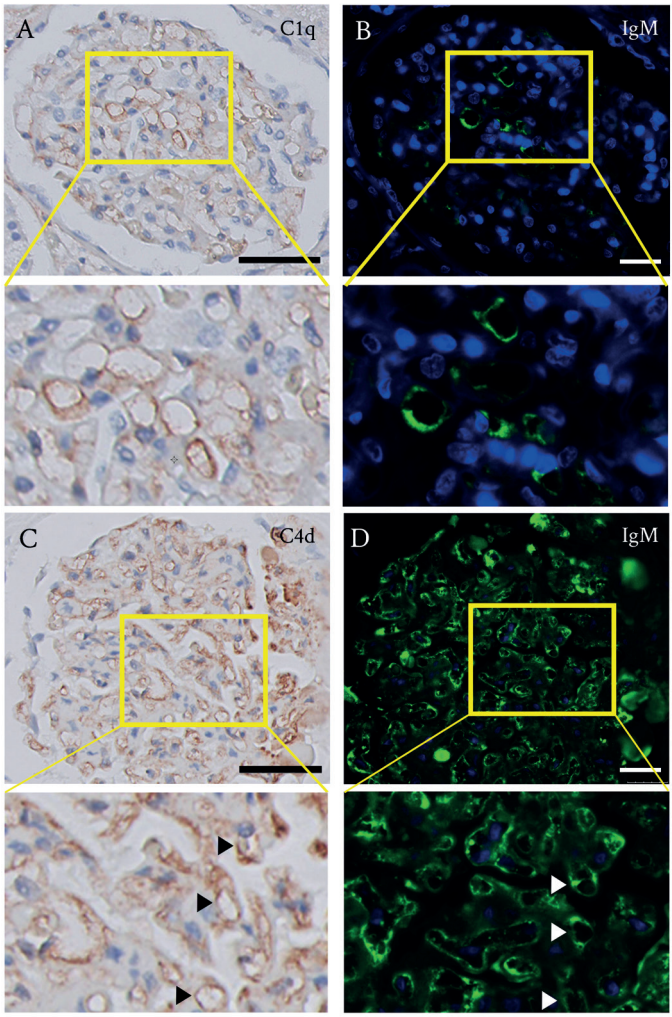


**Figure 6. Glomerular IgM deposits co-localize with glomerular C1q and C4d.** Adjacent sections of an autopsied kidney from a case with diabetic nephropathy were stained for C1q (A) or IgM (B). Adjacent sections of an autopsied kidney from a case with diabetic nephropathy were stained for C4d (C) or IgM (D); the arrowheads indicate co-localization between C4d and IgM. The scale bars in (A) and (C) represent 50 μm; the scale bars in (B) and (D) represent 25 μm.

# DISCUSSION

DN is a microvascular complication that affects 20% to 40% of patients with diabetes, making it the leading cause of end-stage renal disease.[1-3] An increasing body of evidence has suggested that complement activation via the lectin and/or classical complement pathway plays a role in the development of DN.[4-7, 14] We investigated the deposition of complement proteins in a relatively large cohort of cases with diabetes with DN and without DN. We found that the complement activation marker C4d was correlated with DN, as well as with the severity of DN, microvascular and interstitial lesions, and lower eGFR in cases with DN, suggesting that complement activation might play a role in the development of DN.

Because C4d binds covalently to its target cells, C4d can be observed long after the factors that activated the pathway have dissociated, making it a commonly used biomarker for complement activation.[22] The prevalence of C4d deposits in the glomeruli and arterioles was significantly higher in cases with DN than cases without DN and in nondiabetic controls without renal pathology, both in the autopsy cohort and in the biopsy cohort. Moreover, glomerular C4d and arteriolar C4d were more prevalent in the cases with vascular and chronic renal lesions, and glomerular C4d was correlated with the severity of DN. These data suggest that complement activation, together with the renal microvasculature, might be involved in the development of DN. This notion is supported by the general absence of C4d deposits among the control cases without diabetes.

To investigate which complement pathway could underlie the deposition of C4d, we studied the prevalence and localization of C1q (to measure the classical pathway) and MBL (to measure the lectin pathway) deposits. The presence of C1q, IgM, and C4d deposits were associated with each other, and co-localized in the same renal vascular structures, whereas MBL was detected rarely in our cohort. These findings suggest that the presence of C4d reflected activation of the classical complement pathway. However, because MBL is not covalently bound and because other proteins such as ficolins can initiate the lectin pathway, we could not exclude the possibility that activation of the lectin pathway led to C4d deposition.

C4d deposits were significantly more prevalent in kidneys from cases with type 1 DM than in kidneys from cases with type 2 DM, both in the total cohort

of cases with diabetes and in the subgroup of cases with DN. The presence of DN was not a confounder in this association because DN was not more prevalent in type 1 DM compared with type 2 DM. Rowe et al. showed similar differences in pancreatic C4d deposition among cases with type 1 DM, type 2 DM and control cases without diabetes.[23] The higher C4d prevalence between cases with type 1 DM and type 2 DM could possibly reflect a different pathogenesis. Nevertheless, we could not exclude the possibility that the difference observed in complement deposition might be attributed, at least in part, to the duration of diabetes rather than the type of diabetes.

Glomerular C4d was a common finding in our cases with class III DN. Paueksakon et al. suggested that Kimmelstiel-Wilson lesions, which are a hallmark lesion of DN and a diagnostic requirement for class III DN, might be a form of thrombotic microangiopathy. Specifically, they found that a subset of cases with DN had fragmented red blood cells exclusively in Kimmelstiel-Wilson lesions.[24] Recently, we reported that complement factor C4d was a common denominator in several thrombotic microangiopathies.[25] Thus, our data further supported the hypothesis that Kimmelstiel-Wilson lesions might be a form of thrombotic microangiopathy that arises from complement activation. The underlying cause of C4d deposits in the arterioles, arteries, and glomerular hilus (the junction between afferent and efferent arterioles) is currently unknown. Because both renal afferent arterioles and renal efferent arterioles play a role in regulating renal blood flow, these vessels can be exposed to extremely turbulent blood flow and high shear stress conditions,[26-29] which may increase vulnerability to vascular injury, complement deposition, and/or the development of renal pathology.

We found that C5b-9 deposits were more prevalent in our cases with diabetes than in control cases without diabetes; however, the clinical relevance of these deposits in our cohort is difficult to interpret, because C5b-9 was relatively frequently prevalent in control cases without diabetes (albeit to a lesser extent than in the cases with diabetes). This finding might be related in part to our use of autopsy samples because we did not observe these deposits in renal biopsies obtained from living donors, which was consistent with findings reported by Qin et al.[11] Nevertheless, in our cohort of cases with diabetes with DN, the presence of glomerular C5b-9 was correlated with the severity of DN, which suggested that C5b-9 might play a role in the progression of renal damage. Our

data suggests that the process that leads to C4d deposition also leads to C5b-9 deposition. However, C5b-9 could have also been activated due to a direct effect of hyperglycemia on regulatory proteins in the complement system. Hyperglycemia can lead to the glycation of CD59, which inhibits C5b-9 under physiological conditions.[11, 18] This glycation-induced inactivation of CD59 could lead to the formation of C5b-9.

In the setting of DN, several factors could have led to the deposition of C4d. For example, autoantibodies can activate the complement system.[30] This notion is supported by the report of linear IgG staining along the glomerular basement membrane in 60% of type 2 DM cases with DN.[31] In addition, high glucose levels in cases with DM can lead to increased levels of glycated proteins, including advanced glycation end-products and oxidized proteins,[32, 33] which can activate the classical and/or lectin pathways either directly or by reacting with autoantibodies.[16, 34-36] The complement system can also be activated by the binding of natural (i.e., IgM) antibodies to either hypoxic or apoptotic cells in the setting of DN.[37-39] Natural antibodies play an important role in clearing damaged cells via intracellular antigens that are externalized during apoptosis and/or hypoxic conditions.[37-41] We found a significantly higher prevalence of glomerular IgM deposits in biopsies from cases with DN compared with healthy living transplantation donors. Moreover, glomerular IgM deposits co-localized with—and were significantly correlated with—both C1q and C4d, which supported the hypothesis that IgM antibodies activate the classical complement pathway in cases with DN.

It is currently unknown whether complement activation is a cause and/or consequence of microvascular damage in DN. Our data suggests that complement activation was involved in the progression of renal damage in cases with diabetes mellitus because complement activation was correlated with more severe renal damage, including higher DN class and increased IFTA levels. These findings were consistent with other studies with regard to complement activation in the development of DN and other diabetes-associated microvascular and/or macrovascular complications.[7, 14] Furthermore, type 2 diabetic rats treated with a complement inhibitor had improved renal function and morphology compared with untreated rats,[19] which supports the notion that complement activation plays a role in the progression of diabetes-associated kidney disease. In the context of DM, hyperglycemia can both directly and indirectly lead to complement

deposition, which can then lead to the increased production of reactive oxygen species, activation of protein kinase C, and upregulation of nuclear factor-κB, thereby inducing the release of proinflammatory, prothrombotic cytokines, and growth factors.[7] Both complement-dependent and complement-independent mechanisms can then lead to inflammation, proliferation, and thrombosis, which together characterize the diabetes-associated complications in target organs. In contrast, complement activation may also be a consequence of renal vascular damage, possibly following activation via natural antibodies. This hypothesis is consistent with our findings regarding the presence of C4d and IgM antibodies in other renal microangiopathies.[25, 42] Moreover, we previously reported that C4d deposits were associated with remodelling of the glomerular basement membrane,[43] which was consistent with cases with DN that presented with a remodeled glomerular basement membrane. Nevertheless, despite our relatively large cohort and our ability to examine >100 glomeruli per case, the autopsy-based nature of this study precluded our ability to determine causality. Therefore, future studies should be designed to determine whether complement activation is a cause, consequence, or mediator of DN.

Our study has several limitations. First, the use of autopsy samples precluded our ability to investigate whether the prevalence of complement deposition in the glomeruli, glomerular hili, and/or vessels was associated with patient survival and/or renal survival. Second, our study might have had a selection bias because not all cases with diabetes had an autopsy; generally speaking, most cases that are autopsied were in the hospital at the time of death. Third, because autopsy samples were used, we could not exclude the possibility that post-mortem changes and/or cause of death might have affected the tissue in terms of protein expression and/or tissue morphology. However, to address this possibility, we examined control samples obtained from autopsied cases without diabetes or renal pathology. Furthermore, because cause of death was not significantly correlated with the prevalence of complement deposits, and because our findings were supported by examining renal biopsy samples, we concluded that our data were not likely affected by autopsy-related artifacts. In contrast, the strength of our study was the relatively high number of cases combined with our ability to examine >100 glomeruli per case.

In conclusion, complement activation is associated with DN, and both glomerular C4d and glomerular C5b-9 deposits are correlated with the class of

DN. Furthermore, complement deposits in several renal vascular structures are correlated with more severe renal damage. Our data suggest that complement activation is involved with the development of renal damage in cases with diabetes, and that inhibition or modulation of complement activity could be a promising therapeutic strategy for patients with DN.

**Disclosures**

All authors declared no competing interests.

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**Supplemental Table S1A. C1q deposits and histologic lesions in all diabetic cases**

| | Glom C1q - | Glom C1q + | *P* value | Glom hilus C1q - | Glom hilus C1q + | *P* value | Art C1q - | Art C1q + | *P* value | Art branch C1q - | Art branch C1q + | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Glomerular capsular drop | 10 (9.9) | 7 (12.1) | 0.670 | 6 (12.2) | 11 (10.0) | 0.672 | 9 (7.6) | 8 (19.5) | 0.034 | 13 (10.8) | 4 (10.3) | 0.919 |
| Glomerular hyalinosis | 66 (65.3) | 38 (65.5) | 0.983 | 30 (61.2) | 74 (67.3) | 0.459 | 72 (61.0) | 32 (78.0) | 0.048 | 76 (63.3) | 28 (71.8) | 0.334 |
| FSGS | 9 (8.9) | 5 (8.6) | 0.950 | 2 (4.1) | 12 (10.9) | 0.161 | 10 (8.5) | 4 (9.8) | 0.803 | 11 (9.2) | 3 (7.7) | 0.778 |
| IFTA | | | 0.712 | | | 0.109 | | | 0.027 | | | 0.053 |
| 0 | 26 (25.7) | 13 (22.4) | | 18 (36.7) | 21 (19.1) | | 35 (29.7) | 4 (9.8) | | 33 (27.5) | 6 (15.4) | |
| 1 | 53 (52.5) | 28 (48.3) | | 20 (40.8) | 61 (55.5) | | 59 (50.0) | 22 (53.7) | | 63 (52.5) | 18 (46.2) | |
| 2 | 12 (11.9) | 8 (13.8) | | 5 (10.2) | 15 (13.6) | | 11 (9.3) | 9 (22.0) | | 14 (11.7) | 6 (15.4) | |
| 3 | 10 (9.9) | 9 (15.5) | | 6 (12.2) | 13 (11.8) | | 13 (11.0) | 6 (14.6) | | 10 (8.3) | 9 (23.1) | |
| Arteriosclerosis | 88 (87.1) | 50 (86.2) | 0.869 | 43 (87.8) | 95 (86.4) | 0.811 | 101 (85.6) | 37 (90.2) | 0.449 | 101 (84.2) | 37 (94.9) | 0.086 |
| Arteriolar hyalinosis | 88 (87.1) | 50 (86.2) | 0.869 | 43 (87.8) | 95 (86.4) | 0.811 | 101 (85.6) | 37 (90.2) | 0.449 | 101 (84.2) | 37 (94.9) | 0.086 |
| Cholesterol emboli | 4 (4.0) | 2 (3.4) | 0.870 | 3 (6.1) | 3 (2.7) | 0.300 | 4 (3.4) | 2 (4.9) | 0.667 | 3 (2.5) | 3 (7.7) | 0.139 |

**Supplemental Table S1 (A-D). Complement deposits and histologic lesions shown for C1q (A), MBL (B), C4d (C) and C5b-9 (D).** All data are presented as the number of patients (%). Glom, glomeruli; glom hilus, glomerular hilus; Art, arterioles; Art branch, arterial branches; FSGS, focal segmental glomerulosclerosis; IFTA, interstitial fibrosis and tubular atrophy.

**Supplemental Table S1B. Mannose-binding lectin (MBL) deposits and histologic lesions in all diabetic cases**

| | Glom MBL - | Glom MBL + | *P* value | Glom hilus MBL - | Glom hilus MBL+ | *P* value | Art MBL - | Art MBL + | *P* value | Art branch MBL - | Art branch MBL + | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Glomerular capsular drop | 17 (11.3) | 0 (0) | 0.285 | 17 (10.8) | 0 (0) | 0.622 | NA | NA | NA | NA | NA | NA |
| Glomerular hyalinosis | 98 (65.3) | 6 (66.7) | 0.935 | 102 (65.0) | 2 (100) | 0.301 | NA | NA | NA | NA | NA | NA |
| FSGS | 12 (8.0) | 2 (22.2) | 0.144 | 14 (8.9) | 0 (0) | 0.658 | NA | NA | NA | NA | NA | NA |
| IFTA | | | 0.361 | | | 0.583 | | | NA | | | NA |
| 0 | 38 (25.3) | 1 (11.1) | | 39 (24.8) | 0 (0) | | NA | NA | | NA | NA | |
| 1 | 74 (49.3) | 7 (77.8) | | 79 (50.3) | 2 (100) | | NA | NA | | NA | NA | |
| 2 | 19 (12.7) | 1 (11.1) | | 20 (12.7) | 0 (0) | | NA | NA | | NA | NA | |
| 3 | 19 (12.7 | 0 (0) | | 19 (12.1) | 0 (0) | | NA | NA | | NA | NA | |
| Arteriosclerosis | 130 (86.7) | 8 (88.9) | 0.848 | 136 (86.6) | 2 (100) | 0.579 | NA | NA | NA | NA | NA | NA |
| Arteriolar hyalinosis | 130 (86.7) | 8 (88.9) | 0.848 | 136 (86.6) | 2 (100) | 0.579 | NA | NA | NA | NA | NA | NA |
| Cholesterol emboli | 6 (4.0) | 0 (0) | 0.541 | 6 (3.8) | 0 (0) | 0.778 | NA | NA | NA | NA | NA | NA |

**Supplemental Table S1C. C4d deposits and histologic lesions in all diabetic cases**

| | Glom C4d - | Glom C4d + | *P* value | Glom hilus C4d - | Glom hilus C4d + | *P* value | Art C4d - | Art C4d + | *P* value | Art branch C4d - | Art branch C4d + | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Glomerular capsular drop | 10 (10.1) | 7 (11.7) | 0.757 | 6 (7.2) | 11 (14.5) | 0.140 | 12 (9.7) | 5 (14.3) | 0.436 | 17 (11.2) | 0 (0) | 0.349 |
| Glomerular hyalinosis | 58 (58.6) | 46 (76.7) | 0.020 | 45 (54.2) | 59 (77.6) | 0.002 | 74 (59.7) | 30 (85.7) | 0.004 | 97 (63.8) | 7 (100) | 0.049 |
| FSGS | 8 (8.1) | 6 (10.0) | 0.679 | 6 (7.2) | 8 (10.5) | 0.464 | 11 (8.9) | 3 (8.6) | 0.956 | 12 (7.9) | 2 (28.6) | 0.059 |
| IFTA | | | <0.001 | | | 0.202 | | | <0.001 | | | 0.167 |
| 0 | 34 (34.3) | 5 (8.3) | | 26 (31.3) | 13 (17.1) | | 37 (29.8) | 2 (5.7) | | 39 (25.7) | 0 (0) | |
| 1 | 49 (49.5) | 32 (53.3) | | 39 (47.0) | 42 (55.3) | | 65 (52.4) | 16 (45.7) | | 78 (51.3) | 3 (42.9) | |
| 2 | 9 (9.1) | 11 (18.3) | | 10 (12.0) | 10 (13.2) | | 9 (7.3) | 11 (31.4) | | 18 (11.8) | 2 (28.6) | |
| 3 | 7 (7.1) | 12 (20.0) | | 8 (9.6) | 11 (14.5) | | 13 (10.5) | 6 (17.1) | | 17 (11.2) | 2 (28.6) | |
| Arteriosclerosis | 81 (81.8) | 57 (95.0) | 0.017 | 70 (84.3) | 68 (89.5) | 0.339 | 104 (83.9) | 34 (97.1) | 0.041 | 131 (86.2) | 7 (100) | 0.291 |
| Arteriolar hyalinosis | 81 (81.8) | 57 (95.0) | 0.017 | 70 (84.3) | 68 (89.5) | 0.339 | 104 (83.9) | 34 (97.1) | 0.041 | 131 (86.2) | 7 (100) | 0.291 |
| Cholesterol emboli | 3 (3.0) | 3 (5.0) | 0.528 | 4 (4.8) | 2 (2.6) | 0.470 | 4 (3.2) | 2 (5.7) | 0.495 | 5 (3.3) | 1 (14.3) | 0.135 |

**Supplemental Table S1D. C5b-9 deposits and histologic lesions in all diabetic cases**

| | Glom C5b-9 - | Glom C5b-9 + | *P* value | Glom hilus C5b-9 - | Glom hilus C5b-9 + | *P* value | Art C5b-9 - | Art C5b-9 + | *P* value | Art branch C5b-9 - | Art branch C5b-9 + | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Glomerular capsular drop | 4 (8.0) | 13 (11.9) | 0.457 | 1 (33.3) | 16 (10.3) | 0.200 | NA | NA | NA | NA | NA | NA |
| Glomerular hyalinosis | 30 (60.0) | 74 (67.9) | 0.331 | 1 (33.3) | 103 (66.0) | 0.238 | NA | NA | NA | NA | NA | NA |
| FSGS | 2 (4.0) | 12 (11.0) | 0.148 | 0 (0) | 14 (9.0) | 0.587 | NA | NA | NA | NA | NA | NA |
| IFTA | | | 0.008 | | | 0.802 | | | NA | | | NA |
| 0 | 20 (40.0) | 19 (17.4) | | 1 (33.3) | 38 (24.4) | | NA | NA | | NA | NA | |
| 1 | 24 (48.0) | 57 (52.3) | | 2 (66.7) | 79 (50.6) | | NA | NA | | NA | NA | |
| 2 | 3 (6.0) | 17 (15.6) | | 0 (0) | 20 (12.8) | | NA | NA | | NA | NA | |
| 3 | 2 (6.0) | 16 (14.7) | | 0 (0) | 19 (12.2) | | NA | NA | | NA | NA | |
| Arteriosclerosis | 40 (80.0) | 98 (89.9) | 0.087 | 2 (66.7) | 136 (87.2) | 0.299 | NA | NA | NA | NA | NA | NA |
| Arteriolar hyalinosis | 40 (80.0) | 98 (89.9) | 0.087 | 2 (66.7) | 136 (87.2) | 0.299 | NA | NA | NA | NA | NA | NA |
| Cholesterol emboli | 3 (6.0) | 3 (2.8) | 0.318 | 0 (0) | 6 (3.8) | 0.729 | NA | NA | NA | NA | NA | NA |

# Chapter 7

## Summary and general discussion

# SUMMARY AND GENERAL DISCUSSION

## On the definition of thrombotic microangiopathy

Patients presenting with thrombotic microangiopathy (TMA) may provide a wide range of physicians with diagnostic and therapeutic challenges: the differential diagnosis of TMA is broad and includes multifactorial diseases affecting multiple organs; the clinical, laboratory or pathological features can be insufficiently sensitive or specific; advanced diagnostic tests may be unavailable; overlapping diagnostic criteria can complicate the diagnostic procedure; and even among patients with the same diagnosis, such as atypical hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP), the clinical presentation, course, and outcome are variable.[1-10] It is important to overcome these challenges because rapid identification of not only the presence of TMA, but also of the underlying mechanism is required for targeted treatment, which can be life-saving and may prevent irreversible renal failure in some of these patients.[5, 11-13]

It has to be noted, however, that the term TMA is used with slightly different connotations in different contexts. As was mentioned in the introduction of this thesis (Chapter 1), TMA is used as a term which refers to a syndrome characterized by thrombocytopenia, microangiopathic hemolytic anemia and signs of organ injury (systemic TMA, also described as clinical TMA);[5, 6, 14] as a term which refers to a histopathologic pattern of lesions (morphologic TMA) which would typically be present in the affected organs of a patient with systemic TMA, but can also be observed in the absence of such clinical features (local TMA);[15-19] and as an 'umbrella term' for a broad category of disorders that can present with evidence of severe microvascular endothelial injury and microthrombi (the thrombotic microangiopathies; TMAs).[3, 9, 20, 21] It is likely that systemic and local TMA are manifestations of specific causes, such as mutations in complement regulatory genes, deficiencies in A Disintegrin-like And Metalloprotease with ThromboSpondin type 1 motif, member 13 (ADAMTS-13) or the presence of antiphospholipid antibodies, which can be found if they are meticulously looked for, but are not be routinely tested in all patients.

These issues should be taken into consideration in the evaluation of

the patient groups with TMA described in this thesis. Several patients were diagnosed with 'HUS', 'atypical HUS' or 'HUS/TTP', rather than TTP, even if the underlying causes that define these diseases, such as the levels of ADAMTS-13 or genetic deficiencies of complement regulatory proteins, were not examined in the clinical setting. These diagnoses likely reflect the historic association between HUS and the clinical predominance of renal manifestations, as well as the contemporary difficulties in distinguishing HUS from TTP. Furthermore, as all patients described in this thesis were selected from the renal pathology databases, there was a selection bias towards morphologic TMA. Patients who, for various reasons, did not undergo renal biopsy were not included in our studies. This may have excluded patients with a straightforward clinical presentation, a self-limiting course, and patients with contraindications to renal biopsy, such as children with Shiga toxin-producing *Escherichia coli*-associated HUS (STEC-HUS), patients with TTP who lack renal manifestations, or patients with an unacceptably high risk of bleeding due to thrombocytopenia.

Throughout the study period of this thesis, various definitions of morphologic TMA have been suggested,[9, 16, 17, 22-25] but at the present time, no gold standard exists to identify morphologic TMA. Although the characteristic light microscopic and ultrastructural features of morphologic TMA are well documented,[5, 9, 12, 16, 17, 22-29] the definition of morphologic TMA remains based on expert opinion and there is no consensus on the criteria that could be used to define the histopathologic spectrum of lesions.[9, 10] Like in many other fields of immunopathology, what is regarded as TMA is influenced by inter-observer variation and local practices. A survey among pathologists diagnosing renal biopsies with morphologic TMA has shown that there are differences in the use of stains, histopathological criteria, value attribution to clinical features and family history, and availability of advanced diagnostic tools such as genetic analysis of complement proteins, ADAMTS-13 levels, and electron microscopy.[30]

The setting of research may also give nuances to the definition of TMA and this is the reason why different definitions of morphologic TMA have been used in the work described in this thesis. In the work described in Chapter 2, we focused on active lesions. The definition of TMA in Chapter 2 required the presence of one or more microthrombi obstructing vessel lumina on renal biopsy and patients typically had multiple microthrombi throughout the renal tissue specimen. Concomitant chronic microangiopathic lesions were also observed.

For example, eight cases also had a duplicated glomerular basement membrane, and organizing microthrombi with recanalization were occasionally observed. In the work described in Chapter 3, we aimed to compare our results to findings by El Karoui et al, who described that 53% of biopsies with IgA nephropathy had morphologic TMA.[24] In their study, TMA was described as "acute TMA", defined by the presence of fibrin deposits, or as "organized TMA", which did not require microthrombi but was defined by the presence of "evident fibrosis and recanalization and narrowing of the lumen at the arterial and arteriolar levels".[24] We made a similar distinction between acute and chronic cases, but we followed the recent conclusions from the "Kidney Disease: Improving Global Outcomes" (KDIGO) Controversies Conference.[17] Consequentially, we used the term 'microangiopathy' rather than 'TMA' and distinguished cases with active and chronic lesions. In the work described in Chapter 4, we focused on chronic glomerular changes in the setting of transplant glomerulopathy. We included a control group of native biopsies diagnosed with chronic morphologic TMA which had glomerular basement membrane duplication; several cases also had microthrombi in glomeruli, arterioles, or both, but these lesions were not systematically studied.

The grey area of morphologic TMA includes biopsies with local TMA, biopsies with a single microthrombus, and biopsies with chronic microangiopathic lesions that lack microthrombi. Several patients described in this thesis had local TMA, and the microangiopathic lesions of the cases described in Chapter 3 were generally more difficult to identify than the microangiopathic lesions in the cases with disseminated microthrombi, as described in Chapter 2. Nevertheless, the findings described in Chapter 3 suggest that patients with local microangiopathy can have a substantial risk for disease progression, even if the lesions are scarcely present. In comparison with 'full-blown' morphologic TMA, scarcely present microangiopathic lesions could possibly reflect a less severe phenotype, an earlier stage of a disease process, or sampling error. Local TMA has been described in relation to disease progression in several kidney diseases, including lupus nephritis,[31, 32] anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis,[33] and diabetic nephropathy.[34] In a study among kidney transplant recipients, *de novo* local TMA was compared to systemic TMA.[35] In nearly all patients with local TMA, renal function improved with reduction, conversion, or temporary discontinuation of calcineurin inhibitors,

and patients with local TMA had a better short-term prognosis than patients with systemic TMA. However, local TMA was not benign: patients with local TMA also had a worse prognosis than patients without TMA, and long-term graft survival of grafts with local or systemic TMA was similarly poor. In a study on biopsies from deceased donor kidneys taken before engraftment, the presence of fibrin thrombi was a significant risk factor for the development of reduced graft function at 6 months after transplantation and graft loss within the first year after transplantation.[36] Two other studies had a longer follow-up period and found no significant differences in renal outcome after 1 year of transplantation between cases with and without microthrombi in donor kidneys.[37, 38] Although these studies may differ with regard to the clinical setting, study population, causal mechanism, and therapeutic management, the findings suggest that local TMA is an important risk factor for disease progression among various kidney diseases, but that this injury is potentially reversible.

In the studies described in this thesis, microangiopathy with or without thrombosis was scored as a histopathological pattern. Key questions that remain unanswered include: which of the individual microangiopathic lesions are clinically relevant and which criteria should define TMA? Which lesions indicate further examination of risk factors such as deficiencies in genes encoding complement regulatory proteins or ADAMTS-13 levels? What is the optimal treatment strategy for patients with local TMA? To what extent do active and chronic microangiopathic lesions share morphological features, signaling pathways, and mechanisms of injury and repair among patients with different diseases? Differences in the definitions of TMA limit the comparison between the cases reported across individual studies. Therefore, there is room for improvement. Refined definitions may lead to clearer scientific dialogue, improved accuracy for diagnosis and prediction of prognosis, a better understanding of the etiologies underlying similar manifestations, and advances in therapeutic management. For these definitions to be useful, there is a need for consensus among pathologists on what establishes morphologic TMA, and a need for multidisciplinary studies on the clinical outcome and response to therapy in patients with systemic and local TMA, integrating genetic profiles, clinical features, laboratory parameters, and tissue pathology in both native and allograft tissue samples.

## On complement-mediated microangiopathy

The work described in this thesis provides evidence for the involvement of complement activation in the development or progression of various renal microangiopathies. Compared to control groups, complement deposits along the renal microvasculature, including C4d deposits, were observed more frequently in patients with various renal diseases that presented with systemic or local TMA (described in Chapters 2, 3, and 4), transplant glomerulopathy (described in Chapter 4), preeclampsia, (described in Chapter 5) and diabetic nephropathy (described in Chapter 6). Our findings demonstrate that complement activation has taken place along the renal microvasculature of these patients, as demonstrated by the presence of C4d which is a cleavage product that follows from C4 activation that remains covalently bound to the surrounding tissue long after complement-pathway initiating factors have dissociated.[39] Deposits of C5b-9 were also observed in approximately 75% of patients with microangiopathy with or without thrombosis and diabetic nephropathy, suggesting that complement activation via any of the complement pathways proceeded to the formation of terminal complement complexes in these patients. Nevertheless, we also found C5b-9 deposition in a substantial proportion of control samples, which complicates the clinical use of this staining and will be discussed below. Our findings suggest that activation of the lectin and classical pathways could possibly contribute to the development of renal microvascular injury. Furthermore, our data suggest that in a subset of patients, complement-mediated microangiopathy is involved in disease progression, as renal complement deposits were correlated with clinical progression towards end-stage renal disease (described in Chapter 3), or the severity of histopathological lesions (described in Chapters 3, 4 and 6). We hypothesize that these patients are particularly susceptible to complement-mediated microangiopathic injury due to defective complement regulation and that complement-modifying therapy could be beneficial to a wider group of patients than is currently recognized. However, the molecular mechanisms underlying our findings remain to be determined. Future studies need to address whether complement activation along the renal microvasculature is a cause or consequence of injury, if complement-mediated microvascular injury is related to an underlying genetic or acquired defect in diseases characterized by endothelial injury or dysfunction, and if complement staining patterns are

useful in the workup of patients who may benefit from complement-inhibiting therapy.

The work described in Chapter 2 is focused on the role of complement activation in kidneys from patients with various renal diseases, including atypical HUS, who presented with morphologic TMA in the renal biopsy specimens. The data described in Chapter 2 show that C4d deposition is associated with the presence of microthrombi in renal biopsies from patients with various renal diseases that presented with systemic or local TMA. Complement deposits along the renal microvasculature were studied in 42 renal tissue samples obtained from a heterogeneous patient group with morphologic TMA in the renal biopsy and in 53 renal control samples without TMA. C4d deposits were observed in nearly 90% of the kidneys with TMA, indicating that complement activation took place in most kidneys with microthrombi, regardless of the underlying clinical condition. To explore which complement pathway could have led to C4d deposition and whether complement activation led to C5b-9 deposition, deposits of mannose-binding lectin (MBL) (lectin pathway), C1q and IgM (classical pathway), and C5b-9 (terminal pathway) were studied. In most patients, evidence for classical pathway activation was observed, but we also observed evidence for lectin pathway activation. C5b-9 deposits were observed in more than 75% of kidneys with TMA, suggesting that complement-inhibiting therapy could be beneficial for these patients.

The findings on complement deposition in the controls described in Chapter 2 warrant discussion. The control group consisted of healthy control samples without TMA from donor kidneys that were unsuitable for transplantation because of technical deficits, and biopsies from patients with focal segmental glomerulosclerosis (FSGS), Alport syndrome, and kidney transplant recipients with a variety of lesions other than antibody-mediated rejection. C5b-9 deposits were significantly more prevalent in kidneys with TMA than controls, and co-localized with microthrombi. However, C5b-9 deposits were also observed in 40% of controls, predominantly in arterioles. This suggests that the presence of C5b-9 staining alone cannot discriminate cases with microthrombi from cases without microthrombi. In contrast, C4d deposition was infrequently observed in controls, suggesting that a combination of C4d and C5b-9 could possibly be useful in the diagnostic workup of patients with TMA because it could help identify lesions that may have been missed

otherwise, and possibly indicate patients with complement-mediated TMA. C4d deposits were not observed in any of the controls, except for 3 of the 19 control patients with FSGS. In routine diagnostics, complement deposits in FSGS are typically considered a consequence of non-specific entrapment in sclerotic lesions.[40] However, our cases also had staining in glomerular segments that appeared non-sclerotic. This observation is consistent with recent studies demonstrating that IgM and C3 deposits can be observed in unaffected glomeruli of patients with primary FSGS,[41] and that plasma and urine levels of complement factor Ba, C4a, and sC5b-9 are significantly higher in patients with primary FSGS than control groups.[42] Following the observations described in Chapter 2, we studied the glomerular deposition of C4d in native biopsies obtained from patients with primary FSGS, allograft biopsies from patients with native FSGS who would develop recurrent FSGS, and the Munich Wistar Frömter rat model of FSGS. We found that glomerular C4d deposition is associated with FSGS lesions.[43] Moreover, we found that glomerular C4d deposition can precede the development of FSGS and indicate early lesions of FSGS. These data suggest that complement activation is involved in the pathogenesis of FSGS.

The study described in Chapter 2 validates previous findings from our group indicating that C4d deposition can be useful to identify morphologic TMA in renal biopsies from patients with systemic lupus erythematosus (SLE) and patients with antiphospholipid syndrome.[25] Antiphospholipid antibodies and immune-complexes in lupus nephritis can activate the classical pathway, causing endothelial injury and local inflammation.[44, 45] Complement activation has been recognized as an important mechanism for antiphospholipid syndrome-associated thrombosis in experimental models and in patients.[45] Others have shown that renal TMA in patients with SLE was associated with poor renal outcome, and that renal outcome was even poorer among SLE-associated TMA patients with glomerular C4d deposition and decreased serum levels of complement factor H.[32, 46] These data suggest that activation of both the classical and alternative pathways of complement might be involved in the development of TMA in these patients. It is possible that immune-complexes or auto-antibodies activated the classical pathway, but that uncontrolled alternative pathway activation amplified endothelial cell injury. The hypothesis that complement can be involved in SLE-associated TMA is also supported by case

reports describing the efficacy of the anti-C5 monoclonal antibody, eculizumab, and the anti-inflammatory and anticoagulant agent recombinant human soluble thrombomodulin, in patients with lupus nephritis-associated TMA; these findings are promising because some of the patients described in these case reports were unresponsive to conventional therapy.[46-50]

A similar mechanism of antibody-induced and complement-mediated injury might be responsible for a subset of patients with *de novo* TMA in kidney transplant recipients. A study on *de novo* TMA following kidney transplantation found a mutation in complement factor H, factor I, or both in 29% of patients with *de novo* TMA, and in none of the control kidney transplant recipients or healthy controls, suggesting that a subset of patients with *de novo* TMA in the renal allograft may be particularly susceptible to complement-mediated injury.[51] Several case reports describe a beneficial effect of complement-inhibiting therapy in kidney transplant recipients with *de novo* TMA, including patients with antibody-mediated rejection as well as drug-induced TMA that is refractory to other treatments.[52, 53] Future studies need to address the role of complement activation in the development of *de novo* TMA in allograft kidneys and develop optimal therapeutic guidelines for *de novo* TMA after kidney transplantation.

The mechanisms of endothelial injury in TMA following hematopoietic stem cell transplantation (HSCT-TMA) are poorly understood. HSCT recipients may be exposed to multiple endothelial insults, including chemotherapy, radiation, infections, and calcineurin inhibitors to prevent graft-versus-host-disease.[54] We observed C4d in the arterioles of patients with HSCT-TMA. Other studies on renal biopsies from HSCT-recipients reported similar findings and suggested that arteriolar C4d deposition might be useful to discriminate HSCT-TMA from other causes of renal injury in HSCT recipients.[54-60] Given that HSCT-TMA has been associated with graft-versus-host disease,[61] it is possible that complement-mediated TMA is a manifestation of chronic graft-versus-host disease in HSCT recipients.

In most patients with TMA, C4d deposits were associated with C1q and IgM deposits, and we observed co-localization of these deposits on sequentially sectioned kidney samples. These data suggest that the classical pathway was activated, possibly via the IgM subtype. We found similar associations in patients with microangiopathic lesions in the setting of IgA nephropathy (IgAN) or IgA vasculitis with nephritis (IgAVN), preeclampsia, and diabetic

nephropathy (described in Chapters 3, 5 and 6). We hypothesize that endothelial damage triggered the binding of natural IgM antibodies to injured cells, thereby activating the classical pathway and leading to C1q and C4d deposition. Natural IgM antibodies play an important role in the clearance of damaged cells by binding to hypoxic, necrotic, and apoptotic cells through intracellular antigens that become externalized under these conditions. As was reviewed previously,[62, 63] natural IgM antibodies can prevent harmful autoimmune reactions from damaged cells by generating an immunoregulatory milieu and promoting the resolution of inflammation, in part by activating the complement system.

Furthermore, the complement system may also be activated by antibody-independent mechanisms in renal microangiopathies. Experiments have shown that endothelial cells that were stimulated *in vitro* by inflammatory cytokines or high levels of shear stress can activate the classical pathway up to the formation of C5b-9 by demonstrating the deposition of C4d and C5b-9 on endothelial cells, independent of IgM or IgG.[64, 65] Another *in vitro* study demonstrated that complement activation could be initiated by exposure to subendothelial extracellular matrix, independent of endothelial cell activation.[66] Similarly, platelets that were activated either by chemical means or shear stress were found to induce activation of the alternative pathway involving P-selectin, and the classical pathway involving C1q binding proteins.[67] It has been suggested that the recruitment, adhesion, and activation of platelets during injury and inflammation is mediated by C1q.[68] Recently, a new complement activation pathway was discovered: in C3-deficient mice, thrombin was demonstrated to cleave C5.[69] Subsequently, activated components of the coagulation cascade have also been shown to activate the complement system: plasmin, FIX, FX, and FXI can activate C3 and C5, activated FXII can activate C1 causing classical pathway activation, and both ficolin and MBL can interact with fibrinogen and fibrin, enhancing lectin pathway activation.[70] Other triggers that could activate the classical pathway in the setting of renal microangiopathies include the acute phase C-reactive protein (CRP), danger-associated molecular patterns (DAMPs), neutrophil extracellular traps (NETs), free-heme following intravascular hemolysis, and molecules that become accessible after cell apoptosis, necrosis, or ischemia.[71-76]

Data showing that numerous mechanisms can activate the complement

system may complicate the interpretation of our data, but they are likely a realistic representation of complex biological processes. As was reviewed previously, there is a continuous and dynamic interaction in the microvasculature between the endothelium and the inflammation, coagulation, and immune systems.[70, 77] Excessive activation or inadequate regulation of any one of these processes may disturb homeostasis, leading to complement-mediated endothelial injury, or endothelial dysfunction.[77] Regardless of the initiating factor that causes complement activation, complement dysregulation can cause a positive feedback loop that perpetuates endothelial injury. It is possible that a subset of our patients has an increased susceptibility to complement-mediated injury due to inherited or acquired complement defects. Mutations in genes encoding proteins of the complement system can be identified in more than 50% of patient with atypical HUS.[17] These include genes encoding complement factor H, CD46, factor I, C3, factor B, and thrombomodulin. However, disease penetrance is variable in patients with mutations in complement regulatory genes, not all mutations may be pathogenic, and in many patients with atypical HUS, a complement abnormality cannot be identified.[78] A mutation in complement regulatory genes is often a predisposing factor rather than a direct cause of local or systemic TMA, requiring a trigger for atypical HUS. These triggers include autoimmune diseases, transplantation, pregnancy, infections, and drug-toxicity; several of these clinical conditions were also identified in our TMA cohort. Although mutations in complement regulatory genes were documented in a few of our patients as part of their diagnostic workup, we could not determine the causes of complement activation in most of our patients, due to the unavailability of DNA, serum and urinary samples. Therefore, in most of the patients described in Chapter 2, it is unknown if they have mutations in complement regulatory genes, auto-antibodies against factor H,[79, 80] or other auto-antibodies that can cause endothelial injury and complement activation.[44, 79-82] This limitation also applies to the work described in Chapters 3-6.

The work described in Chapter 3 is focused on complement-mediated microangiopathy in the renal biopsies of patients with IgAN and IgAVN. It was previously shown that in patients with IgAN, disease progression was associated with microangiopathic lesions,[24] and with C4d deposition in separate studies that did not address microangiopathic lesions.[83-85] These studies and the observations described in Chapter 2, led to the hypothesis that there could be

an intricate relationship between complement activation and microangiopathic lesions in patients with IgAN and IgAVN, possibly denoting a subgroup of patients with a relatively poor clinical outcome. We studied 128 renal biopsies from adult and pediatric patients who presented with IgAN or IgAVN. Biopsies were re-evaluated histologically with particular attention for the presence of microangiopathy with or without thrombosis and stained for C4d, C1q, MBL, and C5b-9. Re-examination of the renal biopsies revealed microangiopathic lesions in 20% of the biopsies, suggesting that microangiopathy, with or without thrombosis, could possibly be overlooked in clinical practice. In line with findings from Chapter 2, the presence of microangiopathic lesions in renal biopsies was associated with the presence of C4d and C5b-9 deposits along the renal microvasculature, with evidence for activation through the classical complement pathway.

Interestingly, in the cohort described in Chapter 3, patients with a combination of C4d positivity and microangiopathy comprised a clinical subgroup with a higher number of chronic lesions, lower eGFR, and poorer renal survival compared to patients without microangiopathy or C4d deposits. These findings provide evidence for the involvement of complement activation in the development of severe microvascular injury and suggest that complement-mediated microangiopathy contributes to disease progression in at least a subgroup of patients with IgAN and IgAVN. These findings are in line with previous studies suggesting that complement activation is involved in the pathogenesis of IgAN and that complement-inhibiting therapy may be promising for several patients with IgAN.[84] However, the predictive value of microangiopathy and C4d deposition in our cohort should not be overstated, as the study was underpowered to examine the incremental effect of these variables in addition to other markers of poor prognosis, including parameters of the Oxford classification.[84, 86, 87] Future multi-center studies on prognostic factors in IgAN or IgAVN should include C4d staining patterns and microangiopathic lesions as parameters.

Possible explanations for complement activation in relation to microangiopathic lesions in IgAN or IgAVN are similar to those described above. In addition, complement activation may result from processes that are intrinsic to the pathogenesis of IgAN and IgAVN and involve the lectin and alternative complement pathway, as was reviewed previously.[84, 88] This may explain why

complement deposition was also observed in patients without microangiopathic lesions. Several studies reported on glomerular C4d deposition in IgAN.[83, 85, 89, 90] In a study on complement deposition in fresh-frozen renal biopsies of patients with IgAN, C4d deposition was exclusively associated and co-localized with proteins of the lectin pathway.[85] Other studies on C4d deposition in IgAN assumed lectin pathway activation based on the absence of C1q but did not examine the deposition of lectin proteins.[83, 89, 90] In contrast, in our study C4d was associated with C1q but not MBL deposition. However, it is still possible that C4d was activated via the lectin pathway because we did not examine ficolins, or mannose-associated serine protease 1-3 (MASP1-3). Similarly, the MBL-negative patients with TMA, preeclampsia, and diabetic nephropathy described in Chapters 2, 5 and 6 may also have had C4d deposition resulting from the lectin pathway. Nevertheless, it is very well possible that C4d deposition reflects classical pathway activation in IgAN. Although evidence for classical pathway activation is infrequently reported in IgAN,[84] IgG and C1q deposition have been described in association with disease progression and the prevalence of C1q in some cohorts ranges from 10-45%.[91-102] It has to be noted that the sensitivity of detecting C1q varies between techniques. For example, C1q is detected more often by immunoperoxidase staining on formalin-fixed, paraffin-embedded tissue than by immunofluorescence staining on fresh-frozen tissue, possibly because fixed tissue detects antigens that may be lost or removed in the washing steps of immunofluorescence staining on unfixed tissue.[103] Proteomic analysis of laser-captured microdissected glomeruli found that components of the classical pathway C1q, C1r, and C1s were significantly increased in patients with progressive IgAN as compared with non-progressive IgAN.[104] Taken together, several datasets indicate that C4d is a valuable biomarker for disease progression in IgAN, but in addition to being a footprint of lectin pathway activation, C4d may also reflect classical pathway activation as it does in other areas such as in the setting of renal transplant pathology. Future studies should determine the molecular mechanisms leading to complement activation in IgAN.

The work described in Chapter 4 is focused on C4d deposition along peripheral glomerular capillaries with a duplicated glomerular basement membrane (GBM). In the clinical setting of renal transplantation, C4d along the peritubular capillaries of the allograft kidney is an important biomarker for the diagnosis and classification of antibody-mediated rejection.[10, 39] In contrast,

the significance of glomerular C4d deposits in the allograft kidney is not fully understood. In Chapter 4, 319 consecutively obtained diagnostic kidney allograft biopsies from 219 patients are described, including 100 follow-up biopsies. C4d staining along the GBM (GBM-C4d) was associated with C4d deposition along the peritubular capillaries, suggesting that GBM-C4d could be a manifestation of antibody-mediated rejection. GBM-C4d was also associated with GBM duplication and subendothelial new lamina densa formation, the defining features of transplant glomerulopathy, even after correcting for parameters that indicate antibody-mediated rejection. We confirmed the association between GBM-C4d and GBM duplication in a setting without donor-specific antibodies by studying native biopsies with GBM duplication in the setting of chronic TMA and native biopsies without GBM duplication in the setting of minimal change disease. The GBM-C4d staining pattern mirrored the degree of GBM remodelling and the staining intensity was correlated with the severity of GBM duplication. By immunogold labeling, C4d deposits were mainly detected in architecturally altered subendothelial zones, as well as under podocytes, possibly reflecting subepithelial capillary wall turnover and restructuring originating from visceral epithelial cells. These findings suggest that in transplant biopsies, pseudo-linear GBM-C4d deposits can be interpreted as etiologic markers for active ongoing antibody-induced renal graft rejection, especially when observed with concurrent C4d deposition along the peritubular capillaries, or as structural markers for microangiopathy-like GBM duplication, independent of antibody-mediated rejection.

Our findings were recently concurred by von der Thusen et al., stating that isolated pseudo-linear C4d along the GBM in renal allograft biopsies frequently marks ultrastructural alterations to the GBM, even if it the staining is scant.[105] They also report that this staining pattern can be the only sign marking the necessity for further electron microscopic analyses that might be skipped otherwise, since significant hematuria and proteinuria may be absent. Graft survival in patients with transplant glomerulopathy is particularly poor and identifying patients at an early stage may be an important step towards preventing transplant glomerulopathy and prolonging graft survival.[106]

The mechanism of complement activation in GBM duplication remains to be determined. C5b-9-induced endothelial injury can cause leakage and exposure of the subendothelial cell matrix, activation of platelet aggregation

and activation of the coagulation cascade; the morphological manifestation of this process can be observed as mesangiolysis and microthrombi.[107] Sublytic quantities of C5b-9 can induce endothelial cell activation, causing an upregulation of adhesion molecules such as P-selectin, E-selectin, ICAM-1, and VCAM-1, and cytokines such as IL-6, IL-8, and MCP-1.[77] Endothelial cells exposed to C5b-9 can release heparan sulfate, basic fibroblast growth factor, and platelet-derived growth factor which have been suggested to contribute to the chronic microangiopathic lesions, such as GBM duplication and multilayering of the basal lamina of peritubular capillaries.[107]

The work described in Chapter 5 is focused on the role of complement activation in kidneys from patients with preeclampsia, a pregnancy-specific microangiopathy that affects 5-7% of all pregnancies and is a major cause of maternal, fetal, and neonatal morbidity and mortality worldwide.[8, 20, 108, 109] The pathogenesis of preeclampsia is incompletely understood, but there is compelling evidence that it is caused by an imbalance of circulating angiogenic factors, which results in endothelial dysfunction.[109] This angiogenic imbalance is caused by increased levels of the anti-angiogenic factors, such as soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin, and by decreased levels of pro-angiogenic factors, such as placental growth factor and vascular endothelial growth factor A (VEGF-A). Recent studies also provide evidence for complement activation in the development of preeclampsia and our group previously showed that patients with preeclampsia have classical pathway activation in the placenta.[110-114] Based on these findings, we hypothesized that the classical pathway is also activated in the kidneys of women with preeclampsia.

In Chapter 5, we report on the glomerular deposits of complement and immunoglobulins in a unique cohort of renal autopsy samples from 11 women with preeclampsia, 25 pregnant controls, and 14 hypertensive controls who were not pregnant. Preeclampsia was significantly associated with glomerular C4d, C1q, and IgM deposition, suggesting that the classical complement pathway is activated in the kidneys of patients with preeclampsia, possibly via antibody-mediated injury of the IgM-subtype. These findings were validated in the sFlt-1 mouse model for preeclampsia: compared with control-treated mice, sFlt-1 injected pregnant mice had higher serum levels of circulating activated C3 fragments, and significantly more C4-positive glomeruli, which were co-localized with endothelial cells, suggesting that complement activation can result

from endothelial dysfunction caused by an angiogenic imbalance. Together with previously reported studies,[111-115] the findings described in Chapter 5 suggest that complement activation might contribute to renal injury in preeclampsia and that inhibiting the complement system could possibly reduce both the renal and placental manifestations of preeclampsia.

The experiments in sFlt-1 injected pregnant mice support the hypothesis that complement deposition in renal microangiopathies can result from endothelial dysfunction *in vivo.* As was described in the introduction of this thesis (Chapter 1), sFlt-1 binds to vascular endothelial growth factor (VEGF) and placental growth factor, which reduces the bioavailability of these growth factors for their receptors, and causes endothelial dysfunction. If an angiogenic imbalance is induced, this can manifest as a preeclampsia phenotype with hypertension, proteinuria, and endotheliosis.[116] The importance of VEGF is also shown in other renal microangiopathies. VEGF promotes angiogenesis and is essential for maintaining vascular integrity.[117] Homeostasis is required: both overexpression and inhibition of VEGF can cause disease.[118] Patients who were treated with systemic bevacizumab, a monoclonal VEGF inhibitor, have been reported to develop glomerular TMA and this phenotype could be reproduced in adult mice from a podocyte-specific conditional knock-out model for the VEGF gene.[119] Likewise, podocyte-specific heterozygosity for VEGF-A results in renal failure, proteinuria, and endotheliosis and homozygous deletion causes perinatal death.[120] Glomerular TMA has also been demonstrated in patients treated with VEGFR-2 inhibitors sunitinib and sorafenib, and in mice with reduced VEGFR-2 expression.[121-124] Vice versa, in a rat model of TMA induced by anti-endothelial cell antibodies, VEGF-treatment accelerated renal recovery.[125] Interestingly, a recent study demonstrated that VEGF increases complement regulator factor H synthesis by glomerular endothelial cells, whereas VEGF inhibition leads to a reduced expression of factor H and to increased glomerular deposition of activated complement components C3d and C4d.[126] These data suggest that podocytes regulate local complement activation via VEGF in a paracrine way.

The work described in Chapter 6 is focused on the role of complement activation in patients with diabetic nephropathy, a microvascular complication of diabetes mellitus that affects 30-40% of diabetic patients, making it the leading cause of end-stage renal disease.[127-129] The presence of C4d, C1q, MBL, and C5b-9 deposits was examined in 159 kidney samples from autopsied diabetic patients

for whom the histopathological presence or absence of diabetic nephropathy was confirmed, and in a control group of 41 non-diabetic patients without renal pathology. Findings were validated in 12 kidney biopsies from patients with diabetic nephropathy and 10 kidney biopsies from healthy living transplantation donors. Kidneys from patients with diabetic nephropathy had a significantly higher prevalence of C4d and C5b-9 deposits in glomeruli and in arterioles than kidneys from diabetic patients without nephropathy, and kidneys from non-diabetic patients without renal disease. Moreover, C4d deposition was correlated with higher classes of diabetic nephropathy and C4d deposition was associated with chronic microvascular and interstitial lesions. These data provide evidence for complement activation along the renal microvasculature in the development of diabetic nephropathy.

Our findings in diabetic nephropathy are in line with recent studies, suggesting that complement activation is involved in the development of microvascular and macrovascular complications of diabetes mellitus, as was reviewed previously.[130-132] In diabetic nephropathy, several mechanisms of complement activation were suggested. Under normal conditions, MBL and ficolins do not bind to their receptors on cell surfaces; however, hyperglycemia can cause glycation of proteins resulting in the production of advanced glycation end products and the generation of neo-epitopes to which lectin proteins can bind. Although our findings point towards classical pathway activation rather than lectin pathway activation, we cannot exclude that lectin pathway activation led to C4d deposition, as discussed above. Moreover, it was suggested that hyperglycemia can lead to glycation-induced inactivation of complement regulatory proteins such as CD59, which inhibits C5b-9 under physiological circumstances, and can cause C5b-9 induced injury. In our study, the prevalence of C5b-9 deposits was significantly higher in diabetic patients than in non-diabetic controls, but it was similar between diabetic patients with or without diabetic nephropathy. Within the group of patients with diabetic nephropathy, glomerular C5b-9 was correlated with the histologic markers of disease progression. These data suggest that the glycation-induced CD59 inactivation may indeed lead to the formation of C5b-9 as a common mechanism in diabetes mellitus that it is not specific to diabetic nephropathy, but may contribute to disease progression.

As was discussed above, natural IgM antibodies could explain the

association between C4d, C1q, and IgM in our cohort. In addition, diabetic nephropathy is associated with altered VEGF homeostasis and endothelial dysfunction.[117, 118, 133-135] Hyperglycaemia and hypoxia can cause upregulated VEGF-A expression by the podocyte.[135] Although VEGF upregulation may be a protective response that may limit endothelial injury and dysfunction, increased VEGF may also enhance the progression of diabetic nephropathy by causing foot process effacement, TGF-β activation and collagen IV synthesis in podocytes and mesangial cells, and inducing mesangial cell proliferation.[118, 134] Renal VEGF levels are high at early stages but low at advanced stages of diabetic nephropathy in humans and rodent models, possibly due to podocyte dropout.[135] A recent study showed that low VEGF expression was associated with TMA and end-stage renal disease in patients with diabetic nephropathy.[34] Combined with the finding that the podocyte may regulate local complement activation using VEGF,[126] these data suggest patients with severe diabetic nephropathy may have reduced local complement regulation due to lower VEGF levels, and that this contributes to the development of complement-mediated microvascular endothelial injury.

# Future perspectives

At the dawn of the 20th century, the significance of the complement system in humans was first recognized.[136] More than a century after this discovery, there has been a profound shift in our perception of the complement system in health and disease.[137] We have come to realize that over the evolutionary course of millions of years, the human complement system has developed an impressive versatility of functions that, by far, exceed the task of eliminating microbes.[138-141] We have also come to realize that there is a 'dark side' of complement activation; complement activation has now been implicated in the pathogenesis of a wide range of disorders.[140] One of the major future challenges will be to unravel the molecular mechanisms by which complement activation causes or aggravates microvascular diseases, as well as the mechanisms by which complement activation orchestrates repair. In addition, new therapeutic strategies are emerging to modulate the complement system, but it is still difficult to identify which patients will benefit from such therapy. Although the complement system is typically shown as a linear cascade of pathways, it is now known to be a dynamic, hub-like network of circulating, cell-surface expressed, and intracellular proteins, which are connected with other physiologic systems.[138] Given the complexity of the complement system, studies addressing complement-mediated microangiopathies may benefit from systems biology approaches and technical advancements in the fields of complement assays and artificial intelligence.[137, 140, 142-146] These efforts may not only improve our understanding of the pathophysiology of complement-mediated diseases but also help identify complement profiles in individual patients to facilitate patient-tailored therapy.[137]

### Complement-mediated microangiopathy in other fields

The findings on complement activation in the renal microangiopathies described in this thesis could be relevant to other renal disorders that are characterized by endothelial injury or dysfunction. For example, Timmermans et al. recently studied a cohort of patients with morphologic TMA attributed clinically to malignant hypertension; however, they found that 67% of patients had mutations in complement genes, concomitant evidence of complement activation *in vivo*, and poor renal outcome.[19] Moreover, they validated our finding that C4d

deposition is associated with the presence of microangiopathic lesions, as was described in Chapters 2 and 3. Although we did not examine ADAMTS-13 levels, a recent study demonstrated that C4d deposition was also more prevalent in renal biopsies from patients with the Upshaw-Schulman syndrome, a congenital form of TTP associated with loss-of-function mutations in the ADAMTS13 gene than controls.[147] These findings suggest that complement-mediated injury could also be involved in other diseases in which local TMA manifests, such as the recently described patients with microangiopathic lesions in the setting of ANCA-associated glomerulonephritis, diabetic nephropathy, and anti-GBM glomerulonephritis.[33, 34, 148] It would be interesting to determine if the combined presence of complement deposits and microangiopathic lesions could identify patients with a poor prognosis who may benefit from complement-modulating therapy and if these patients have a genetic predisposition to complement-mediated injury. Furthermore, the finding that patients with preeclampsia and diabetic nephropathy have more glomerular complement deposits than controls, suggests that complement activation could be involved in the pathophysiology of various disorders other than TMA, in which endothelial injury or dysfunction is a key component, such as systemic sclerosis, diffuse intravascular coagulation, or variants of FSGS. In addition, both microangiopathic injury and complement-mediated injury are not limited to the kidney. For example, atypical HUS, TTP, systemic lupus erythematosus, and TMA following hematopoietic stem cell transplantation may affect multiple organs such as the lungs, brain, and gastrointestinal tract.[5, 149] Similarly, the microvascular complications of preeclampsia and diabetes mellitus may affect multiple organs.[109, 150, 151] A recent study from our group demonstrated that C4d and C5b-9 deposits were associated with microthrombi in the brains of patients with systemic lupus erythematosus and neuropsychiatric involvement.[152] Therefore, complement activation may also be involved in the development or progression of microangiopathic injury in the extra-renal manifestations of systemic diseases described in this thesis.

**Complement-inhibiting therapy**

Complement inhibition has greatly reduced morbidity and mortality in patients with atypical HUS, even in patients without demonstrated mutations in complement regulatory genes.[77, 153] Our observations suggest that treatment against components of the complement system could benefit a broad range of

patients with complement-mediated microangiopathy. As described in this thesis, complement activation up to C5b-9 deposition was observed along the renal microvasculature of a heterogeneous group of patients, but not in all, and it is tempting to speculate that patients with severe microvascular injury and complement deposition at the site of injury will benefit most from complement-inhibiting therapy. There is evidence that eculizumab, a monoclonal antibody against C5, can benefit patients with other renal microangiopathies than atypical HUS.[53, 114, 153-169] Although these studies show encouraging efficacy in a subset of patients, there are important concerns preventing the justification of life-long complement-inhibiting therapy for a broader patient group. These concerns include publication bias, the harmful side effects and risks associated with complement-inhibiting therapy, and the financial impact on health care services. Moreover, for many renal microangiopathies, it is still unclear if complement activation is the primary cause of injury, a disease modifier that can contribute to disease progression, or an 'innocent bystander'. Therefore, future studies should systematically address the potential use of complement-inhibiting therapy in various renal microangiopathies, including systemic or local TMA, and microangiopathies such as diabetic nephropathy, preeclampsia, and transplant glomerulopathy. Although the standard maintenance treatment of atypical HUS requires life-long eculizumab therapy, discontinuation seems to be possible in some patients with stable remission.[170] This indicates that in patients with temporary complement-amplifying conditions, such as pregnancy, temporary inhibition could be sufficient to break the vicious cycle of complement-mediated injury. The work described in this thesis suggests that multiple pathways of complement activation could be involved in the pathogenesis of renal microangiopathies. It is, therefore, encouraging that new complement therapies are emerging, targeting various components of the complement system. As was reviewed recently,[137, 171] these therapies cover an impressive scope of approaches, including local and systemic administration, treating acute, chronic and episodic disorders, focusing on initiation, amplification and effector phases of complement activation, targeting specific activation fragments, and intervening at the protein and gene levels.

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# Chapter 8

## Nederlandse samenvatting

# NEDERLANDSE SAMENVATTING

Dit proefschrift gaat over de betrokkenheid van het complementsysteem in ziektebeelden die de kleine bloedvaten van de nieren treffen, de microangiopathieën. Wij bestudeerden hiervoor de nieren van patiënten met:

- *Trombotische microangiopathie* (TMA), een zeldzame maar zeer ernstige aandoening die gekenmerkt wordt door de ontwikkeling van stolsels in de kleine bloedvaten en schade aan het endotheel, de binnenbekleding van bloedvaten (hoofdstukken 2, 3, en 4). TMA kan op zichzelf staan als primair syndroom zoals bij het hemolytisch uremisch syndroom (HUS) en trombotische trombocytopenische purpura (TTP), maar het kan ook secundair ontstaan als een complicatie van ziekte of behandeling, waaronder nier- of stamceltransplantatie, infecties, kanker, en auto-immuunziekten als systemische lupus erythematosus (SLE), antifosfolipide syndroom, anti-neutrofiele cytoplasmatische antistoffen geassocieerde vasculitis en IgA nefropathie;
- *IgA nefropathie en IgA vasculitis met nefritis*, relatief veelvoorkomende auto-immuunziekten die in volwassenen en kinderen kunnen leiden tot vaatlesies die een acuut of chronisch karakter kunnen hebben (hoofdstuk 3);
- *Transplant glomerulopathie,* een ziekteproces dat vaak wordt gezien bij chronische afstoting van de getransplanteerde nier en een zeer slechte prognose kent (hoofdstuk 4);
- *Pre-eclampsie* – in de volksmond *zwangerschapsvergiftiging* genoemd – één van de belangrijkste oorzaken van moedersterfte en neonatale sterfte in de wereld (hoofdstuk 5); en
- *Diabetische nefropathie*, de microvasculaire complicatie in de nieren van patiënten met diabetes mellitus – in de volksmond *suikerziekte* genoemd – die wereldwijd één van de belangrijkste oorzaken is van nierfalen (hoofdstuk 6).

Deze entiteiten zijn in sommige gevallen levensbedreigend en kunnen leiden tot onherstelbaar nierfalen waardoor dialyse of een niertransplantatie nodig is om te overleven. Ze veroorzaken veel leed en wij kunnen veel van deze ziektebeelden tot op de dag van vandaag nog niet genezen. Een beter begrip van deze ziektebeelden is daarom nodig en kan bijdragen aan de ontwikkeling van een betere behandeling. Recente studies geven aanwijzingen dat complementactivatie mogelijk bijdraagt aan het ontstaan of de verergering van de ziektebeelden die beschreven staan in dit proefschrift.

**Het complementsysteem**

Het complementsysteem is een onderdeel van het afweersysteem. Het bestaat uit tientallen eiwitten die al vanaf de geboorte samenwerken om het lichaam te beschermen tegen ziekteverwekkers zoals bacteriën en virussen. Complement is oeroud. Het bestond al miljoenen jaren voordat dinosaurussen op aarde rondliepen en men schat dat het complementsysteem in gewervelde kaakdieren, zoals de mens, ten minste 600 miljoen jaar oud is. Het complementsysteem is dan ook nauw verbonden met andere processen in het lichaam die in de loop van de evolutie zijn ontstaan.

Binnen een paar seconden nadat een ziekteverwekker in het bloed is beland, kan het complementsysteem deze vaak opsporen en doden. Complement maakt daarbij gebruik van receptoren die bepaalde moleculaire patronen herkennen. Nadat een patroon is herkend, raakt complement geactiveerd en ontstaat een kettingreactie. In deze kettingreactie activeren de complementeiwitten elkaar razendsnel als een reeks dominosteentjes die omvallen nadat het eerste steentje is aangetikt. In dit proces worden de meeste complementeiwitten gesplitst in fragmenten waarvan sommige fragmenten werken als enzymen die het volgende eiwit in de cascade activeren, terwijl andere fragmenten diverse biologische functies hebben die bijdragen aan de ontstekingsreactie. Zo kunnen ze ervoor zorgen dat een ziekteverwekker sneller kan worden herkend, dat witte bloedcellen naar de plaats van de infectie komen, en dat een opruimreactie wordt gestart.

Complementactivatie wordt ingedeeld in drie hoofdroutes: de klassieke route, de alternatieve route en de lectine route (zie figuur 1 van Hoofdstuk 1). De klassieke route werd als eerste ontdekt en deze route bestaat uit eiwitten die worden aangeduid met de letter C gevolgd door een nummer (C1 tot en met C9). Klassieke route activatie start na de binding van C1q aan de initiators van deze route. Vaak zijn dat immuuncomplexen waarin antistoffen zoals IgM en IgG zijn gebonden aan antigenen, maar andere initiërende factoren van de klassieke route zijn ook beschreven. De alternatieve route werd als tweede ontdekt en de bijkomende eiwitten van deze route worden aangegeven met 'factor' en een letter (bijvoorbeeld factor H). De alternatieve route wordt spontaan geactiveerd door hydrolyse van C3 en is daardoor continu geactiveerd. Alternatieve route activatie kan worden versterkt, bijvoorbeeld wanneer C3b bindt aan het celoppervlak van ziekteverwekkers of beschadigde cellen. De lectine route werd als laatste

van de drie routes ontdekt en deze route wordt hoofdzakelijk geactiveerd door de binding van mannosebindend lectine (MBL) aan suikergroepen die vaak voorkomen op het celoppervlak van ziekteverwekkers. Ook in de lectine route zijn verschillende andere initiërende factoren beschreven. Alle drie de routes leiden tot een C3 convertase, die C3 activeert, waarna het verloop van de routes gelijk is. Deze route wordt ook wel de terminale route genoemd. Aan het eind van de terminale route ontstaat het 'membrane attack complex', een eiwitcomplex dat bestaat uit C5b, C6, C7, C8 en meerdere C9 moleculen en daarom wordt aangeduid als C5b-9. C5b-9 kan cellen doden door gaten te boren in de celmembraan en de cel daarmee lek te prikken. Om te voorkomen dat complementactivatie buitenproportioneel is, heeft elke route complement regulatoren die de activatie remmen.

De laatste decennia zijn er belangrijke ontdekkingen gedaan over het complementsysteem. Zo zijn er nieuwe triggers gevonden die het complementsysteem kunnen activeren, blijken verschillende complementeiwitten functies te hebben die verder reiken dan het helpen doden van ziekteverwekkers en zijn er aanwijzingen dat complement niet alleen systemisch in het bloed, maar ook lokaal in het orgaan kan functioneren. Ook is ontdekt dat C4d, een afsplitsproduct dat gevormd wordt tijdens de activatie van de klassieke of de lectine route, een stabiele biomarker is voor complementactivatie in de nier. C4d kan zelfs worden gedetecteerd wanneer de oorspronkelijke triggers die de complementcascade activeerden niet meer kunnen worden gezien doordat C4d covalent bindt aan de lokale omgeving. In de transplantatiegeneeskunde wordt C4d wereldwijd gebruikt om een vorm van afstoting te diagnosticeren waarin antistoffen van de ontvanger tegen eiwitten op het donororgaan de klassieke route activeren en schade veroorzaken. Recente studies laten zien dat het complementsysteem betrokken is bij tal van ziekten die niet eerder als complement-gemedieerd werden beschouwd. Te weinig complementactivatie kan leiden tot terugkerende infecties, maar te veel complementactivatie – door te weinig regulatie, te veel stimulatie, of een combinatie hiervan – kan óók leiden tot ziekte. In complement-gemedieerde HUS is aangetoond dat patiënten erfelijke afwijkingen hebben in genen die verantwoordelijk zijn voor de regulatie van het complementsysteem. Hierdoor werken de complement regulatoren onvoldoende waardoor te veel complementactivatie ontstaat en de kleine bloedvaten in de nier worden beschadigd. In klinische trials is aangetoond

dat complement-remmende behandeling leidt tot een belangrijke afname van ziekte en sterfte in deze patiënten. De resultaten van recentere studies doen vermoeden dat complementactivatie ook betrokken is bij de pathogenese van andere nierziekten, waaronder de nierziekten die bestudeerd zijn in dit proefschrift. Vergeleken met controlegroepen hebben verschillende van deze patiëntengroepen vaker erfelijke veranderingen in complement-regulerende genen, of vaker tekenen van complementactivatie in het bloed of de urine. In een aantal gevallen is beschreven dat patiënten baat hebben bij therapie die het complementsysteem remt. Echter, complementremmers zijn alleen in een deel van de beschreven patiënten werkzaam en we begrijpen nog niet precies hoe complementactivatie precies betrokken is in deze ziektebeelden.

**Complementactivatie in de pathogenese van renale microangiopathieën**

Het werk dat beschreven staat in dit proefschrift geeft aanwijzingen dat complementactivatie betrokken is in de pathogenese van verschillende microangiopathieën in de nier. In dit proefschrift onderzochten wij glomeruli, kleine vaatkluwen die in de nier als een zeef het bloed filteren. Ook onderzochten wij de arteriolen en de peritubulaire capillairen, omliggende kleine bloedvaten die zorgen voor de aanvoer van bloed aan de glomeruli en het omringende weefsel. Complementeiwitten, waaronder C4d, werden vaker langs de kleine bloedvaten geobserveerd in de nieren van patiënten met TMA (hoofdstuk 2, 3 en 4), IgA nefropathie of IgA vasculitis met nefritis en microangiopathische lesies (hoofdstuk 3), transplant glomerulopathie (hoofdstuk 4), pre-eclampsie (hoofdstuk 5) en diabetische nefropathie (hoofdstuk 6) dan in de nieren van controlegroepen. Omdat C4d een stabiele biomarker is voor lokale complementactivatie, betekenen onze bevindingen dat complementactivatie daadwerkelijk plaats heeft gevonden rondom de plaats waar het eiwit werd gezien. Omdat C5b-9 in verschillende patiënten werd gezien lijkt het erop dat complementactivatie heeft doorgezet tot aan het niveau van het membrane attack complex. Toch is de klinische relevantie van C5b-9 depositie onduidelijk omdat C5b-9 ook regelmatig in de controlegroepen werd gezien. De bevindingen die beschreven staan in dit proefschrift doen vermoeden dat de activatie van de klassieke of lectine route bijdraagt aan de ontwikkeling van microvasculaire schade in de nier. Ook doen de bevindingen vermoeden dat complement-gemedieerde microangiopathie bijdraagt aan de ziekteprogressie in sommige patiënten. Complement depositie

was geassocieerd met een slechtere prognose of ernstigere schade in het nierweefsel bij lichtmicroscopisch of elektronenmicroscopisch onderzoek. We vermoeden dat sommige patiënten extra vatbaar zijn voor complement-gemedieerde schade door een onderliggend defect in de complementregulatie en dat patiënten met verschillende microangiopathieën baat zouden kunnen hebben bij therapieën die het complementsysteem beïnvloeden.

In het werk dat beschreven staat in hoofdstuk 2, onderzochten wij complementeiwitten in het nierweefsel van patiënten met microtrombi die werden gediagnosticeerd met TMA in het nierbiopt. In de meest fulminante vorm is TMA levensbedreigend en kan het ziektebeeld leiden tot onherstelbaar nierfalen. Patiënten met TMA presenteren zich dan ook vaak met systemische symptomen zoals Coombs-negatieve microangiopathische hemolytische anemie, trombocytopenie en schade aan verschillende organen. TMA kan zich ook enkel lokaal manifesteren in de nier, waardoor patiënten met TMA een verslechtering van de nierfunctie hebben en het nierbiopt tekenen toont van beginnende of chronische microvasculaire schade. De oorzaken van TMA zijn divers en de pathofysiologie is vaak complex en multifactorieel. Desondanks kan gerichte behandeling tegen de onderliggende oorzaak – zoals te veel complementactivatie – voor sommige patiënten levensreddend zijn of de nierfunctie herstellen. Het werk uit hoofdstuk 2 bouwt voort op een eerdere studie van onze groep die liet zien dat C4d depositie TMA kon identificeren in nierbiopten van patiënten met SLE en antifosfolipide syndroom. De bevindingen in hoofdstuk 2 laten zien dat C4d geassocieerd is met TMA in de nierbiopten van patiënten ten gevolge van verschillende onderliggende oorzaken. We bestudeerden de aanwezigheid van complementeiwitten langs de kleine bloedvaten in 42 nieren met TMA en in 53 controles zonder TMA. C4d depositie werd gezien in bijna 90% van alle nieren met TMA. Dit geeft aan dat complementactivatie plaatsvond in deze nieren, ongeacht de klinische context waarin TMA plaatsvond. Om te onderzoeken welke route kon leiden tot C4d depositie en of complementactivatie door kan zetten tot aan het niveau van het membrane attack complex, hebben we een kleuring verricht voor MBL (lectine route), C1q en IgM (klassieke route), en C5b-9. In de meeste patiënten zagen we aanwijzingen voor klassieke route activatie, ook als van de onderliggende ziektebeelden niet bekend was dat antistoffen daarbij een rol speelden. C5b-9 depositie werd gezien in meer dan 75% van de nieren met TMA. De bevindingen die beschreven staan in hoofdstuk

2 geven aanwijzingen dat immunohistochemische kleuringen voor C4d en C5b-9 nuttig zouden kunnen zijn voor de diagnostiek of therapie van patiënten die verdacht worden van complement-gemedieerde TMA.

Het werk dat beschreven staat in hoofdstuk 3 richt zich op complement-gemedieerde microangiopathie in de nierbiopten van patiënten met IgA nefropathie en IgA vasculitis met nefritis (ook bekend als Henoch-Schönlein purpura nefritis). IgA nefropathie is de meest voorkomende primaire glomerulaire ziekte ter wereld, en IgA vasculitis met nefritis is een systemische ziekte die op het biopt niet te onderscheiden is van IgA nefropathie en voor een deel een overlappend ziekteproces kent. Wij bestudeerden 128 nierbiopten van volwassenen en kinderen met IgA nefropathie of IgA vasculitis met nefritis, zochten naar tekenen van acute of chronische TMA – we noemden dit microangiopathische lesies volgens recente internationale aanbevelingen omdat er niet altijd stolsels te zien waren – en onderzochten de nierbiopten op de aanwezigheid van C4d, C1q, MBL, en C5b-9 langs de kleine bloedvaten. Microangiopathische lesies, met of zonder stolsels, werden gevonden in 20% van de biopten, vaker dan we hadden verwacht. Een eerdere studie toonde aan dat microangiopathische lesies vaak voor kunnen komen bij IgA nefropathie en dat deze patiënten dan een slechtere prognose hebben dan patiënten zonder een microangiopathische lesie. Onze bevindingen suggereren dat dit patroon van vaatschade mogelijk wordt onderschat in de dagelijkse diagnostiek van patiënten met IgA nefropathie en IgA vasculitis met nefritis. Net als in hoofdstuk 2, was de aanwezigheid van microangiopathische lesies geassocieerd met de aanwezigheid van C4d en C5b-9 depositie langs de kleine bloedvaten, en vonden wij hoofdzakelijk aanwijzingen voor klassieke route activatie. In ons cohort vormden patiënten die zowel microangiopathie als C4d deposities hadden een klinische subgroep met meer chronische schade in het nierbiopt, een slechtere nierfunctie ten tijde van het nierbiopt, en een slechtere prognose dan patiënten zonder microangiopathie en C4d depositie. De klinische relevantie van microangiopathische lesies en C4d in het nierbiopt moet alleen niet worden overschat. Er zijn ook andere factoren die de prognose voorspellen en onze studie was te klein om de toegevoegde waarde te onderzoeken van C4d en microangiopathie bovenop alle andere parameters. De bevindingen die beschreven staan in hoofdstuk 3 geven aanwijzingen dat complement betrokken is bij de ontwikkeling van ernstige microvasculaire schade, dat complement-

gemedieerde microangiopathie bijdraagt aan de ziekteprogressie in ten minste een subgroep van patiënten met IgA nefropathie of IgA vasculitis met nefritis, en dat complement-remmende therapie mogelijk van meerwaarde is in deze patiënten.

Het werk dat beschreven staat in hoofdstuk 4 richt zich op C4d depositie in de glomeruli van patiënten die een niertransplantatie hebben ondergaan en transplant glomerulopathie ontwikkelen. Transplant glomerulopathie is een morfologisch patroon waarbij in het nierbiopt een verdubbeling van de glomerulaire basaalmembraan wordt gezien. Dit patroon komt voor bij verschillende ziektebeelden die gekenmerkt worden door chronische endotheelschade. Vaak hebben patiënten met transplant glomerulopathie een chronische antistof-gemedieerde afstoting. De prognose van patiënten met transplant glomerulopathie in het nierbiopt is slecht. In de context van een niertransplantatie wordt C4d langs de peritubulaire capillairen gebruikt om antilichaam-gemedieerde afstoting aan te tonen. Soms wordt C4d ook in glomeruli gezien, maar het is onduidelijk wat de betekenis is van glomerulair C4d, zeker als C4d niet in de peritubulaire capillairen wordt gezien. In hoofdstuk 4 beschrijven wij een onderzoek verricht op 319 nierbiopten van 219 patiënten met een getransplanteerde nier. Wij vonden dat de aanwezigheid van C4d langs de glomerulaire basaalmembraan geassocieerd was met tekenen van antilichaam gemedieerde afstoting. Wij vonden ook dat C4d depositie geassocieerd was met transplant glomerulopathie, zowel met lichtmicroscopie als met elektronenmicroscopie, zelfs wanneer er in de patiënt geen aanwijzingen waren voor antilichaam-gemedieerde afstoting. Om de bijdrage van antilichaam-gemedieerde afstoting uit te sluiten, onderzochten we ook een controlegroep van 35 patiënten die geen niertransplantatie hadden ondergaan maar bij wie wel een biopt was genomen van de eigen nier. We selecteerden patiënten met én zonder een verdubbeling van de glomerulaire basaalmembraan en zagen daarbij dat C4d vaker voorkwam langs de glomerulaire basaalmembraan als deze verdubbeld was dan als er geen afwijking was. Hoe ernstiger de basaalmembraan was aangedaan, des te meer C4d we zagen. Middels immunogold labeling, konden we op ultrastructureel niveau kijken naar C4d depositie. C4d werd hoofdzakelijk subendotheliaal gezien op plaatsen waar de glomerulaire basaalmembraan ultrastructurele veranderingen had, en C4d werd ook onder de podocyten gezien, wat zou kunnen betekenen dat complementactivatie rondom

deze cellen betrokken is bij de heropbouw van de glomerulaire basaalmembraan na endotheelschade. Onze bevindingen suggereren dat C4d depositie langs de glomerulaire capilairlissen enerzijds kan worden gezien als een biomarker voor actieve antilichaam-gemedieerde afstoting, zeker als dit ook samen voorkomt met C4d in de peritubulaire capillairen, maar dat het anderzijds ook als een biomarker kan worden gezien voor afwijkingen in de structuur van de glomerulaire basaalmembraan die kenmerkend is voor chronische endotheelschade en onafhankelijk kan worden gezien van antilichaam-gemedieerde afstoting. C4d kan daarom soms de enige aanwijzing zijn om nader elektronenmicroscopisch onderzoek te verrichten naar tekenen van transplant glomerulopathie.

Het werk dat wordt beschreven in hoofdstuk 5 richt zich op complementactivatie in de nieren van patiënten met pre-eclampsie, wat in de volksmond zwangerschapsvergiftiging wordt genoemd. Het is een syndroom dat in 1-4% van de Nederlandse zwangere vrouwen voorkomt, en wordt gekenmerkt door een hoge bloeddruk, eiwitverlies in de urine door schade in de glomeruli, of andere tekenen van orgaanschade. Vaak verloopt pre-eclampsie mild, maar het ziektebeeld kan zich snel ontwikkelen tot een levensbedreigende situatie voor moeder en kind, zoals bij het HELLP syndroom (een acronym van Hemolysis Elevated Liver enzymes and Low Platelets) en bij eclampsie. Het is niet duidelijk hoe pre-eclampsie precies ontstaat maar er wordt gedacht dat pre-eclampsie mede wordt veroorzaakt door een disbalans tussen pro-angiogene en anti-angiogene factoren. Angiogene factoren zijn verantwoordelijk voor de aanmaak en de groei van bloedvaten. De placenta maakt grote hoeveelheden anti-angiogene factoren tijdens pre-eclampsie die de angiogene factoren remmen en hierdoor kan endotheelschade ontstaan. Recente studies tonen ook aanwijzingen voor de betrokkenheid van complementactivatie in dit ziektebeeld. De manifestaties van het ziektebeeld in de nier zijn moeilijk te onderzoeken omdat deze patiënten zelden worden gebiopteerd. In hoofdstuk 5 beschrijven we de observaties in een uniek obductiecohort. Wij bestudeerden de glomeruli van 11 patiënten die zijn overleden met pre-eclampsie of aan de gevolgen hiervan, 25 controle patiënten die overleden zijn aan een andere oorzaak tijdens de zwangerschap en 14 niet-zwangere controle patiënten die overleden zijn met een hoge bloeddruk. Patiënten met pre-eclampsie hadden vaker C4d, C1q, en IgM depositie dan controlegroepen, wat suggereert dat de klassieke route mogelijk via IgM is geactiveerd. In hoofdstuk 5 hebben wij ook

de nieren van een muismodel voor pre-eclampsie bestudeerd. Deze zwangere muizen hebben te veel sFlt-1, een anti-angiogene factor, en ontwikkelen daarop een beeld dat lijkt op pre-eclampsie. Vergeleken met controle muizen hadden de sFlt-1 geïnjecteerde zwangere muizen hogere bloedwaarden van geactiveerde C3 fragmenten en significant meer C4 positieve glomeruli ter plaatse van de endotheelcellen. Deze data toont aan dat complementactivatie waarschijnlijk een gevolg is van endotheelschade in dit ziektebeeld en kan bijdragen aan de nierschade die ontstaat in pre-eclampsie. Het remmen van complement in patiënten met pre-eclampsie verdient nader onderzoek.

Het werk dat beschreven wordt in hoofdstuk 6 gaat over complementactivatie in patiënten met diabetische nefropathie, een vorm van nierschade die voorkomt bij patiënten met diabetes mellitus, ook wel bekend als suikerziekte. Diabetes wordt veroorzaakt doordat de suikerwaarden in het bloed onvoldoende gereguleerd worden door insuline. Dit komt doordat de insuline producerende cellen – de β cellen in de alvleesklier – onvoldoende insuline maken, doordat de cellen die normaal gesproken ontvankelijk zijn voor insuline ongevoelig worden, of door een combinatie van beide processen. Mede door de hoge suikerspiegels raken zowel de grote als de kleine bloedvaten beschadigd, waaronder die van de nieren. Diabetische nierschade kan zorgen voor een hoge bloeddruk, verlies van eiwit in de urine, een verhoogde kans op het krijgen van hart- en vaatziekten, en verlies van de nierfunctie. Diabetische nefropathie kan optreden bij zowel diabetes mellitus type 1 als diabetes mellitus type 2 en komt in 20-40% van de patiënten met diabetes mellitus voor. Wij onderzochten de aanwezigheid van C4d, C1q, MBL, en C5b-9 deposities in 159 obductie samples van patiënten met diabetes mellitus en een controle groep van 41 patiënten zonder diabetes of een nierziekte. Ook onderzochten we 12 nierbiopten van patiënten met diabetische nefropathie en 10 nierbiopten van gezonde individuen die hun nier doneerden. Wij zagen C4d en C5b-9 depositie vaker in de glomeruli en de arteriolen van patiënten met diabetische nefropathie dan van diabetische patiënten zonder diabetische nefropathie of patiënten zonder diabetes. C4d depositie was gecorreleerd aan een hogere klasse van diabetische nefropathie, en C4d depositie werd vaker gezien bij patiënten met chronische microvasculaire en interstitiële lesies. Deze data suggereren dat complementactivatie langs de kleine bloedvaten betrokken is bij de ontwikkeling van diabetische nefropathie. Onze bevindingen sluiten aan bij andere studies die aanwijzingen geven dat

complementactivatie betrokken is bij de complicaties van diabetes mellitus in de kleine bloedvaten van andere organen en in de grote bloedvaten.

Ons onderzoek heeft een aantal beperkingen die in acht moeten worden genomen bij de interpretatie van onze data. Zo zijn de studies retrospectief en observationeel van aard, is er een selectiebias van patiënten waarvan het nierweefsel is onderzocht, konden we slechts een relatief klein aantal patiënten onderzoeken door de zeldzaamheid van de ziekten of van het nierweefsel, waren de patiëntengroepen heterogeen in klinische presentatie en behandeling, en waren serum, urine, en DNA materiaal niet beschikbaar voor verdere analyse. Hoewel onze bevindingen aanwijzingen geven dat het complementsysteem betrokken is bij de ziektebeelden die beschreven zijn in dit proefschrift, tonen ze geen causaal verband aan. Toch zijn de resultaten hoopvol omdat een beter begrip van de rol van complement in deze ziektebeelden kan bijdragen aan een betere behandeling.

De mechanismen die ten grondslag liggen aan onze bevindingen verdienen daarom verdere aandacht. Toekomstig onderzoek is nodig om vast te stellen of de betrokkenheid van het complementsysteem in de hier beschreven ziektebeelden een oorzaak of een gevolg is van microvasculaire schade, wat de relatieve bijdrage van het complementsysteem is aan het ontstaan, verergeren, of verbeteren van microangiopathieën in de nier, en welke moleculaire mechanismen hiervoor verantwoordelijk zijn. Toekomstige studies zijn ook nodig om vast te stellen of complement-gemedieerde microangiopathie belangrijk is in andere ziekten die gekenmerkt worden door schade of dysfunctie van de kleine bloedvaten, of dit gerelateerd is aan een onderliggend defect in complementregulatie, en of het leven van patiënten met ziekten van de kleine bloedvaten beter wordt door therapie die het complementsysteem beïnvloedt.

# Appendices

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# CURRICULUM VITAE (NEDERLANDS)

Jamie Shaun Chua werd geboren op 31 oktober 1991 in Johor Bahru, Maleisië. In 2008 behaalde hij zijn VWO-diploma cum laude en het International Baccalaureate English diploma met de maximale score aan het Haarlemmermeer Lyceum. Op zestienjarige leeftijd begon hij met de studie Geneeskunde aan de Universiteit Leiden in het Leids Universitair Medisch Centrum (LUMC). In zijn tweede jaar werd hij toegelaten tot het Koninklijk Conservatorium in Den Haag om Jazz Piano te studeren via het Practicum Musicae traject. Tijdens zijn studie werd Jamie geselecteerd voor het Excellente Studententraject van het LUMC en werd de basis gelegd voor het huidige proefschrift bij de onderzoeksgroep immunopathologie onder begeleiding van prof. dr. Jan Anthonie Bruijn en dr. Ingeborg Bajema aan de afdeling Pathologie van het LUMC (hoofd: prof. dr. V.T.H.B.M. Smit).

In 2011 behaalde hij zijn bachelordiploma met diverse verdiepingsstages waaronder Tropical Medicine aan de Universitas Muhammadiyah in Yogyakarta, Indonesië. Van 2012-2016 verrichtte hij fulltime onderzoek waarin hij werd ondersteund middels de Kolff studentonderzoeker beurs van de Nierstichting en een tweejarige MD/PhD aanstelling. Een deel van het onderzoek werd verricht aan de University of North Carolina in Chapel Hill, VS op het laboratorium van prof. J. Charles Jennette onder supervisie van prof. Volker Nickeleit en prof. Harsharan Singh. Tussen 2016 en 2018 liep hij coschappen, waaronder oogheelkunde in de Himalaya Eye Hospital in Pokhara, Nepal. Na het behalen van het artsexamen in 2018, heeft hij het proefschrift voltooid. Hij presenteerde zijn onderzoek op diverse congressen zoals de Nederlandse Nefrologiedagen, het Dutch Complement Symposium, the American Society of Nephrology (ASN) Kidney Weeks (2010-2015) en the Oxford conference on IgA Nephropathy. Voor deze presentaties ontving hij beurzen en prijzen waaronder de ASN Kidney STARS Grant, de KNAW Van Walree beurs, de Pirani Award en de Liliane Striker Young Investigator Award van de Renal Pathology Society.

Jamie was tijdens zijn promotietraject voorzitter van de LUMC Association for PhD candidates (LAP). In die functie behartigde hij de belangen van promovendi op lokaal niveau bij de Raad van Bestuur en de Graduate School commissie van het LUMC; op regionaal niveau in samenwerking met leden van de Universiteitsraad en de verenigingen van andere faculteiten; en op nationaal

niveau als lid van het promovendi netwerk Nederland. Ook organiseerde hij netwerkgelegenheden, academische lezingen en debatten. Hij werd aangewezen als vertrouwenspersoon van het LUMC en hielp bij het oplossen van conflicten en integriteitskwesties. Hij gaf onderwijs in het curriculum van studenten Geneeskunde en Biomedische Wetenschappen en begeleidde geneeskundestudenten tijdens wetenschappelijke stages. Jamie was lid van de Boerhaave Commissie voor postacademisch onderwijs en ontving in 2017 de gouden Boerhaave-penning voor zijn inspanningen. Jamie is de oprichter van stichting GIG Haarlemmermeer. Deze stichting ontplooit het kunstzinnig talent van kinderen en jongeren en leert hen om met dat ontplooide talent een steentje bij te dragen aan de lokale samenleving door het lokale hospice en inloophuis voor kankerpatiënten en hun naasten te ondersteunen. In 2011 en 2017 ontving Jamie diverse gemeentelijke onderscheidingen voor zijn werk. Daarnaast volgde hij lessen in het Mandarijn Chinees, richtte hij met twee partners een e-commerce bedrijf op en behaalde hij de zwarte band in Judo.

# CURRICULUM VITAE (ENGLISH)

Jamie Shaun Chua was born in Johor Bahru, Malaysia on the 31st of October 1991. In 2008, Jamie graduated from the Haarlemmermeer Lyceum and obtained both the VWO diploma and the International Baccalaureate English certificate with distinction, concluding his bilingual education. At the age of 16, he started medical school at Leiden University at the Leiden University Medical Center. During the second year of medical school, Jamie was admitted to the Royal Conservatoire of The Hague, combining his medical study with Jazz Piano studies as part of the Practicum Musicae program. During his studies, Jamie was awarded the Excellent Student Program research scholarship from the LUMC. He started as a junior research student at the immunopathology group under the guidance of Prof. Dr. Jan Anthonie Bruijn and Dr. Ingeborg Bajema and built the foundations of this thesis at the Department of Pathology (head Prof. Dr. V.T.H.B.M. Smit).

Jamie earned his bachelor's degree in 2011 and enriched his curriculum with various courses, including Tropical Medicine at the Universitas Muhammadiyah in Yogyakarta, Indonesia. From 2012-2016 he worked full time with the support of the Kolff Student Grant from the Dutch Kidney Foundation and the MD/PhD scholarship from the LUMC. A part of the research described in this thesis was performed at the Unversity of North Carolina, Chapel Hill, the USA at the laboratory of Prof. J. Charles Jennette under supervision of Prof. Volker Nickeleit and Prof. Harsharan Singh. From 2016-2018 he completed his clinical rotations, including Ophthalmology at the Himalaya Eye Hospital in Pokhara, Nepal. Jamie completed his thesis after obtaining his master's degree in 2018. He presented the research at various conferences, including the Dutch Nephrology days, the Dutch Complement Symposium, the American Society of Nephrology (ASN) Kidney Weeks (2010-2015) and the Oxford conference on IgA Nephropathy. He obtained several grants and awards for these presentations, including the ASN Kidney STARS Grant, The Academy Van Walree Grant, the Pirani Award and the Liliane Striker Young Investigator Award from the Renal Pathology Society.

During his PhD studies, Jamie was the chairman of the LUMC Association for PhD candidates (LAP). In this function, he represented the interests of PhD candidates locally at the Board of Directors and the Graduate

School Committee of the LUMC, regionally in cooperation with the members of the University Council and associations of other faculties, and nationally as a member of the PhD Candidates Network of the Netherlands. He also organized networking events, academic lectures and debates. Jamie was appointed as the LUMC confidant, helping to solve conflicts and issues concerning scientific integrity. He lectured in the curriculum of Medicine and Biomedical Sciences and supervised medical students during their research internships. He was a member of the Boerhaave Committee for post-academic education and was awarded the golden Boerhaave medal in 2017 for his work. Jamie is the founder of the GIG Haarlemmermeer Foundation. This charity has the dual aim of developing young artistic talents and using their developed talents to contribute to society by supporting the local hospice and home for cancer patients and their families. In 2011 and 2017, Jamie received various municipal awards for his work in this charity. In addition, he took Mandarin Chinese lessons, founded an e-commerce business with two partners and earned a black belt in Judo.

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