The complement system and obesity-associated metabolic disorders

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The complement system and obesity-associated metabolic disorders: The CODAM study

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Chapter 1

General Introduction

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1 The complement system

The complement system is an essential part of the innate immune system. It consists of approximately 50 plasma and membrane proteins that are widely expressed in various tissues and are derived from different cell types (1, 2). Activation of the complement cascade occurs both in plasma and at the tissue level, and recently it was demonstrated to occur also within cells (3).

1.1 The Complement Pathways

The complement cascade includes three activation pathways, i.e. the classical, the lectin, and the alternative pathway, and one effector pathway, the terminal pathway (Figure 1). Activation of the classical and lectin pathways follows a similar pattern-recognition process (4). Classical pathway activation starts with the interaction of the C1 complex with its ligands, usually an immune complex. C1 complex consists of six C1q molecules, as well as two C1r and two C1s, which are the serine proteases. Interaction between C1q and the crystallisable (Fc) part of the IgG or IgM in the immune complex leads to a structural change in the C1q molecule, which causes the subsequent activation of C1r and C1s. The activated C1s can then induce the cleavage of C2 and C4 to form classical pathway C3 convertase, C4b2a. Activation of the lectin pathway starts with the recognition of its ligands, usually a carbohydrate structure in pathogens, by mannose-binding lectin (MBL), ficolins, or collectins. Similar to the classical pathway, this receptor-ligand binding causes the activation of MBL-associated serine proteases (MASPs), which form a complex with MBL, and then interact with C2 and C4 to generate the lectin pathway C3 convertase (C4b2a). The alternative complement pathway can be initiated via two routes. In the circulation, the alternative complement pathway is continuously activated at a low level via the so-called “tick-over” mechanism (5). This process starts with spontaneous hydrolysis of C3, which turns it into a bioactive form, C3(H_2O). C3(H_2O) binds with factor B and makes it available for cleavage by factor D to form C3(H_2O)Bb, the fluid phase alternative pathway C3 convertase. C3b, which is generated during the “tick-over” process, or is derived from

classical and lectin complement pathways, can deposit on the pathogen surface or cell membrane and lead to the activation of the alternative pathway. This is considered to be the amplification loop (6). Similar to C3(H_2O) in the fluid phase, surface-bound C3b binds to factor B and interacts with factor D to generate another form of alternative pathway C3 convertase, C3bBb. C3 convertases generated from the different complement activation pathways can cleave C3 into C3a and C3b. Activation of C3 leads to the generation of C5 convertases, which cleave C5 into C5a and C5b. C5b triggers the terminal pathway activation by recruiting C6, C7, C8, C9 to form C5b-9, the membrane attack complex (MAC), in the surface of the targets (4) .

Activation of the complement system results in the generation of several functional products. By the interaction with their specific receptors on the target surface, these functional complement fragments can contribute to the immune regulation (7), for instance by removal of pathogens and/or damaged host cells for C3b and C4b, via e.g. phagocytosis; by direct lysis of pathogens for C5b-9; and by promotion of the inflammatory process for C3a and C5a (also known as anaphylatoxins).

Recently, a novel finding of intracellular complement activation was demonstrated. Intracellular complement activation was first identified in T-cells (3). In that study, the authors found that inside the T-cells, C3 can be directly cleaved by cathepsin L into C3a and C3b, independent of the previously reported C3 convertases, and this was implicated in T-cell survival and differentiation. Although not extensively studied, the endogenous production and the uptake of C3(H_2O) from plasma was considered the main source of intracellular C3 (3, 8). Besides C3, intracellular presence of C5 and its degradation product C5a has also been demonstrated, although the cleavage process is still unclear (9). Intracellular complement activation was not only seen in immune cells but may also occur in other non-immune cell types, e.g. epithelial cells, endothelial cells, and fibroblasts; however, the underlying mechanisms and the downstream biological effect(s) still need to be further studied (9).

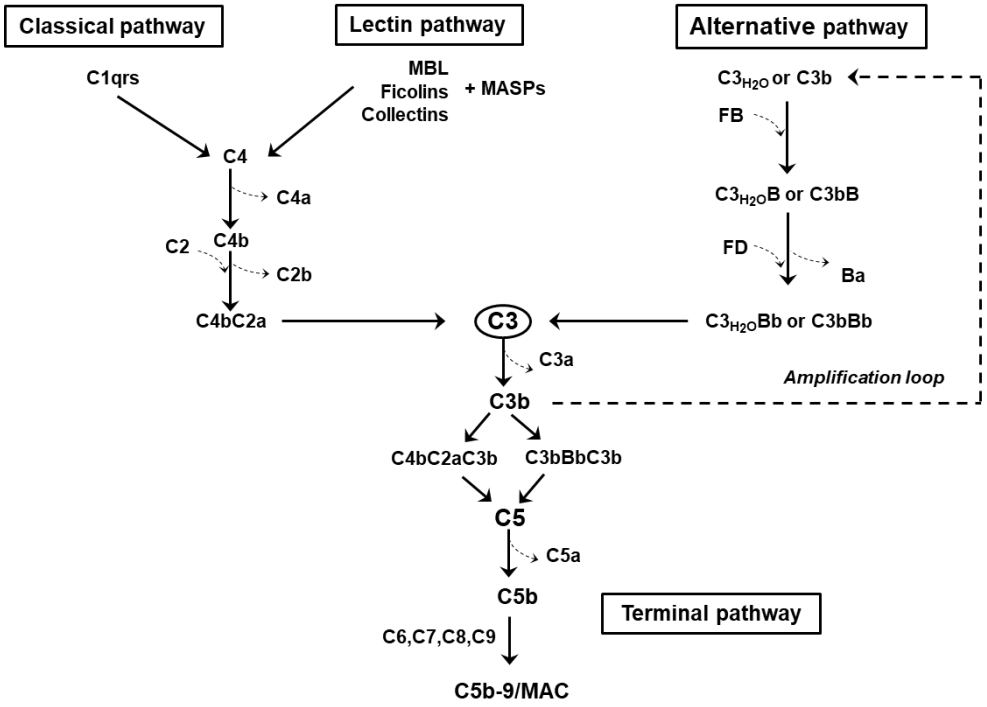

Figure 1. Schematic overview of the activation of the complement system. In response to corresponding ligands, the complement system can be activated via the classical pathway, with the participation of C1 complex; via the lectin pathway, in the presence of mannan-binding lectin (MBL)/ficolins/collectins; or via the alternative pathway, with the participation of C3(H_2O) (in fluid phase) or C3b (on the surface of target). Activation of the classical and lectin pathway can consequently activate C1r and C1s in C1 complex and MBL-associated serine proteases (MASPs), which then help to cleave C2 and C4 to form the classical and lectin pathway C3 convertase (C4b2a). C3(H_2O) and C3b from the alternative pathway can bind factor B (FB), which then is cleaved by factor D (FD) to form the alternative pathway C3 convertase (C3(H_2O)Bb or C3bBb). These C3 convertases can cleave C3 into C3a and C3b. C3b produced by different complement pathways can, in turn, contribute to activation of the alternative pathway, through which the amplification loop is formed. C3 convertases generated from the three activation pathways can bind to C3b to form the C5 convertase, which cleaves C5 into C5a and C5b and triggers the terminal pathway to recruit C6, C7, C8, C9 and form the membrane attack complex, C5b-9.

1.2 Regulation of complement activation

Activation of the complement system is under strict control by several circulating and membrane-bound regulatory proteins. In the circulation, factor H can inhibit the activation of the alternative pathway by preventing the formation and accelerating the decay of the C3 convertase, and by inactivating C3b as a cofactor for factor I (10). In contrast, properdin is a stabilizer for the alternative pathway C3 convertase that helps to enhance the activation (11). In addition, MASP-3, which is part of the lectin pathway, was reported to be an

activator of factor D (12). The soluble protein C1 inhibitor can inactivate the classical and lectin pathway by interfering with the C1 complex and the MBL complex, while C4b-binding protein controls the activation of the classical and the lectin pathway by accelerating the decay of the C3 convertase. The terminal pathway is also controlled by several circulating proteins, such as clusterin and vitronectin (13). There are also other circulating complement regulators, for instance, factor I, which can inactivate C3b and C4b in the presence of other cofactors (e.g. factor H, complement receptor 1 [CR1], C4b-binding protein) and inhibit the formation of C3 convertases from different activation pathways (14). To avoid excessive complement activation or escape from the complement surveillance, host cells, tumour cells, and some pathogens can also produce membrane inhibitors, including decay accelerating factor (DAF, also known as CD55), membrane cofactor protein (also known as CD46), complement receptor 1 (receptor of C3b and C4b, also known as CD35), and CD59 (also known as protectin) (15). DAF can prevent the formation of the complement convertases and accelerate their decay. Membrane cofactor protein and complement receptor 1 mainly function as cofactor for factor I to inhibit C3 activation. Complement receptor 1 was also reported to have decay accelerating activity. CD59 is a specific inhibitor of C5b-9 and prevents its formation by blocking the interaction between C5b-8 and C9. These soluble and membrane-bound complement regulators are essential to maintain the homeostasis of the complement cascade, and defects in these regulatory proteins can lead to imbalanced complement activation, resulting in severe diseases, such as infection or autoimmune disease (16).

The role of innate immunity in metabolism has been well established (17). As an essential part of the immune system, the complement system has also been implicated in cell and tissue homeostasis (18). Specifically, a potential role of complement components in obesity and related metabolic disorders, for instance, dyslipidaemia, insulin resistance, and metabolic syndrome, has been demonstrated.

2 The complement system in obesity-associated metabolic disorders

Nowadays, obesity is a worldwide health issue. According to data from the world health organization, the number of individuals with obesity has nearly tripled since 1975, and it now affects more than 650 million adults and over 124 million children and adolescents worldwide (19). Obesity can directly cause health problems, such as labored breathing, muscle or joint problems, but also can contribute to the development of chronic metabolic disorders, for instance, dyslipidaemia, type 2 diabetes mellitus (T2DM), metabolic syndrome, and cardiovascular disease (CVD). Therefore, the investigation of the etiology of obesity and related metabolic disorders is much needed.

2.1 The complement system in obesity

The association between obesity and the complement system has been long discussed (20). Obesity is characterized by excessive accumulating of body fat (adiposity) in peripheral and particularly, visceral adipose tissue. Adipose tissue is a complex organ that consists not only of adipocytes, but also of other cell types (e.g. endothelial, immune, and fibroblast-like cells) (21). This makes it a multifunction organ for storage of fat and energy, but also an endocrine organ that is capable of synthesizing and secreting a number of bioactive compounds that contribute to whole-body metabolic homeostasis (22). In the last decades, the presence of complement proteins and regulators in adipose tissue has been demonstrated (2). The alternative complement pathway component factor D was even reported to be specifically derived from adipose tissue (23). Also, likely as a consequence of adipose tissue expansion, increased local (adipose tissue) and circulating levels of complement components were observed in individuals with obesity (24). In agreement with this, decreased complement levels were reported in individuals after weight loss (25, 26). Obesity is accompanied by low-grade inflammation that is caused by induced production of pro-inflammatory adipokines/cytokines from dysfunctional adipose tissue (27). Several *in vitro* studies showed that some cytokines, i.e. interleukin-6 and tumour necrosis factor, of which the expression

is increased in obese individuals (28) can induce the production of complement components (29, 30).

At the same time, complement components can affect adipose tissue function and homeostasis, which may, in turn, contribute to obesity. Several lines of experimental data demonstrated a possible role of the C3-C3a-C3adesarg axis in obesity. C3adesarg (also known as acylation stimulating protein [ASP]) is the degradation product of C3a. Several *in vitro* studies demonstrated that ASP can increases fat storage in human and murine adipocytes by stimulating the triglyceride synthesis and preventing intracellular lipolysis (31, 32). Similarly, factor D was reported to induce lipid accumulation and cell differentiation in murine pre-adipocytes, probably via its influence on the C3-C3a-C3a receptor (C3aR) pathway (33). In line with this, less weight gain was often observed in mice that lack of the C3 gene (and therefore are deficient in not only C3 protein but also C3a and ASP) (34, 35). Mice that are deficient in C3aR were also reported to be resistant to high-fat diet induced obesity (36). Gene-deficient mouse models also showed effects of other complement components, mainly those from the alternative and terminal complement pathway, on obesity, but these data were less consistent. Absence of factor B and factor D did not affect body weight (37, 38), while lack of properdin led to either increased weight gain or no consistent effect on body weight (39, 40). More weight gain was reported in mice deficient for C5a receptor 1 (C5aR1, the main receptor for C5a) (41), while lack of effect on body weight was observed in mouse knockout models for C5 or C5L2 (the complement receptor that may bind C3a, C5a, as well as ASP) (42, 43). In addition, a decrease in circulating levels of factor D was observed in obese mouse models (44, 45), while increased plasma factor D was often reported in humans (46, 47). This discrepancy between murine and humans raises the necessity to explore the association of various complement components with obesity in humans. Also, although evidence from these experimental data indicated a possible causal role of the complement system in adipose tissue homeostasis and the development of obesity, the contribution of complement components in obesity has been rarely evaluated in humans. Enhanced or abnormal complement activation was observed in some forms of human lipodystrophy, which are characterized by progressive atrophy of subcutaneous fat (48). In addition, circulating levels of some specific complement components were reported

to be associated with obesity in a substantial number of cohort studies (49). Also, one previous study has demonstrated a positive association between plasma C3 and incident obesity (50).

Taken together, most of the complement proteins can be produced locally by the adipose tissue. Expansion of adipose tissue can induce the abnormality in local production and activation of the complement system, which may, in turn, affect adipose tissue function, homeostasis and/or the development of obesity. Abnormality in production and activation of complement components in obese individuals can also contribute to various metabolic disorders (21).

2.2 The complement system in dyslipidaemia

Dyslipidaemia, including elevated levels of triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and decreased high-density lipoprotein cholesterol (HDL-C), as well as the presence of small dense LDL and HDL, is the most common comorbidity of obesity (51). In obese individuals, excessive lipid accumulation in adipose tissue leads to increased levels of free fatty acids in the circulation. The flux of these free fatty acids to non-adipose tissue, such as liver, and muscle, causes lipotoxicity in these organs which may, in turn, contribute to further abnormalities in lipid metabolism (52). For instance, increased free fatty acids uptake by the liver induces the production of VLDL (very low-density lipoprotein), which contribute to higher TG levels (53). Elevated lipid content in skeletal muscles and liver can also lead to insulin resistance, causing imbalance in lipogenesis and lipolysis and lead to disorders in lipid metabolism (54). In addition, as an endocrine organ, adipose tissue is able to produce a large number of cytokines/adipokines that are involved in lipid metabolism. The dysregulation of these secretory factors in obese individuals may also play a role in dyslipidaemia. As mentioned above, most complement components can be produced by the adipose tissue, and a link between complement system and lipid metabolism has been proposed (55).

The role of complement components, mainly those involved in the alternative complement pathway activation, in lipid metabolism has been demonstrated in several *in*

vitro and *in vivo* studies. Among them, C3adesarg/ASP is the most widely studied component. For instance, mice lacking the C3 gene (and therefore lack not only C3 protein but also C3a and ASP) were shown to have a delayed postprandial triglyceride clearance and increased fasting and postprandial free fatty acids (56). Although the underlying mechanism has not been fully clarified, the effect of C3adesarg/ASP on lipid accumulation in adipocytes may have played a role herein (31, 32). Effects on lipid metabolism were also observed for some other components from the alternative complement pathway. Data from *in vivo* studies showed that mice that lack factor B (37) or properdin (39) showed delayed postprandial lipid clearance and altered systemic lipid levels. Indeed, in this latter study, an inhibitory effect of properdin on insulin-induced fatty acid uptake was observed in differentiated murine adipocytes (39). Inactivation or overexpression of the factor D gene was also demonstrated to influence lipid accumulation and cell differentiation in murine pre-adipocytes *in vitro*, via the activation of C3-C3a-C3adesarg/ASP axis (33), but lipid levels were not affected in mice that were deficient in factor D (38).

In humans, a role of complement components in lipid metabolism is in part implicated by the observation that complement components are carried by lipoproteins in the circulation. Several complement factors (i.e. C3, C4a, C4b, and C9) and regulators (i.e. clusterin, vitronectin, C4b-binding protein, and complement factor H–related protein) were found to be present on VLDL, LDL, and/or on HDL lipoproteins in human plasma (57-59). In addition, increased amounts of these complement components on HDL lipoproteins were reported in CVD patients, and in patients with cholesteryl ester transfer protein deficiency (58, 59). These observations suggest that there may be an interplay between the complement components and metabolism and/or function of circulating lipoproteins on which they reside. This may contribute to the development of metabolic disorders. In line with this, several human studies have described the associations of circulating complement components with abnormal lipid and lipoproteins levels. To date, these studies are preliminary, limited to the major lipid classes, and generally focus on one or only a few individual complement components. For instance, higher circulating levels of C3 and C3adesarg/ASP were reported to be associated with an adverse lipid- and lipoprotein profile, e.g. higher TG, total cholesterol, and LDL-C, combined with lower HDL-C (60-62).

Altogether, evidence from experimental studies suggests a role of the alternative pathway components in lipid and lipoprotein metabolism, probably via the C3-C3a-C3adesarg/ASP axis. Human data also revealed a possible interplay between complement proteins and plasma lipoproteins, which is reflected by the associations for the complement components with plasma lipid and lipoproteins.

2.3 The complement system in metabolic syndrome

The metabolic syndrome is a cluster of metabolic disorders that mainly includes central obesity and obesity-related chronic metabolic diseases, such as dyslipidaemia, hyperglycaemia, and hypertension. There are different definitions of metabolic syndrome from various organizations worldwide, among which the most commonly used is the joint interim statement proposed by the International Diabetes Federation, American Heart Association, National Heart, Lung, Blood Institute, and other organizations that was published in 2009 (63). According to this definition, participants who meet three or more of the following criteria are classified as having the metabolic syndrome: (1) waist circumference ≥88 cm in women and ≥102 cm in men; (2) TG ≥ 1.7 mmol/L, and/or use of medication for elevated TG; (3) HDL-C < 1.0 mmol/L in men, and < 1.3 mmol/L in women, and/or use of medication for reduced HDL; (4) Systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg, and/or use of antihypertensive medication; (5) fasting glucose ≥ 100 mg/dl , and/or use of glucose-lowering medication.

As mentioned above, the complement system is closely related to obesity and dyslipidaemia. The role of central complement component, C3, in insulin resistance has also been reported in humans (64-66). Thus, the complement system may also be involved in the pathogenesis of the metabolic syndrome. Indeed, some human studies provided the association of some complement components with the prevalence of the metabolic syndrome. Plasma C3 level was reported to be higher in individuals with metabolic syndrome at different age and from various ethnic groups (67-70). Similarly, a higher ASP level was also found to be related to a higher number of metabolic syndrome components in Chinese children and adolescents (71). It has been suggested that the prospective

associations between C3 and development of cardiometabolic diseases, such as T2DM and CVD, is related to activation of the complement system, and particularly the alternative pathway (72, 73). Metabolic syndrome is a strong risk factor for cardiometabolic diseases, such as CVD and T2DM. To date, prospective associations of complement components with the metabolic syndrome have, so far, only been evaluated in two human observational studies (74, 75). The results implied higher risks for the development of the metabolic syndrome in individuals with higher circulating C3 and C4 levels.

Altogether, the associations between complement system and metabolic profiles suggest a role of complement system in the metabolic syndrome. Available human evidence mostly focuses on C3 and C3a-desarg/ASP. The information on the associations of other complement components that were reported to be involved in metabolic homeostasis, e.g. factor D, factor H, properdin, and C1q (33, 39, 44, 76, 77), with the metabolic syndrome is still scarce in humans. Prospective human data on the association between complement system and metabolic syndrome is also limited.

2.4 The complement system in type 2 diabetes mellitus

Obesity is regarded as the major risk factor for T2DM. T2DM is a chronic metabolic disease that mainly characterized by an increased blood glucose concentration which results from insulin resistance and pancreatic β-cell dysfunction. Insulin resistance is a pathologic condition in which peripheral tissues, such as adipose tissue and muscle, are less sensitive to the effects of insulin. In the condition of insulin resistance, more insulin must be produced by β-cells to control the glucose concentration in the circulation. Once the β-cells fail to secrete sufficient amounts of insulin to compensate for insulin resistance, T2DM occurs (78).

The complement system may be involved in the pathophysiological process of insulin resistance. *In vitro* studies showed that degradation product of C3, C3adesarg/ASP, can induce the accumulation of lipid and the uptake of glucose in different cell types (79). Plasma ASP was reported to be associated with postprandial TG clearance in humans (80). ASP resistance, characterized by increased ASP level with impaired clearance of

postprandial TG was observed in individuals with obesity, T2DM, or CVD, and was attributed to the disturbance in ASP-C5L2 (ASP receptor) signalling pathway (81). Therefore, ASP resistance may lead to the redistribution of lipid and glucose in liver, adipose tissue, or muscles, and cause insulin resistance in these tissues, thus contributing to obesity and (obesity-associated) morbidities such as T2DM (66, 81). In line with this, improved insulin sensitivity with increased energy expenditure was reported in ob/ob mice lacking C3 (also deficient in ASP) (35). It was also demonstrated that mice that do not have C5L2 developed more severe insulin resistance on a diabetogenic diet (82). This may have occurred via altered body substrate partitioning, fat distribution, and increased inflammation. C3a, the activation product of C3 and the precursor of ASP, is well-known for its pro-inflammatory properties. A higher circulating concentration of C3a is generally associated with more (low-grade) inflammation (83, 84), which is known to induce insulin resistance (2, 85). Less high-fat-diet-induced macrophage infiltration and less insulin resistance were observed in mice deficient in C3aR (36). In addition, deletion of the gene C1QA (an important component of the C1 complex) can protect mice from high-fat-diet/obesity-induced hepatic insulin resistance and glucose intolerance, via prevention of complement activation (86). Association of complement components, particularly the central complement component C3, with (obesity-associated) insulin resistance has also been shown in human studies (87). Data from our group (66) and others (88) have identified C3 as an independent contributor to insulin resistance and T2DM. Also, higher circulating levels of C3a and/or its derived peptide C3a-desarg were shown to be associated with metabolic conditions that are characterized by insulin resistance, such as fatty liver disease (89) and obesity (71, 90).

In recent years, a role for complement components in β-cell function, e.g. insulin secretion, has also been demonstrated. Most of the evidence comes from experimental studies. For instance, it was reported that C3a and C5a, as well as their receptors, were expressed by murine and human islets, and activation of C3a-C3aR and C5a-C5aR1 pathways may contribute to potentiation of glucose-depended insulin secretion (91). In line with this, a beneficial role for factor D on β-cell function in mouse models of obesity-induced diabetes was reported, which may act via the induction of C3a-C3aR pathway-related insulin secretion (44). In agreement with these experimental data, a positive association was

reported for the circulating concentration of C3 with insulin secretion in individuals without diabetes, independent of adiposity and insulin resistance (92).

Taken together, previous experimental data and some human studies suggested associations of complement proteins, mainly the central component C3 and components involved in C3 activation, with the two main underlying pathways of T2DM, i.e. worse (peripheral) insulin resistance but at the same time improved insulin secretion by the β-cells. Future investigations are required to explore which part of these associations play a dominant role in humans.

3 Possible impact of dicarbonyl stress on complement activation

As mentioned above, the complement system is under strict control by a group of soluble and membrane regulatory proteins. DAF and CD59 are two key membrane inhibitors for complement activation (for details see page 3). Inhibition or loss of function of these may cause excessive complement activation and induce severe diseases. Absent or decreased expression of DAF and/or CD59 on erythrocytes can cause excessive complement activation on cell surface and induce cell lysis, leading to haemolysis in paroxysmal nocturnal haemoglobinuria (93). Deletion of CD59 in mice on a pro-atherogenesis genetic background can accelerate atherosclerosis via enhanced complement activation and C5b-9/MAC formation (94). Interestingly, one recently identified pathway that may affect the functionality of the complement regulation is glycation of these regulatory proteins (95-98).

Glycation is a non-enzymatic modification process that is initiated by the interaction between reducing sugars (e.g. glucose and fructose) with the arginine or lysine residues on proteins (99). In the early stage of glycation, a Schiff base is quickly formed. Via several rearrangements this then turns into a more stable compound, an Amadori product. The formation of Schiff base and Amadori product is reversible. Further dehydration, condensation and fragmentation of the Amadori product leads to the generation of the irreversible advanced glycation end products (AGEs). The classical glycation process (also called “Maillard reaction”) occurs slowly *in vivo.* During glucose metabolism, a group of

intermediate products, known as α-dicarbonyl compounds, is produced. These α-dicarbonyl compounds can interact directly with the amino acid residuals on proteins and form AGEs in a much faster way and this process is considered a major source of AGEs (100). Glycation of proteins may change their activity and/or function and/or their susceptibility to enzymatic degradation (101). Pathological conditions, such as diabetes and renal failure, can induce the accumulation of α-dicarbonyl compounds, causing enhanced glycation of proteins and increased formation of AGEs, a process that is regarded as dicarbonyl stress (102). It is increasingly clear that dicarbonyl stress can contribute to the dysfunction and damage in tissues and to the progression of diseases, by modification of cellular proteins and nucleic acids via glycation.

Presence of glycated CD59 in the membrane of endothelial and epithelial cells, as well as in the circulating and urine has been demonstrated in humans, especially in individuals with T2DM (96, 97, 103). Glycated DAF was also found in erythrocytes from diabetic patients (95). Several lines of evidence, mostly from *in vitro* studies, suggested that glycation can impair the inhibitory function of CD59 and DAF on complement activation (95, 98, 103). In line with this, in one of the studies mentioned above, glycated CD59 was found to co-localize with C5b-9 in the kidney and nerves from diabetes patients (97). The authors suggested that presence of glycated, less functional CD59 may cause increased C5b-9 deposition in these organs, which contribute to the pathological process of vascular complications in diabetes patients. Glycation of complement C3 and factor B have also been reported previously, but the effect of glycation on their metabolism or function is still not clear (101, 104-108).

Overall, dicarbonyl stress can enhance the glycation process by inhibiting the inhibitors CD59 and DAF and usually occurs in pathological situations such as diabetes. Previous human studies reported presence of glycated complement regulators in diabetes patients. Experimental data showed impaired regulatory function as well as altered complement activation by glycation. The role of dicarbonyl stress on glycation-inactivation of complement regulatory proteins and related changes in complement activation still needs to be explored in humans.

4 Outline of the thesis and study population

4.1 Aim and outline of the thesis

Beyond the pathogen defence capacity, increasing evidence suggests multiple functions of the complement system, including its role in metabolic processes (109). Until now, these results were mostly obtained from experimental studies. Human studies investigating the association between the complement system and various metabolic disorders are quite limited and the available data often focus on only one or a few complement factors. The research presented in this thesis therefore, focuses on detailed associations of a wide range of classical complement proteins from various pathways with obesity and its most commonly related metabolic disorders in humans. The studies described in this thesis were performed with data from an observational human cohort study (see more details below).

Figure 2 illustrates a schematic overview of the studies included in this thesis. In **Chapter 2,** we investigated the longitudinal association of the circulating concentrations of complement components from alternative (i.e. C3, C3a, Bb, factor D, factor H, and properdin) and terminal (i.e. C5a and sC5b-9) pathway and adiposity (body mass index [BMI] and waist circumference). In **Chapter 3** and **Chapter 5**, we focused in detail on the role of complement components of the alternative pathway (i.e. C3, C3a, Bb, factor D, factor H, properdin, and MASP-3) in dyslipidaemia and T2DM, two major comorbidities of obesity. In **Chapter 4** we performed a systematic evaluation of the associations of complement components involved in alternative and classical activation (i.e. C3, C3a, Bb, factor D, factor H, properdin, C4, C1q, and C1 inhibitor) with prevalence and incidence of the metabolic syndrome. In **Chapter 6,** we explored the influence of dicarbonyl stress on systemic complement activation.

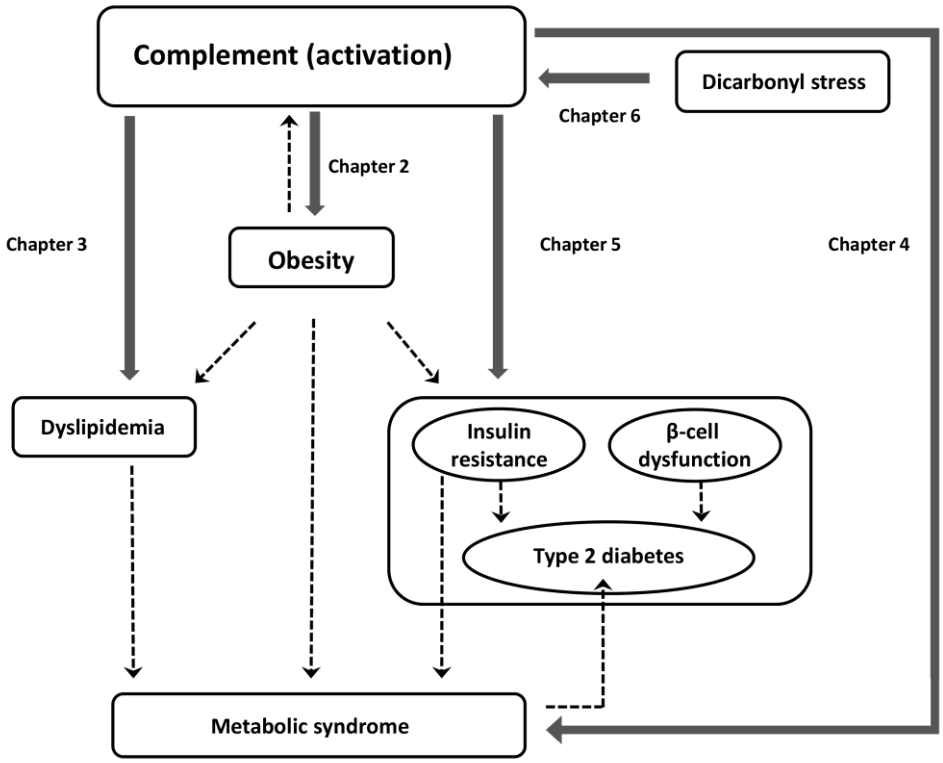

Figure 2. Schematic overview of the studies included in this thesis. The bold grey arrows indicate associations investigated in this thesis.

4.2 The CODAM Study

The work described in this thesis is conducted with data obtained from an ongoing human observational cohort study, the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) study (110, 111). The CODAM study was designed to investigate the possible contributors to the natural development of T2DM and cardiometabolic disease. An oversampling strategy for IGM and T2DM was used for the recruitment of participants. Participants of a large population-based cohort study (112) were invited for screening of the CODAM study if they were of Caucasian descent and > 40 years of age, and had one or more of the following characteristics: BMI > 25 kg/m^2; use of antihypertensive medication; positive family history of T2DM; postprandial blood glucose level > 6.0 mmol/L; history of

gestational diabetes and/or glycosuria. Thus, participants of the CODAM were characterized by increased risks for T2DM and CVD. At the start, 2715 individuals who met the selection criteria participated in the screening and underwent an oral glucose tolerance test (OGTT). According to the results of the OGTT test, 226 individuals were newly diagnosed with T2DM, 600 had impaired glucose metabolism (IGM), and 1889 had normal glucose metabolism (NGM) based on the 1999 WHO criteria (113). Of note, among individuals with IGM, 385 were impaired glucose tolerant and 215 had impaired fasting glucose (individuals had both impaired glucose tolerant and impaired fasting glucose were classified as impaired glucose tolerant). Among these, all individuals with newly diagnosed T2DM, 291 individuals with IGM, and 728 randomly selected individuals with NGT were invited for the CODAM study. To ensure sufficient power for analyses in individuals with T2DM, an additional 134 individuals with known T2DM were also invited. Eventually, 574 individuals (42% participation rate, NGM=301, IGM=127, T2DM=146) were recruited for the first (baseline) evaluation of the CODAM study. The mean age of the participants was 59 ± 6 years, 62% were men, and their BMI was, on average, 28.5 ± 4.3 kg/m^2.

Thus far, participants of the CODAM study have been evaluated at the Maastricht University's metabolic research unit twice, i.e. at baseline (1999-2001) and after a median of 7 years [interquartile range 6.9-7.1] follow-up (2006-2009). Of the 574 individuals who were included at baseline, 79 (14%) were lost to follow-up, leaving 495 (86%) individuals available at the second evaluation. The reasons for loss to follow-up were death (N=37) and others, including do not longer willing/able to participate, moved to other city/country, not able to re-establish contact, etc. (N=42). During the evaluations, participants were asked to fill in self-administrated questionnaires to provide information on their lifestyle habits, such as smoking habits, alcohol intake, diet, physical activity, and self-perceived health status. Clinical measurements, including adiposity, blood pressure, and several vascular measurements were performed. A second OGTT test was also conducted and venous blood samples were collected for the biochemical measurements of several metabolic parameters, e.g. complement components, glucose, insulin, lipids, liver enzymes, as well as some inflammation markers. Several common polymorphisms were also investigated with a

genome-wide assay. In addition, blood metabolomic profile was measured at baseline by using high-throughput ^{1}H-NMR metabolomics.

The CODAM study is an observational study with detailed phenotyping of the participants. With the measurement for an array of complement factors, as well as various metabolic profiles in one cohort, we can comprehensively investigate the metabolic effects of complement, to further translate the existing animal studies and *in vitro* data to 'the human situation'.

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Chapter 2

Longitudinal associations of the alternative and terminal pathways of complement activation with adiposity: The CODAM study

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Abstract

Objective: To investigate longitudinal associations of components of the alternative (C3, C3a, Bb, factor D [FD], factor H [FH], and properdin) and the terminal complement pathway (C5a, sC5b-9) with adiposity.

Methods: A prospective human cohort study (n = 574 at baseline, n = 489 after 7 years follow-up) was analysed. Generalized estimating equations were used to evaluate the longitudinal associations between complement components (standardized values) and adiposity (main outcome BMI [kg/m^2]). Multiple linear regression models were used to investigate the associations between change in complement levels and change in BMI. Analyses were adjusted for age, sex, medication and lifestyle.

Results: Over the 7-year period, baseline C3 was positively associated with BMI (β = 1.72 [95% confidence interval (CI): 1.35; 2.09]). Positive associations were also observed for C3a (β = 0.64 [0.31; 0.97]), FD (β = 1.00 [0.59; 1.42]), FH (β = 1.17 [0.82; 1.53]), and properdin (β = 0.60 [0.28; 0.92]), but not for Bb, C5a or sC5b-9. Moreover, changes in C3 (β = 0.52 [0.34; 0.71]) and FH (β = 0.51 [0.32; 0.70]) were significantly associated with changes in BMI.

Conclusions: The complement system, particularly activation of the alternative pathway, may be involved in development of adiposity. Whether individual aspects of alternative pathway activation have a causal role in human obesity, remains to be investigated.

1 Introduction

Adipose tissue shows adaptive reactions such as expansion or atrophy in response to changes in the microenvironment (as reviewed in (1)). Obesity is an ultimate consequence of adipose tissue expansion. It is characterized by remodelling of adipocytes in size (hypertrophy) and numbers (hyperplasia) which is accompanied by local vascularization, infiltration of inflammatory cells, and enhanced extracellular matrix production (as reviewed in (2)).

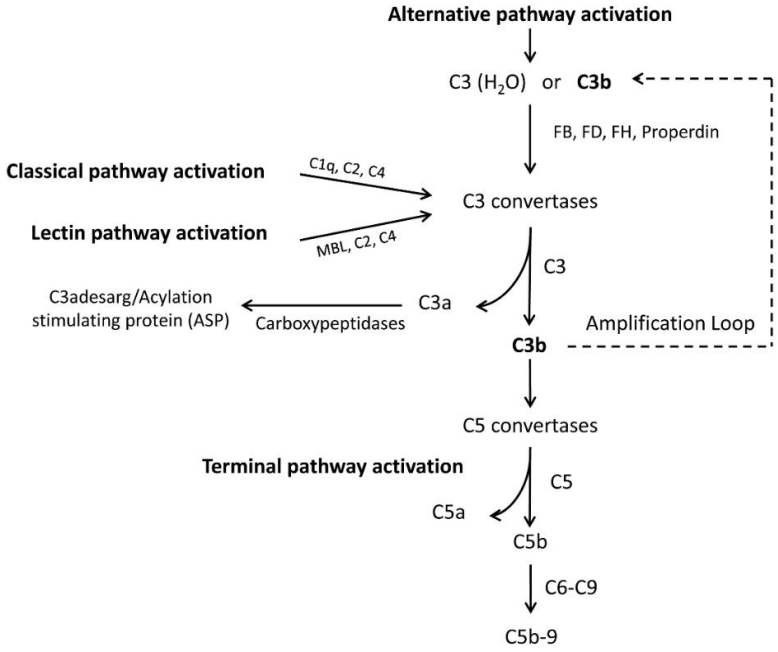

Figure 1. Activation of the complement system with focus on components that are most relevant for the current study. Complement activation can be initiated via the classical, lectin, or alternative pathway. Classical pathway activation starts with interaction between C1q and its ligands, lectin pathway activation starts with the recognition of its ligands by mannose binding lectin (MBL), collectins, or ficolins. Both can result in the cleavage of C2 and C4 to form C3 convertase (C4b2a). The alternative pathway can be initiated via two routes. Activation can start with spontaneous hydrolysis of C3 which generates C3(H_2O) or with C3b that is generated by the classical or lectin pathways. C3b and C3(H_2O) make factor B available for cleavage by factor D (FD). This yields Bb which combines with C3b and C3(H_2O) to generate the alternative pathway C3 convertase (C3bBb and C3(H_2O) Bb). The alternative pathway functions as an amplification loop for all activation pathways. Activation of the alternative pathway is under strict control of several regulatory proteins, including factor H (FH), which acts as a destabilizer of the C3 convertase, and properdin which is a C3 convertase stabilizer. C3 convertases generated via either pathway cleave C3 into C3a and C3b. The anaphylatoxin C3a is rapidly degraded to C3a-desarg, also called acylation stimulating protein (ASP). C3b can contribute to the amplification loop to produce more C3 convertases and, by combining with the C3 convertase, generate a C5 convertase, which cleaves C5 into C5a and C5b and triggers the terminal pathway to recruit C6, C7, C8, C9 to form the membrane attack complex, C5b-9.

The complement system is a complex protein network that is extensively expressed in adipose tissue (3). It plays an important role in the innate immune system, but has in recent years also been implicated in cell and tissue homeostasis. It has three main activation pathways (classical, lectin, and alternative pathway). Activation of any of these pathways will generate C3 convertases that can cleave C3 into C3a and C3b. Activation of C3 triggers the generation of C5 convertases, which cleave C5 into C5a and C5b and with subsequent activation of the main effector pathway, i.e. the terminal pathway (for details see **figure 1**) (as reviewed in (4)).

A growing body of data suggests that complement activation is involved in adipose tissue function and homeostasis. First, in vitro studies demonstrated that the C3a degradation product C3adesarg (also known as acylation stimulating protein [ASP], see **figure 1**) is able to stimulate the synthesis of triglycerides in adipocytes. As such, C3adesarg/ASP may be directly involved in adipose tissue metabolism and biology, and possibly advance differentiation of pre-adipocytes into mature adipocytes (as reviewed in (5)). In line with this, less weight gain was often observed in mice that were deficient for the C3 gene (and therefore lack not only C3 protein but also C3a and ASP), or for the receptor for C3a (C3aR) (6-8). Thus, several lines of experimental data suggest that the C3-C3a-C3adesarg/ASP axis may causally contribute to adipocyte biology and total body weight. The reported effects of other components of the alternative and terminal complement pathway on body weight in mouse models were less consistent. Absence of factor B did not affect body weight (8, 9), while lack of properdin resulted in either increased weight gain or no consistent effect on body weight (10, 11). Mice deficient for the receptor for C5a (C5aR1) showed more weight gain (12), while lack of C5 or C5L2 (the alternative C5a receptor that may bind both C3a and C5a) did not affect body weight (13, 14). In addition to these experimental data, some forms of human lipodystrophy, which are characterized by progressive atrophy of subcutaneous fat, are associated with enhanced or abnormal complement activation (15). Moreover, a substantial number of studies, mostly focusing on only one or very few complement components, reported on cross-sectional associations between circulating complement and adiposity (as reviewed in (16)). One small case-control study in elderly individuals with age-related macular degeneration reported positive cross-

sectional associations with body mass index (BMI), for a larger number of complement factors (17). Only a few studies have reported associations between changes in complement and changes in body weight in humans (18, 19), and to the best of our knowledge only one study showed that complement C3 was positively associated with incident obesity (20).

Taken together, current experimental and human data suggest a link between complement and obesity that may be related to disturbed adipose tissue homeostasis. However, longitudinal human data on this relationship are still scarce. We herein investigated the longitudinal associations between complement factors of the alternative pathway [C3, C3a, Bb, factor D (FD), factor H (FH), and properdin], as well as terminal pathway (C5a and sC5b-9) and adiposity in a human observational cohort.

2 Materials and methods

2.1 Participants

Participants of the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) were selected from a large population-based study as previously described (21). The inclusion criteria included Caucasian descent and age > 40 years plus one or more of the following: body mass index (BMI) > 25 kg/m^2; use of anti-hypertensive medication; positive family history of type 2 diabetes mellitus (T2DM); postprandial blood glucose level > 6.0 mmol/L; history of gestational diabetes and/or glycosuria. At baseline 574 participants were included and 495 participants (> 85% of baseline participants) were re-evaluated after a median follow-up of 7 [interquartile range (IQR) 6.9 – 7.1] years. A flowchart for inclusion of participants in the current study is shown in **supplementary Figure 1 (Fig. S1)**. The study was approved by the medical ethics committee of Maastricht University. All participants gave written informed consent.

2.2 Measurements

Complement factors: After an overnight fast, blood samples were collected and stored at –80 °C before use. FH was measured in EDTA plasma using a commercially available ELISA kit (DuoSet, R&D Systems, Minneapolis, MN, USA) at a 1/10,000 dilution according to the manufacturer's instructions, inter-assay variation was 13.5 %. Measurements of other complement factors (i.e. C3, C3a, Bb, FD, properdin, C5a, sC5b-9) were as previously described (22, 23). C3a, Bb, and properdin were measured only at baseline, while C3, FD, FH, C5a, and sC5b-9 were measured at baseline and at follow-up.

Adiposity and other variables: BMI (kg/m^2) and waist circumference (waist, cm), as previously described (21) were used as continuous measures of adiposity. Measurements for other variables were also obtained as described before. Briefly, information on medication use (lipid-modifying, glucose-lowering, and/or anti-hypertensive, yes/no), smoking status (current or previous tobacco smoking, yes/no), physical activity (METs/week) and total energy intake (kJ/d) was obtained using several questionnaires (21). Fasting plasma glucose, C-reactive protein (CRP), alanine aminotransferase (ALT), aspartate aminotransferase (AST); gamma-glutamyl transferase (GGT) concentrations were obtained as previous described (24). Prevalence of cardiovascular disease (CVD, yes/no) was defined as previously described. Impaired glucose metabolism (IGM) (yes/no) and T2DM (yes/no) were defined according to the 1999 WHO criteria as previously described (21). All these variables were measured at baseline and at follow-up.

2.3 Statistical analyses

Variables with normal distribution are presented as mean ± standard deviation (SD). Those with skewed distribution are presented as median (IQR) and were normalized by $\log_2$ transformation before any further analyses. Standardized values were calculated ([individuals' observed values – population mean]/standard deviation of the population) for the various complement components to allow direct comparison of their effect sizes. T-test and chi-square test were used to compare two groups, as indicated. All analyses were performed using IBM SPSS statistics version 22 and a 2-tailed *P*-value of <0.05 was considered significant.

Generalized estimating equations (GEE) with an exchangeable correlation structure were used to evaluate the longitudinal associations between complement factors (main independent variables) and BMI or waist (outcomes). BMI was used as primary outcome in the analyses. Waist was evaluated as a secondary measure of adiposity that represents a more central accumulation of fat. Analyses were initially adjusted for stratification factors (age [years], sex [male/female]), for sampling characteristics (glucose metabolism status: IGM [yes/no], T2DM [yes/no]), and also for follow-up time (years) and time-point (baseline/follow-up). The fully adjusted model additionally included medication use (glucose-, blood pressure- and/or lipid-lowering, each yes/no), smoking status (current and previous tobacco smoking, yes/no), physical activity (METs/week) and energy intake (kJ/d) to control for potential confounding. Subsequently, linear regression analyses were done to investigate the associations between the within-individual changes in complement concentration and adiposity, over time.

In addition, as various glucose-lowering medications may affect body weight (25), sensitivity analyses were performed by repeating the analyses after excluding glucose-lowering medication users at baseline (n = 73). Also, some disease conditions may affect plasma concentrations of complement. Therefore, similar sensitivity analyses were performed in which the main analyses were repeated after excluding (1) participants with acute or chronic infections (CRP>10 mg/L, n = 36), (2) patients with a (suspected) history of autoimmune disease (defined as self-reported current chronic joint inflammation/ rheumatoid arthritis or a severe intestinal disorder that lasted for the past 3 months or longer, n = 61), or (3) patients with a self-reported current malignant condition / cancer (n = 19).

3 Results

3.1 General characteristics of the study population

Table 1. General characteristics of the study population at baseline and follow-up.

	N = 541 [a]	N = 460 [b, c] Attended follow-up	
Variables	Baseline	Baseline	Follow-up
Age (years)	59.5 ± 7.0	59.1 ± 7.0	66.2 ± 7.0*
Sex (male %)	61	61	61
BMI (kg/m^2)	28.6 ± 4.4	28.5 ± 4.2	28.6 ± 4.3
Waist circumference (cm)	99.4 ± 12.1	98.8 ± 11.8	100.5 ± 12.1*
Plasma glucose (mmol/l)	5.60 (5.20−6.46)	5.56 (5.18−6.32)	5.40 (5.00−6.20)*
HOMA2-IR	1.62 (1.11−2.56)	1.61 (1.09−2.47)	1.45 (1.02−2.19)
ALT (U/l)	22.2 (17.2−28.3)	22.3 (17.2−28.4)	--
AST (U/l)	19.9 (16.4−24.2)	19.9 (16.3−24.2)	--
GGT (U/l)	24.0 (17.0−37.8)	24.0 (17.0−37.0)	--
CRP (mg/l)	2.07 (0.94−3.96)	1.89 (0.90−3.70)	1.98 (0.91−3.92)
Lipid-modifying medication (%)	20	19	44*
Glucose-lowering medication (%)	14	12	25*
Anti-hypertensive medication (%)	39	36	60*
Ever smoking (%)	22	22	16*
Cardiovascular disease (%)	28	27	42*
Type 2 diabetes (%)	26	23	35*
Physical activity (10^3·METs/week)	6.66 ± 4.12	6.73 ± 4.18	7.11 ± 4.57
Energy intake (10^3·kJ/day)	9.28 ± 2.78	9.39 ± 2.80	8.89 ± 2.60*
C3 (g/l)	1.01 ± 0.16	1.01 ± 0.16	1.15 ± 0.20*
C3a (μg/l)	59.2 (49.9−72.6)	58.6 (49.3−71.9)	--
Factor Bb (mg/l)	0.72 ± 0.19	0.71 ± 0.19	--
Factor D (mg/l)	1.00 ± 0.24	1.00 ± 0.24	1.110.31*
Factor H (mg/l)	324.9 ± 77.9	320.8 ± 78.1	324.8 ± 74.9
Properdin (mg/l)	6.05 ± 1.29	6.05 ± 1.25	--
C5a (μg/l)	7.58 ± 3.85	7.47 ± 3.72	7.68 ± 3.87*
sC5b-9(μg/l)	112.7 ± 32.8	112.9 ± 33.1	156.3 ± 74.9*

Abbreviations: BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; CRP, C-reaction protein. [a] 541 participants were included in GEE analyses. At baseline, data were available for all but missing for plasma glucose (n = 1), HOMA2-IR (n = 13), ALT (n = 6), AST (n = 6), and/or for GGT (n = 1). [b] 460 participants were included in GEE analyses. At baseline, data for complement factor and covariates were available for all but missing for BMI (n = 1), waist circumference (n = 1), plasma glucose (n = 1), HOMA2-IR (n = 9), ALT (n = 5), AST (n = 5), and/or for GGT (n = 1). At follow up, data for BMI were available for all but missing for either plasma glucose (n = 1), HOMA2-IR (n = 38), CRP (n = 5), lipid-modifying medication (n = 1), glucose-lowering medication (n = 1), anti-hypertensive medication (n = 1), ever smoking (n = 1), physical activity (n = 2), energy intake (n = 6), C3 (n = 21), factor D (n = 6), factor H (n = 7), C5a (n = 6) or sC5b-9 (n = 5). ALT, AST, GGT, C3a, Bb, and properdin were not available at follow-up. [c] Paired t-test and chi-square test were used for comparison of baseline and follow-up. *$p < 0.05$.

At baseline, complete data were available for 541 participants [59.5 ± 7.0 years old, 61% men, BMI 28.6 ± 4.4 kg/m^2; **Table 1**]. Among them, 22% were ever-smokers, 28% had CVD, and 26% had T2DM. The percentage of lipid-modifying, glucose-lowering, and anti-hypertensive medication users was 20%, 14%, 39%, respectively. Participants included in the follow-up analyses (N = 460) had complete data for complement and other variables at baseline, and for BMI and waist at baseline and follow-up. Waist, medication use,

prevalence of CVD and T2DM and concentrations of C3, FD, C5a, and sC5b-9 increased significantly during the 7-year period, while no significant changes in BMI, HOMA2-IR, CRP, and FH concentrations were observed. Habitual energy intake and plasma glucose level were lower at follow-up. The individuals without follow-up data (N = 114; **Table S1**) were at baseline slightly older, had higher FH concentration, poorer health status (more likely to have T2DM and more likely to be glucose-lowering and anti-hypertensive medication users), and had lower habitual energy intake than those with follow-up data.

3.2 Longitudinal associations between plasma complement and adiposity over a 7-year follow-up period

A significant positive correlation between baseline C3 concentration (standardized value) and BMI (in kg/m^2, baseline and follow-up) was observed in the unadjusted model (Model 1; N = 541 at baseline, N=460 at follow-up; **Table 2**). This association remained basically unchanged in the minimally adjusted model (Model 2) and in the fully adjusted model (Model 3, C3 β = 1.72 [95% confidence interval (CI): 1.35; 2.09]). Thus 1 SD higher baseline concentration of C3 was associated with, on average, 1.72 kg/m^2 higher BMI over the 7-year period. Positive associations were also observed for other factors of the alternative pathway: C3a (β = 0.64 [95% CI 0.31; 0.97]), FD (β = 1.00 [95% CI 0.59; 1.42]), FH (β = 1.17 [95% CI 0.82; 1.53]), and properdin (β = 0.60 [95% CI 0.28; 0.92]). In contrast, the associations of Bb (β = −0.16 [95% CI −0.49; 0.17]), which is also part of the alternative pathway, and C5a (β = 0.18 [95% CI −0.19; 0.54]) and sC5b-9 (β = 0.10 [95% CI −0.25; 0.45]), which are part of the terminal pathway, with BMI were not significant.

Table 2. Longitudinal associations between plasma complement factor concentrations (*baseline*) and BMI over the 7-year follow-up period (GEE analyses, N = 541 at baseline and N = 460 at follow-up)

Variables[e]	Association with BMI (kg/m^2)					
	Model 1[a]		Model 2[b]		Model 3[c]	
	β 95% CI	*P* value	β 95% CI	*P* value	β 95% CI	*P* value
C3	1.96 [1.61; 2.30]	< 0.001	1.78 [1.41; 2.15]	< 0.001	1.72[1.35; 2.09]	< 0.001
C3a[d]	0.83 [0.18; 1.17]	< 0.001	0.76 [0.43; 1.09]	< 0.001	0.64 [0.31; 0.97]	< 0.001
Factor D	0.91 [0.53; 1.29]	< 0.001	1.11 [0.68; 1.54]	< 0.001	1.00 [0.59; 1.42]	< 0.001
Factor H	1.46 [1.11; 1.80]	< 0.001	1.25 [0.90; 1.59]	< 0.001	1.17 [0.82; 1.53]	< 0.001
Properdin	0.72 [0.37; 1.06]	< 0.001	0.63 [0.30; 0.95]	<0.001	0.60 [0.28; 0.92]	<0.001
Bb	−0.26 [−0.60; 0.08]	0.135	−0.16 [−0.50; 0.18]	0.351	−0.16 [−0.49; 0.17]	0.341
C5a	0.32 [−0.06; 0.70]	0.098	0.20 [−0.18; 0.58]	0.298	0.18 [−0.19; 0.54]	0.345
sC5b-9	0.19 [−0.19; 0.58]	0.322	0.14 [−0.22; 0.51]	0.442	0.10 [−0.25; 0. 45]	0.580

Abbreviations: BMI, body mass index; 95% CI, 95% confidence interval; β, regression coefficient. [a] Model 1: unadjusted. [b] Model 2: adjusted for baseline age, sex, baseline glucose metabolic status, follow-up time and time point. [c] Model 3: Model 2 additionally adjusted for baseline medication use and lifestyle (smoking status, physical activities and energy intake). [d] Data were $\log_2$ transformed prior to analyses. [e] Data were standardized to allow direct comparison of their effect sizes.

Next, follow-up data of complement concentrations (available for C3, FD, FH, C5a, and sC5b-9) were added to the GEE model (N = 546 at baseline and N = 442 at follow-up; **Table 3**). As for the GEE analyses with complement factors measured only at baseline, positive associations were observed for the markers of the alternative pathway (Model 1, Model 2), which were significant for C3 and FH, but not for FD. The regression coefficients were basically unchanged in the fully adjusted models (C3: β = 1.04 [95% CI 0.81; 1.26], FH: β = 0.88 [95% CI 0.66; 1.11], FD: β = 0.48 [95% CI −0.08; 1.05]). The associations for C5a and sC5b-9 were, again, non-significant (Model 1, Model 2). The regression coefficients remained basically unchanged in the fully adjusted models (C5a: β = 0.12 [95% CI −0.18; 0.42], sC5b-9: β = 0.08 [95% CI −0.07 0.24]). When waist instead of BMI was used as a measure of adiposity, most associations were consistent. A few additional statistically significant associations were observed in the unadjusted models for waist that were not seen with BMI, i.e. a significant inverse association for baseline Bb (β = −1.11 [95% CI −2.13; −0.09]), and significant positive associations for FD (β = 1.69 [95% CI 0.16; 3.21]) and sC5b-9 (β = 0.74 [95% CI 0.11; 1.37]) at baseline and follow-up. These associations all disappeared in the minimally and fully adjusted models (**Tables S2, S3**).

By performing the GEE analyses, we obtain an indication of the overall longitudinal association between complement factors and adiposity, which consists of a combination of associations within and between individuals. To further evaluate the associations within

individuals, linear regression was used to evaluate the associations between changes in complement factors and changes in adiposity, over time.

As shown in **Table 4**, change (Δ) in C3 (baseline to follow-up) was positively associated with Δ BMI in the unadjusted and minimally adjusted model, and remained virtually unchanged in the fully adjusted model (Δ C3: β = 0.52 [95% CI 0.34; 0.71] N = 429). A 1 SD larger Δ C3 was associated with 0.52 kg/m^2 larger Δ BMI. A positive association was also observed for FH (Model 3, Δ FH: β = 0.51 [95% CI 0.32; 0.70]. The associations of changes in the other complement factors with Δ BMI were non-significant (Model 3, Δ FD: β = 0.12 [95% CI −0.09; 0.33], Δ C5a: β = −0.00 [95% CI −0.20; 0.19], Δ sC5b-9: β = 0.12 [95% CI −0.07; 0.32]). When waist instead of BMI was used as a measure of adiposity, again, similar results were observed (**Table S4**).

Table 3. Longitudinal associations between plasma complement factor concentrations (*baseline and follow-up*) and BMI over a 7-year follow-up period (GEE analyses, N = 546 at baseline and N = 442 at follow-up)

Variables[d]	Association with BMI (kg/m^2)					
	Model 1[a]		Model 2[b]		Model 3[c]	
	β 95% CI	*P* value	β 95% CI	*P* value	β 95% CI	*P* value
C3	0.80 [0.63; 0.96]	< 0.001	1.11 [0.88; 1.34]	< 0.001	1.04 [0.81; 1.26]	< 0.001
Factor D	0.41 [−0.07; 0.90]	0.097	0.53 [−0.05; 1.11]	0.071	0.48 [−0.08; 1.05]	0.094
Factor H	0.97 [0.75; 1.19]	< 0.001	0.90 [0.68; 1.12]	<0.001	0.88 [0.66; 1.11]	<0.001
C5a	0.18 [−0.13; 0.48]	0.251	0.10 [−0.20; 0.40]	0.495	0.12 [−0.18; 0.42]	0.424
sC5b-9	0.10 [−0.04; 0.24]	0.151	0.09 [−0.08; 0.26]	0.294	0.08 [−0.07; 0.24]	0.291

Abbreviations: BMI, body mass index; 95% CI, 95% confidence interval; β, regression coefficient. [a] Model 1: unadjusted. [b] Model 2: adjusted for baseline age, sex, baseline glucose metabolic status, follow-up time and time point. [c] Model 3: Model 2 additionally adjusted for medication use and lifestyle (smoking status, physical activities and energy intake) at baseline and follow-up. [d] Data were standardized to allow direct comparison of their effect sizes.

Table 4. Linear regression analyses between change in complement factor and change in BMI (follow-up − baseline) N = 429

Variables[d]	Association with Δ BMI (kg/m^2)					
	Model 1[a]		Model 2[b]		Model 3[c]	
	β 95% CI	*P* value	β 95% CI	*P* value	β 95% CI	*P* value
Δ C3	0.58 [0.40; 0.77]	< 0.001	0.58 [0.39; 0.77]	< 0.001	0.52 [0.34; 0.71]	< 0.001
Δ Factor D	0.10 [−0.09; 0.30]	0.303	0.17 [−0.04; 0.37]	0.108	0.12 [−0.09; 0.33]	0.246
Δ Factor H	0.52 [0.33; 0.71]	< 0.001	0.53 [0.34; 0.72]	< 0.001	0.51 [0.32; 0.70]	<0.001
Δ C5a	−0.04 [−0.23; 0.15]	0.686	−0.03 [−0.23; 0.16]	0.745	−0.00 [−0.20; 0.19]	0.987
Δ sC5b-9	0.10 [−0.09; 0.30]	0.292	0.12 [−0.08; 0.32]	0.233	0.12 [−0.07; 0.32]	0.213

Abbreviations: Δ BMI, change in BMI (all changes refer to change from baseline to follow-up); Δ C3, change in C3 level; Δ C5a, change in C5a level; sC5b-9, change in sC5b-9 level; Δ factor D, change in factor D level; Δ factor H, change in factor H level, β, regression coefficient. [a] Model 1: unadjusted. [b] Model 2: adjusted for baseline age, sex, baseline glucose metabolic status, follow-up time. [c] Model 3: Model 2 additionally adjusted for medication use and lifestyles (smoking status, physical activities and energy intake) at baseline and follow-up. [d] Data were standardized to allow direct comparison of their effect sizes.

3.3 Additional analyses

In the sensitivity analyses, all analyses were repeated after excluding participants using glucose-lowering medication at baseline. This did not materially change the above results **(Tables S5-S7)**. Also when participants with acute or chronic infections, with a (suspected) history of autoimmune disease, or with a self-reported current malignant condition / cancer, were excluded from the analyses, the results remained similar to what was observed in the main analyses (data not shown).

4 Discussion

In this Caucasian cohort we investigated the longitudinal associations of systemic factors of complement and complement activation with development of obesity, as represented by progression of adiposity. Our study has three main findings. First, over the 7-year follow-up period, higher baseline concentrations of C3, C3a, FD, FH, and properdin, but not factor Bb, were associated with more adiposity, and these associations remained for C3 and FH, but not for FD, after additionally considering their concentrations at follow-up. Second, greater changes in C3 and FH were positively associated with greater changes in BMI and waist. Third, no associations were observed between components of the terminal pathway, i.e. C5a and sC5b-9, and adiposity.

Previously-published studies mainly showed positive cross-sectional association of individual complement components — including C3 (26, 27), Bb (17), FD (28, 29), and FH (17, 30) — with human adiposity. However, until now, systematic evaluations of the associations of complement system with adiposity within human cohorts have been scarce. Also, the number of studies that provide longitudinal data is limited. Moreover, human information on the relation of properdin, an important regulator for alternative pathway, with adiposity was lacking, while associations for C5a and sC5b-9 were evaluated only in very small cross-sectional studies (17).

The positive association of C3 we observed with adiposity over time is in line with the results in previous cross-sectional and prospective studies (20, 26, 27). These human data

are also supported by the findings of less weight gain observed in C3- and C3aR-deficient mice (6, 7). Indeed, we also observed that C3a, the cleavage product of C3, was positively associated with adiposity, albeit weaker than C3. This weaker association may, at least partly, be explained by the lower concentration and shorter half-life of C3a in plasma (as reviewed in (31)).

Over the 7-year follow-up period, a positive association was also observed between adiposity and FD, the rate-limiting protease of the alternative complement pathway activation. Noteworthily, changes in FD were not associated with changes in adiposity. Thus, the positive association between FD and adiposity appeared to reflect inter-individual associations that remained consistent over time rather than within-individual effects. Existing mouse models of obesity suggest that the relation between FD and obesity in mice may differ from that in man because, in contrast to the positive association between FD and obesity in man, several obese mouse models have very low concentrations of FD (32, 33). Notwithstanding these apparent differences between man and mice, the observation that FD gene knock-out in mice did not affect body weight (34) seems to be consistent with the current lack of an association between FD and adiposity within individuals. The strong between-individual and the absence of within-individual associations suggest a complex relation between FD and adiposity. We did not observe any associations between the activated complement factor, Bb, and adiposity. This was consistent with the results of the above-mentioned cross-sectional study and previous mouse models (8, 9, 17).

FH and properdin are two important regulators of alternative complement pathway activation. FH acts as an inhibitor by dissociating the C3 convertase, whereas properdin can function as a stabilizer of this convertase which results in prolonged activation (as reviewed in (35)), (36). Despite these opposite biological functions, systemic concentrations of both FH and properdin could reflect alternative pathway activation: properdin via a direct effect on prolonged activation and FH as a compensatory response to enhanced alternative pathway activation. This corroborates and expands the previous cross-sectional findings of a positive association between FH and obesity (17, 30). Until now, no information was available for properdin in human obesity and effects of properdin in mouse models were not fully consistent. Properdin–deficient mice showed more weight gain on a high fat diet,

(10) while no effect on body weight in male mice on an Ldlr$^{-/-}$ background (11). Our current data add evidence for a potential role of properdin in human adipose tissue metabolism and obesity, but possible underlying mechanisms remain to be identified. Effect-sizes for FH were slightly larger than for properdin. This can be explained by several phenomena, including higher plasma concentration of FH and differences in measurement error and/or biological variability, but also to different functional properties since they have different binding sites with the activated C3 fragment (as reviewed in (37)), as well as to actual differences in their mutual effects on human adiposity.

In contrast to the above-mentioned results for the alternative pathway, the terminal pathway (as represented by C5a and sC5b-9) was not longitudinally associated with adiposity. This is in line with the cross-sectional study mentioned above (17). It is also in line with experimental data that showed that absence of C5 in mice, which basically abolishes the terminal pathway, did not affect weight gain (13). Effects of absence of the receptors for terminal pathway activation products showed mixed results since C5L2 knock-out did not affect body weight (14) whereas more weight gain was observed in C5aR1 deficient mice (12).

Taken together our data suggest that, of the various components and regulators of the alternative and terminal pathway of complement activation we evaluated, it is primarily the C3-C3a-C3adesarg/ASP axis that may contribute to the development of obesity in humans. A possible explanation for the association between C3-C3a-C3adesArg/ASP axis activation and obesity lies in its proposed effects on lipid accumulation and adipocyte differentiation. Activation of the C3-C3a-C3adesArg/ASP axis starts by cleavage of C3 by C3 convertase under the control of FD, FH, and properdin. In this process C3a is generated, which is unstable and can be rapidly cleaved into C3adesArg/ASP. Thus, C3adesArg/ASP is a primary end-product of this axis. Previous in experimental studies showed that C3adesarg/ASP was implicated in lipid metabolism via promotion of the uptake adipocyte fatty acid by adipocytes and stimulation of triglyceride synthesis in adipocytes (as reviewed in (5)). Via such effects, C3adesArg/ASP may contribute to body fat storage. It has also been shown that chylomicrons, the intestinal lipoproteins that are produced upon food ingestion, can induce the production and activation of C3 and C3a by adipocytes, *in vitro* (38). Promotion

and activation of C3-C3a-C3adesArg/ASP axis by chylomicrons may as such contribute to the development of high-fat-diet-induced obesity. Moreover, gain or loss of function of Factor D in adipocytes, *in vitro*, was also reported to have an effect on lipid accumulation and cell differentiation via the activation of C3-C3a-C3adesarg/ASP axis (39). In addition to its role as stabilizer of the C3 convertase, properdin might also affect adipocyte metabolism via other routes. By itself, properdin did not directly affect adipocyte biology. Rather, it inhibited insulin-induced fatty acid uptake, which was independent of C3adesarg/ASP (10). Moreover, properdin is homologous to thrombospondin-1, which is expressed in adipose tissue and may affect pre-adipocytes differentiation (40) (as reviewed in (41)).

The main strength of our present study is that we are the first to investigate the longitudinal association of adiposity and obesity with complement activation, and were able to perform a comprehensive analysis that extends beyond the complement components usually measured in human cohorts. Another key strength of this study is the comprehensive adjustment for potential confounders. Nevertheless, some limitations should be highlighted as well. Firstly, selection of the study population may limit generalizability. Besides, the BMI of our study population was on average (28.6± 4.4 kg/m^2) slightly elevated, which may also weaken the generalizability of our findings. However, considering the wide range of BMI (from 18.22 to 47.37 kg/m^2) at baseline, and the fact that we still had enough cases of participants (70%) with normal BMI and overweight (BMI<30 kg/m^2), CODAM does represent the middle–aged to older Caucasian population with moderately increased risk of T2DM and CVD. Other limitations of our study are the relatively small sample size, the fact that 15% of the original cohort was lost-to-follow-up, and the lack of information on for C3a, Bb, and properdin at follow-up. However, the fact that our results are consistent with previous data, substantiates our current observations. In addition, we cannot completely exclude the possibility of reversed causality. In other words, because of the intricate association between complement, low-grade inflammation and obesity in adipose tissue, it is not easy to disentangle the order of events, in this observational study with two available time-points. However, absence of complement genes in several mouse models did affect body weight. This suggests that the associations we report here may, at least in part, be due to effects of complement on human obesity,

rather than the reverse. We do acknowledge that the reverse may occur as well contributing to a vicious, potentially accelerating cycle of obesity and complement activation in adipose tissue. Further experimental data are needed to pin-point the exact mechanisms that underlie the observed associations.

In conclusion, in this study we evaluated a broad range of factors and regulators of the alternative and terminal pathways of complement activation in humans and showed that primarily activation of C3-C3a-C3adesarg/ASP axis pathway might contribute to the development of adiposity and eventually obesity, possibly via effects on fat storage in the adipocytes. Notably, in our analyses we focused on development of adiposity and obesity rather than on adipose tissue function. Our current data therefore do not exclude that other aspects of complement activation in adipose tissue may act as relevant potential contributors to adipose tissue dysfunction in individuals overweight or with obesity. To further extend our knowledge on the intricate relation between the complement activation and human obesity, more human prospective and intervention studies with detailed information on complement activation and detailed phenotyping of adipose tissue and metabolism are needed.

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Supplementary data

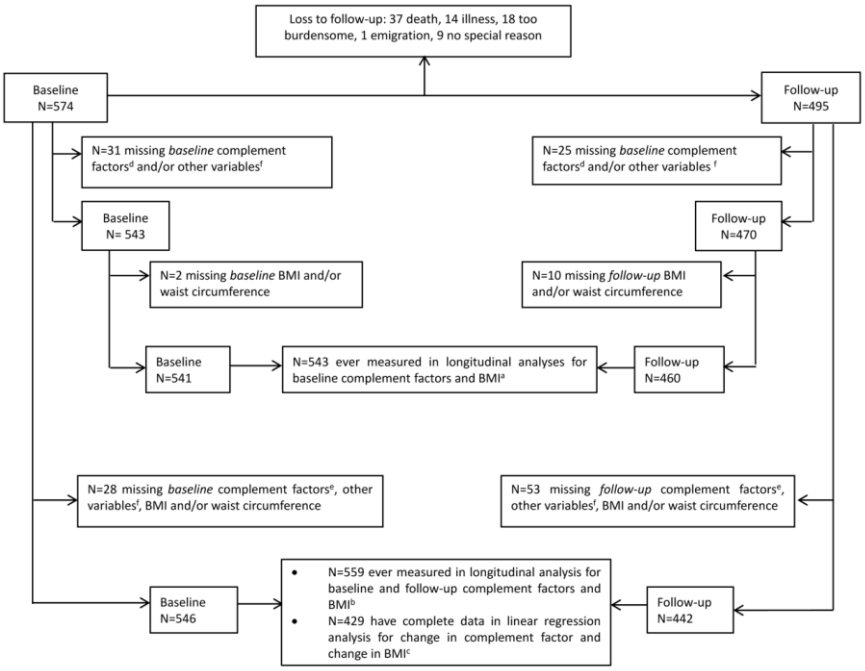

Figure S1. Flowchart of the individuals included in the main and in the additional analyses in this study. [a] The GEE analyses for baseline complement factors included 1001 observations: complete data for complement and other variables (at baseline) were available for all while BMI and/or waist circumference were available for 458 individuals at baseline and at follow-up, for 83 individuals only at baseline, and for 2 individuals only at follow-up. [b] The GEE analyses for complement factors over 7-year follow-up included 988 observations: complete data for complement factors, other variables, BMI and waist circumference were available for 429 individuals at baseline and follow-up, for 117 individuals only at baseline, and for 13 individuals only at follow-up. [c] The linear regression analyses included 429 individuals who had complete data for complement factors, other variables, BMI and waist circumference (baseline and follow-up). [d] *Baseline* complement factors referred to are C3, C3a, Bb, FD, FH, properdin, C5a and sC5b-9, [e] *Baseline* complement factors referred to are C3, C5a, factor D, factor H and sC5b-9, [f] Other variables referred to are age, sex, glucose metabolic status, follow-up time, medication use, smoking status, physical activity and energy intake.

Table S1. General characteristics of the study population at baseline

Variables	Whole population N=574[a]	Loss to follow-up N=114[b]	Attend follow-up N=460[c, d]
Age (years)	59.6 ± 7.0	61.9 ± 6.5	59.1 ± 7.0*
Sex (male %)	61	61	61
BMI (kg/m^2)	28.6 ± 4.3	28.8 ± 4.6	28.5 ± 4.2
Waist circumference (cm)	99.3 ± 11.9	101.2 ± 12.1	98.9 ± 11.8
Lipid-modifying medication (%)	19	17	19
Glucose-lowering medication (%)	13	19	12*
Anti-hypertensive medication (%)	38	47	36*
Cardiovascular disease (%)	28	28	27
Type 2 diabetes (%)	25	36	23*
Ever smoking (%)	22	23	22
Physical activity (10^3·METs/week)	6.71 ± 4.21	6.61 ± 4.33	6.73 ± 4.18
Energy intake (10^3·kJ/day)	9.25 ± 2.80	8.69 ± 2.73	9.39 ± 2.80*
C3 (g/l)	1.02 ± 0.16	1.03 ± 0.16	1.01 ± 0.16
C3a (μg/l)	59.1 (49.4–72.6)	63.4 (50.1–75.9)	58.6 (49.3–71.9)
Factor Bb (mg/l)	0.72 ± 0.19	0.74 ± 0.20	0.71 ± 0.19
Factor D (mg/l)	1.00 ± 0.24	1.01 ± 0.25	1.00 ± 0.24
Factor H (mg/l)	325.4 ± 77.3	346.7 ± 70.4	320.8 ± 78.1*
Properdin (mg/l)	6.06 ± 1.28	6.11 ± 1.39	6.05 ± 1.25
C5a (μg/l)	7.61 ± 3.86	8.25 ± 4.37	7.47 ± 3.72
sC5b-9(μg/l)	113.7 ± 34.8	116.8 ± 41.4	112.9 ± 33.1

Abbreviations: BMI, body mass index. [a] 574 participants were included in the cohort study at baseline, data were missing for BMI (n=1), waist circumference (n=1), cardiovascular disease (n=1), physical activity (n=10), C3a (n=3), factor D (n=12), factor H (n=13), porperdin (n=6), C5a (n=12), and/or for sC5b-9 (n=5) concentrations. [b] 114 participants either have missing data for cardiovascular disease (n=1), physical activity (n=10), C3a (n=3), factor D (n=12), factor H (n=13), porperdin (n=6), C5a (n=12), or for sC5b-9 (n=5) concentrations at baseline. [c] 460 participants have complete data for complement factor and other independent variables, but missing data for BMI (n=1) and/or for waist circumference (n=1) at baseline. [d] Independent sample t-test and chi-square test were used to compare the baseline variables with and without follow-up data. *$p < 0.05$.

Table S2. Longitudinal associations between plasma complement factor concentrations (*baseline*) and waist circumference over the 7-year follow-up period (GEE analyses, N=541 at baseline and N=460 at follow-up)

Variables[e]	Association with waist circumference (cm)					
	Model 1[a]		Model 2[b]		Model 3[c]	
	β [95% CI]	*P* value	β [95% CI]	*P* value	β [95% CI]	*P* value
C3	5.19 [4.28; 6.09]	< 0.001	4.92 [4.01; 5.83]	< 0.001	4.83[3.91; 5.75]	< 0.001
C3a[d]	1.82 [0.78; 2.87]	< 0.001	2.10 [1.19; 3.00]	< 0.001	1.86 [0.97; 2.75]	< 0.001
Factor D	2.60 [1.59; 3.61]	< 0.001	2.57 [1.51; 3.63]	< 0.001	2.32 [1.28; 3.35]	< 0.001
Factor H	3.99 [3.06; 4.92]	< 0.001	3.36 [2.49; 4.24]	< 0.001	3.18 [2.29; 4.07]	< 0.001
Properdin	2.01 [1.10; 2.93]	< 0.001	1.69 [0.86; 2.52]	<0.001	1.58 [0.75; 2.42]	<0.001
Bb	–1.11 [–2.13; –0.09]	0.032	–0.48 [–1.40; 0.43]	0.303	–0.50 [–1.39; 0.39]	0.268
C5a	0.71 [–0.36; 1.78]	0.195	0.85 [–0.13; 1.83]	0.088	0.78 [–0.18; 1.73]	0.110
sC5b-9	0.10 [–0.94; 1.14]	0.850	0.27 [–0.65; 1.18]	0.566	0.15 [–0.74; 1.04]	0.745

Abbreviations: 95% CI, 95% confidence interval; β, regression coefficient. [a] Model 1: unadjusted. [b] Model 2: adjusted for baseline age, sex, baseline glucose metabolic status, follow-up time and time point. [c] Model 3: Model 2 additionally adjusted for baseline medication use and lifestyle (smoking status, physical activities and energy intake). [d] Data were $\log_2$ transformed prior to analyses. [e] Data were standardized to allow direct comparison of their effect sizes.

Table S3. Longitudinal associations between plasma complement factor concentrations (*baseline and follow-up*) and waist circumference over a 7-year follow-up period (GEE analyses, N=546 at baseline and N=442 at follow-up)

Variables[d]	Association with waist circumference (cm)					
	Model 1[a]		Model 2[b]		Model 3[c]	
	β [95% CI]	*P* value	β [95% CI]	*P* value	β [95% CI]	*P* value
C3	3.12 [2.60; 3.64]	< 0.001	3.72 [3.06; 4.38]	< 0.001	3.59 [2.93; 4.25]	< 0.001
Factor D	1.69 [0.16; 3.21]	0.030	1.37 [–0.016; 2.89]	0.079	1.25 [–0.26; 2.75]	0.104
Factor H	2.58 [1.93; 3.23]	< 0.001	2.35[1.72; 2.98]	<0.001	2.30 [1.67; 2.94]	<0.001
C5a	0.62 [–0.27; 1.50]	0.171	0.64 [–0.18; 1.47]	0.126	0.64 [–0.18; 1.46]	0.127
sC5b-9	0.74 [0.11; 1.37]	0.022	0.34 [–0.32; 0.99]	0.314	0.32 [–0.29; 0.93]	0.302

Abbreviations: 95% CI, 95% confidence interval; β, regression coefficient. [a] Model 1: unadjusted. [b] Model 2: adjusted for baseline age, sex, baseline glucose metabolic status, follow-up time and time point. [c] Model 3: Model 2 additionally adjusted for medication use and lifestyle (smoking status, physical activities and energy intake) at baseline and follow-up. [d] Data were standardized to allow direct comparison of their effect sizes.

Table S4. Linear regression analyses between change in complement factor and change in waist circumference (follow-up − baseline, N= 429)

Variables[d]	Association with Δwaist (cm)					
	Model 1[a]		Model 2[b]		Model 3[c]	
	β [95% CI]	*P* value	β [95% CI]	*P* value	β [95% CI]	*P* value
ΔC3	1.99 [1.39; 2.58]	< 0.001	1.97 [1.37; 2.56]	< 0.001	1.86 [1.24; 2.47]	< 0.001
ΔFactor D	0.06 [–0.57; 0.68]	0.854	0.35 [–0.31; 1.01]	0.301	0.20 [–0.47; 0.88]	0.554
ΔFactor H	1.09 [0.48; 1.71]	0.001	1.15 [0.53; 1.77]	< 0.001	1.11 [0.49; 1.73]	0.001
ΔC5a	–0.08 [–0.71; 0.54]	0.791	–0.07 [–0.70; 0.55]	0.822	–0.07 [–0.70; 0.57]	0.839
ΔsC5b-9	0.33 [–0.29; 0.96]	0.297	0.44 [–0.19; 1.07]	0.171	0.45 [–0.19; 1.09]	0.166

Abbreviations: Δwaist, change in waist circumference (all changes refer to change from baseline to follow-up); ΔC3, change in C3 level; ΔC5a, change in C5a level; ΔsC5b-9, change in sC5b-9 level; Δfactor D, change in factor D level; Δfactor H, change in factor H level; β, regression coefficient. [a] Model 1: unadjusted. [b] Model 2: adjusted for baseline age, sex, baseline glucose metabolic status, follow-up time. [c] Model 3: Model 2 additionally adjusted for medication use and lifestyles (smoking status, physical activities and energy intake) at baseline and follow-up. [d] Data were standardized to allow direct comparison of their effect sizes.

Table S5. Longitudinal associations between plasma complement factor concentrations (*baseline*) and BMI over the 7-year follow-up period among participants without glucose-lowering medication use at baseline (GEE analyses, N=468 at baseline and N=406 at follow-up)

Variables[e]	Association with BMI (kg/m^2)					
	Model 1[a]		Model 2[b]		Model 3[c]	
	β [95% CI]	*P* value	β [95% CI]	*P* value	β [95% CI]	*P* value
C3	1.99 [1.61; 2.36]	<0.001	1.88 [1.49; 2.28]	<0.001	1.86 [1.47; 2.24]	<0.001
C3a[d]	0.75 [0.40; 1.09]	<0.001	0.66 [0.32; 1.01]	<0.001	0.55 [0.21; 0.89]	0.002
Factor D	0.93 [0.55; 1.31]	<0.001	1.15 [0.77; 1.54]	<0.001	1.07 [0.70; 1.43]	<0.001
Factor H	1.50 [1.13; 1.86]	<0.001	1.36 [0.99; 1.73]	<0.001	1.30 [0.92; 1.68]	<0.001
Properdin	0.65 [0.30; 1.00]	<0.001	0.56 [0.23; 0.90]	0.001	0.55 [0.21; 0.89]	0.001
Bb	–0.12 [–0.48; 0.25]	0.534	–0.08 [–0.45; 0.28]	0.652	–0.08 [–0.43; 0.27]	0.656
C5a	0.30 [–0.11; 0.71]	0.149	0.21 [–0.20; 0.61]	0.323	0.19 [–0.21; 0.58]	0.351
sC5b-9	0.22 [–0.17; 1.25]	0.264	0.15 [–0.23; 0.52]	0.439	0.12 [–0.25; 0.48]	0.541

Abbreviations: BMI, body mass index; 95% CI, 95% confidence interval; β, regression coefficient. [a] Model 1: unadjusted. [b] Model 2: adjusted for baseline age, sex, baseline glucose metabolic status, follow-up time and time point. [c] Model 3: Model 2 additionally adjusted for baseline medication use and lifestyle (smoking status, physical activities and energy intake). [d] Data were $\log_2$ transformed prior to analyses. [e] Data were standardized to allow direct comparison of their effect sizes.

Table S6. Longitudinal associations between plasma complement factor concentrations (*baseline and follow-up*) and BMI over a 7-year follow-up period among participants without glucose-lowering medication use at baseline (GEE analyses, N=473 at baseline and N=391 at follow-up)

Variables[d]	Association with BMI (kg/m^2)					
	Model 1[a]		Model 2[b]		Model 3[c]	
	β [95% CI]	*P* value	β [95% CI]	*P* value	β [95% CI]	*P* value
C3	0.76 [0.58; 0.94]	<0.001	1.10 [0.85; 1.35]	<0.001	0.99 [0.74; 1.24]	<0.001
Factor D	0.31 [–0.23; 0.85]	0.265	0.39 [–0.25; 1.03]	0.229	0.27 [–0.35; 0.89]	0.389
Factor H	0.94 [0.70; 1.19]	<0.001	0.90 [0.65; 1.15]	<0.001	0.85 [0.59; 1.11]	<0.001
C5a	0.11 [–0.22; 0.43]	0.531	0.05 [–0.27; 0.38]	0.754	–0.06 [–0.40; 0.29]	0.741
sC5b-9	0.11 [–0.04; 0.26]	0.166	0.09 [–0.09; 0.27]	0.322	0.09 [–0.07; 0.26]	0.270

Abbreviations: BMI, body mass index; 95% CI, 95% confidence interval; β, regression coefficient. [a] Model 1: unadjusted. [b] Model 2: adjusted for baseline age, sex, baseline glucose metabolic status, follow-up time and time point. [c] Model 3: Model 2 additionally adjusted for medication use and lifestyle (smoking status, physical activities and energy intake) at baseline and follow-up. [d] Data were standardized to allow direct comparison of their effect sizes.

Table S7. Linear regression analyses between change in complement factor and change in BMI (follow-up – baseline) among participants without glucose-lowering medication use at baseline (N= 379)

Variables[d]	Association with ΔBMI (kg/m^2)					
	Model 1[a]		Model 2[b]		Model 3[c]	
	β [95% CI]	*P* value	β [95% CI]	*P* value	β [95% CI]	*P* value
ΔC3	0.61 [0.40; 0.81]	<0.001	0.60 [0.39; 0.80]	<0001	0.55 [0.34; 0.76]	<0.001
ΔFactor D	0.02 [–0.20; 0.23]	0.875	0.08 [–0.15; 0.30]	0.502	0.03 [–0.20; 0.26]	0.784
ΔFactor H	0.55 [0.35; 0.76]	<0.001	0.55 [0.35; 0.76]	<0.001	0.54 [0.33; 0.74]	<0.001
ΔC5a	–0.09 [–0.30; 0.13]	0.437	–0.09 [–0.31; 0.13]	0.423	–0.05 [–0.27; 0.17]	0.661
ΔsC5b-9	0.10 [–0.11; 0.31]	0.339	0.12 [–0.09; 0.33]	0.257	0.13 [–0.08; 0.34]	0.228

Abbreviations: ΔBMI, change in BMI (all changes refer to change from baseline to follow-up); ΔC3, change in C3 level; ΔC5a, change in C5a level; ΔsC5b-9, change in sC5b-9 level; Δfactor D, change in factor D level; Δfactor H, change in factor H level; β, regression coefficient. [a] Model 1: unadjusted models. [b] Model 2: adjusted for baseline age, sex, baseline glucose metabolic status, follow-up time. [c] Model 3: Model 2 additionally adjusted for medication use and lifestyles (smoking status, physical activities and energy intake) at baseline and follow-up. [d] Data were standardized to allow direct comparison of their effect sizes.

Chapter 3

Complement C3 and other components of the alternative pathway are associated with an adverse lipoprotein subclass profile: The CODAM study

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Submitted

Abstract

Objective

Presence of complement components on lipoproteins may affect their composition and metabolism. We investigated the associations of complement C3, as well as other components of the alternative complement pathway, with the plasma lipoprotein subclass profile.

Approach and Results

Plasma complement concentrations (C3, properdin, factor H, factor D, MASP-3, C3a, and Bb), and lipoprotein subclass profile (as measured by nuclear magnetic resonance spectroscopy) were obtained in 523 participants (60±7 years, 61% men) of the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) study. Multiple linear regression was used to investigate the associations of C3 (primary determinant) and the other alternative pathway components (secondary determinants) with characteristics (particle concentration, size [main outcomes]), and lipid contents (secondary outcomes) of 14 lipoprotein subclasses, ranging from extremely large VLDL to small HDL (all standardized [std] values).

Participants with higher C3 concentrations had more circulating VLDLs (stdβs ranging from 0.27 to 0.36), IDL and LDLs (stdβs ranging from 0.14 to 0.17), and small HDL (stdβ = 0.21). In contrast, they had fewer large and very large HDL particles (stdβs = –0.36). Also, in those with higher C3 concentrations, all lipoprotein subclasses were enriched in triglycerides. All false discovery rate corrected P-values were <0.05.

Similar but weaker associations were observed for properdin, factor H, factor D, and MASP-3, but not for the activated products of the alternative pathway (C3a and Bb).

Conclusions

The alternative complement pathway, and most prominently C3, is associated with an adverse lipoprotein subclass profile that is characterized by more triglyceride-enriched lipoproteins but fewer large HDL.

1 Introduction

Components of the complement system are present on plasma lipoproteins (1-3), and the levels of these complement components may vary with disease status (2, 3). In patients with chronic cardiometabolic diseases, such as the metabolic syndrome, type 2 diabetes mellitus (T2DM) and related cardiovascular diseases (CVD), altered complement expression and activation (4), as well as dyslipidaemia (5) are present. This suggests a possible relationship between the complement system and metabolism and/or function of circulating lipoproteins.

The complement system is a complex protein network. Besides its canonical function in the immune response, a role for the complement system in metabolic disease processes has been demonstrated (4). Activation of the complement system is initiated via three pathways: the classical, the lectin, and the alternative pathway (**Supplementary Figure 1 [Figure S1])** (4). Particularly C3, the central component of the complement system, has consistently been shown to be higher in plasma of patients with cardiometabolic diseases (6). C3 was also shown to be present on very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) (1, 2). Recently, a number of human studies reported associations, mainly in an adverse direction, of complement components with systemic lipid profile (7). These studies primarily focused on C3 and its degradation products, C3a/C3adesarg, and were generally limited to the major lipids and lipoprotein classes, i.e. triglycerides (TG), LDL-cholesterol (LDL-C), and HDL-cholesterol (HDL-C). For instance, in several human cohort studies, circulating levels of C3 and C3a/C3adesarg were reported to be positively associated with plasma TG, total cholesterol, and LDL-C levels, and inversely associated with HDL-C levels (8-10). Human data for other complement regulators and activated products are still scarce, but it has been shown that not only C3, but also e.g. C4 and C9 are present on VLDL, LDL, and HDL (1-3).

Experimental findings suggest that the complement system, mainly the alternative complement pathway, may be causally involved in lipid metabolism. For instance, male mice that are deficient in C3 showed delayed postprandial triglyceride clearance and increased fasting and postprandial free fatty acids compared to their wild type controls (11).

In line with this, mice that lack other components of the alternative pathway such as factor B (12) or properdin (13), also showed delayed postprandial lipid clearance and altered systemic lipid levels. In contrast, lipid levels were not affected in mice that were deficient in the alternative pathway protease factor D (FD) (14). In addition, *in vitro* studies in murine adipocytes showed that exogenous properdin inhibited insulin-induced fatty acid uptake (13). Also, the desargenated derivative of C3a (C3adesarg, also known as acylation simulating protein), stimulated triglyceride synthesis in human and murine adipocytes (7). These data suggest that particularly the alternative pathway may be involved in lipid homeostasis. To date, no studies are available on the possible effects on lipid metabolism of other regulators of the alternative pathway, such as factor H (FH), a key regulator of alternative pathway C3 activation, and mannan-binding lectin-associated serine proteases 3 (MASP-3), which activates FD.

Taken together, experimental studies suggest a link between the complement system and lipoprotein metabolism, but the available human data are limited to the major lipid classes, and generally focus on one or a few individual complement components. To gain more insight in the relationship between the complement system and lipoprotein metabolism, we investigated the associations of complement C3, as well as additional components, regulators, and activated products of the alternative pathway (i.e. properdin, FH, FD, MASP-3, C3a, and Bb), with detailed lipoprotein characteristics as measured by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), in a Caucasian cohort with a moderately increased risk of cardiometabolic disease.

2 Materials and Methods

2.1 Study population

Participants of the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM, n = 574) were selected from a large population-based observational study as described previously (15). They were of Caucasian descent and > 40 years of age with one or more of the following characteristics: body mass index (BMI) > 25 kg/m^2; use of antihypertensive

medication; positive family history of T2DM; postprandial blood glucose level > 6.0 mmol/L; history of gestational diabetes and/or glycosuria. This study was approved by the medical ethics committee of Maastricht University. All participants gave written informed consent.

In the current analyses, we excluded individuals with missing data on complement concentrations, ^{1}H-NMR measured lipoprotein profile, and/or important covariates (n = 51), leaving 523 participants for the main analyses.

2.2 Biochemical and clinical measurements

Participants were asked to stop their lipid-modifying medication 14 days, and any other medication one day prior to the measurements. Blood samples were collected after an overnight fast, processed and stored at –80 °C until use. Total cholesterol, TG, LDL-C, HDL-C, and glucose concentrations, as well as concentrations of markers for low-grade inflammation (i.e. haptoglobin, ceruloplasmin, C-reactive protein [CRP], serum amyloid A [SAA], interleukin-6 [IL-6], interleukin-8 [IL-8], tumour necrosis factor-a [TNF-a], and soluble intercellular adhesion molecule 1 [sICAM-1]) and liver enzymes (i.e. aspartate aminotransferase [AST], alanine aminotransferase [ALT], and gamma–glutamyl transferase [GGT]) in the fasting plasma samples were obtained as previously described(16, 17). An estimate of insulin resistance (homeostasis model assessment insulin resistance [HOMA2-IR]) was obtained as described before (18). Concentrations of complement factors (i.e. C3, properdin, FH, FD, MASP-3, C3a, and Bb) were measured as previously described (16, 17).

Measures for other variables were obtained as described before. Briefly, BMI (kg/m^2), waist circumference (waist, cm) were measured at the research facility (15). Information on medication use (glucose-lowering, lipid-modifying, and/or anti-hypertensive, each yes/no), smoking status (current or previous tobacco smoking, yes/no), physical activity (METs/week), total energy intake (kJ/d), and alcohol consumption (g/d) was obtained using questionnaires (15). Prevalence of CVD (yes/no) was defined as previously described using information on self-reported history, electrocardiograms and the ankle-brachial index (15). Normal glucose metabolism (NGM, yes/no), impaired glucose metabolism (IGM, yes/no)

and T2DM (yes/no) were defined according to the 1999 WHO criteria (19) based on oral glucose tolerance test data as previously described (15).

2.3 Lipoprotein characteristics measured by ^{1}H-NMR metabolomics

Fourteen lipoprotein subclasses were quantified from fasting EDTA plasma samples using high-throughput ^{1}H-NMR metabolomics (Nightingale Health Ltd, Helsinki, Finland). Details of the ^{1}H-NMR metabolomics platform have been described (20). The lipoprotein subclasses were defined by their sizes as follows: extremely large VLDL (XXL-VLDL) with particle diameters from 75 nm upwards and a possible contribution of chylomicrons, five VLDL subclasses (i.e. very large [XL], large [L], medium [M], small [S], very small [XS] VLDL, with average particle diameters of 64.0, 53.6, 44.5, 36.8, and 31.3 nm, respectively), intermediate-density lipoproteins (IDL, particle diameter: 28.6 nm), three LDL subclasses (i.e. L-, M-, S-LDL, with particle diameters of 25.5, 23, and 18.7 nm, respectively) and four HDL subclasses (i.e. XL-, L, M-, S-HDL, with particle diameters of 14.3, 12.1, 10.9, and 8.7 nm). Next to the particle concentration of each subclass, the mean sizes of VLDL, LDL and HDL particles were calculated by weighting the corresponding subclass diameters with their particle concentrations, measurements of IDL were included in the LDL particles (21). The following lipid components were quantified within each of the 14 lipoprotein subclasses: phospholipids (PL), free cholesterol (FC), cholesteryl esters (CE), and TG.

2.4 Statistical analysis

Variables with a normal distribution are presented as mean ± SD, and discrete variables as percentages. Variables with a skewed distribution (^{1}H-NMR measured lipid characteristics, HOMA2-IR, alcohol intake, concentrations of plasma glucose, total TG, C3a, CRP, SAA, IL-6, IL-8, TNF-a, sICAM-1, AST, ALT, GGT) are presented as median (interquartile range) and were LN-transformed prior to further analyses. For the various lipoprotein characteristics and complement components, standardized values ([individual observed values − population

mean]/standard deviation of the population) were calculated to allow direct comparison of their effect sizes in the regression analyses. All analyses were performed using R software (version 3.5.0). A false discovery rate (22) adjusted *P*-value (q-value) of <0.05 was regarded significant.

Main analyses: multiple linear regression analyses were used to investigate the associations of systemic concentrations of complement C3 (primary determinant) and other alternative pathway components (secondary determinants) with particle concentrations of various lipoprotein subclasses and mean particle sizes of the major lipoprotein classes (main outcomes). All analyses were initially adjusted for age and sex **(Model 1)**, then additionally for glucose metabolism status (NGM/IGM/T2DM, as dummy variables), medication use (lipid-modifying, antihypertensive, and/or glucose-lowering), smoking status, alcohol consumption, physical activity, and energy intake to control for potential confounding **(Model 2)**.

Markers of obesity (BMI and waist), low-grade inflammation (haptoglobin, ceruloplasmin, CRP, SAA, IL-6, IL-8, TNF-a, and sICAM-1), insulin resistance (HOMA2-IR), and liver disease (represented by increased levels of liver enzymes, i.e. AST, ALT, and GGT) were added in separate groups to model 2, as these variables could potentially confound, but also potentially mediate associations between the complement system and lipoprotein profile. Possible effect modification by sex or glucose metabolism status was also evaluated by adding interaction terms in the fully adjusted models (**Model 2**). Further, to exclude possible residual confounding, we repeated the analyses while excluding all participants who used lipid-modifying medication (n = 99).

Additional analyses: for those complement components that were found to be significantly associated with lipoprotein concentrations and sizes, we investigated their associations with the lipid composition of the individual lipoproteins. For this, multiple linear regression analyses were performed to evaluate the associations of complement components with the absolute and relative lipid content (PL, FC, CE, and TG) of the 14 lipoprotein subclasses (secondary outcomes).

3 Results

3.1 Characteristics of the study population

General characteristics of the 523 participants are shown in **Table 1.** Overall, the study population consisted of middle-aged to older individuals (59.6 ± 6.9 years, 60.8 % men). They were, on average, overweight (BMI, 28.6 ± 4.4 kg/m^2; waist, 99.4 ± 12.1 cm), with relatively normal lipid levels (total cholesterol, LDL-C, HDL-C, and total TG concentrations were 5.2 ± 1.0, 3.3 ± 0.9, 1.2 ± 0.3 and 1.4 [1.0-2.0] mmol/l, respectively). Among them, 26% had T2DM, 27% had CVD, and the percentages of glucose-lowering, lipid-modifying, and anti-hypertensive medication users were 14%, 19%, 38%, respectively. Detailed information on the lipoprotein characteristics as analysed by high-throughput ^{1}H-NMR is provided in **Table S1**.

Table 1. Characteristics of the study population

	Total study population N = 523*
Age (years)	59.6 ± 6.9
Sex (% men)	60.8
BMI (kg/m^2)	28.6 ± 4.4
Waist circumference (cm)	99.4 ± 12.1
Fasting plasma glucose (mmol/L)	5.6 (5.2-6.4)
HOMA2-IR	1.6 (1.1-2.6)
Glucose metabolism status	
NGM (%)	52
IGM (%)	22
Type 2 Diabetes (%)	26
Cardiovascular Disease (%)	27
Medication use	
Glucose-lowering medication (%)	14
Lipid-modifying medication (%)	19
Antihypertensive medication (%)	38
Lifestyle variables	
Current or previous smokers (%)	23
Alcohol intake (g/d)	8.5 (1.2-21.9)
Energy intake (10^3·kj/day)	9.3 ± 2.8
Physical activity (10^3·METs/week)	6.7 ± 4. 1
Inflammation markers	
Haptoglobin (g/l)	1.3 ± 0.5
Ceruloplasmin (g/l)	0.3 ± 0.1
CRP (mg/l)	2.1 (1.0-4.0)
SAA (mg/l)	1.4 (1.0-2.3)
IL-6 (pg/ml)	1.6 (1.2-2.3)

Table 1. Characteristics of the study population (Continued)

	Total study population N = 523*
IL-8 (pg/ml)	4.4 (3.6-5.6)
TNF-α (pg/ml)	6.2 (5.3-7.6)
sICAM-1 (μg/l)	213 (188-244)
Liver enzymes	
ALT (U/L)	22.2 (17.2-28.3)
AST (U/L)	19.8 (16.3-24.2)
GGT (U/L)	24.5 (17.0-37.0)
Lipid measures	
Total triglycerides (mmol/l)	1.4 (1.0-2.0)
Total cholesterol (mmol/l)	5.2 ± 1.0
LDL-cholesterol (mmol/l)	3.3 ± 0.9
HDL-cholesterol (mmol/l)	1.2 ± 0.3
Complement components	
C3 (g/l)	1.0 ± 0.2
Properdin (mg/l)	6.1 ± 1.3
Factor H (mg/l)	325 ± 79
Factor D (mg/l)	1.0 ± 0.2
MASP-3 (mg/l)	7.1 ± 2.3
Bb (mg/l)	0.72 ± 0.19
C3a (μg/l)	59.2 (49.9-72.6)

* Data for BMI were available for n = 522, waist circumference available for n = 522, fasting plasma glucose available for n = 522, HOMA2-IR available for n = 511, haptoglobin available for n = 518, ceruloplasmin available for n = 521, ALT available for n = 517, AST available for n = 517, GGT available for n = 522, and for LDL-cholesterol available for n = 514. HOMA2-IR, homeostasis model assessment insulin resistance; NGM, normal glucose metabolism; IGM, impaired glucose metabolism; CRP, C-reactive protein; SAA, serum amyloid A; IL-6, interleukin-6; IL-8, interleukin-8; TNF-a, tumour necrosis factor-a; sICAM-1, soluble intercellular adhesion molecule-1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; HDL, high-density lipoproteins; LDL, low-density lipoproteins.

3.2 Associations of C3 and other components of the alternative pathway with lipoprotein concentrations and sizes

Figure 1 and Figure S2 show the associations of C3 and other components of the alternative pathway with lipoprotein concentrations and sizes. In the age- and sex-adjusted models (**Model 1**), C3 was positively associated with the particle concentrations of all VLDL classes.

Positive trends were observed for C3 with the concentrations of IDL/LDL lipoproteins, which did not reach statistical significance. Significant inverse associations were observed for particle concentrations of the larger (XL-, L-) HDL lipoproteins, while there was no association with medium-sized (M)-HDL lipoproteins and, in contrast, positive and significant associations were observed with the small (S)-HDL lipoproteins**.** C3 was significantly and positively associated with the mean particle size of the VLDL lipoproteins and inversely with the mean particle sizes of LDL and HDL lipoproteins**.** In the fully adjusted models (**Model 2**), the positive associations between C3 and IDL/LDL lipoproteins were somewhat stronger and significant (βs range from 0.14 to 0.17), while the other associations remained unchanged (particle concentrations: VLDLs, βs range from 0.27 to 0.36, XL-, L-HDL, βs = –0.36, S-HDL, β = 0.21; sizes: VLDL, β = 0.31, LDL, β = –0.13, HDL, β = –0.39). Similar trends, although less pronounced, were observed for the associations of properdin, FH, FD, and MASP-3 (in descending order of the respective effect sizes). C3a was not associated with lipoprotein concentration or size, except for the inverse association with XL-HDL concentration. In contrast, for factor Bb, inverse associations with VLDLs were observed.

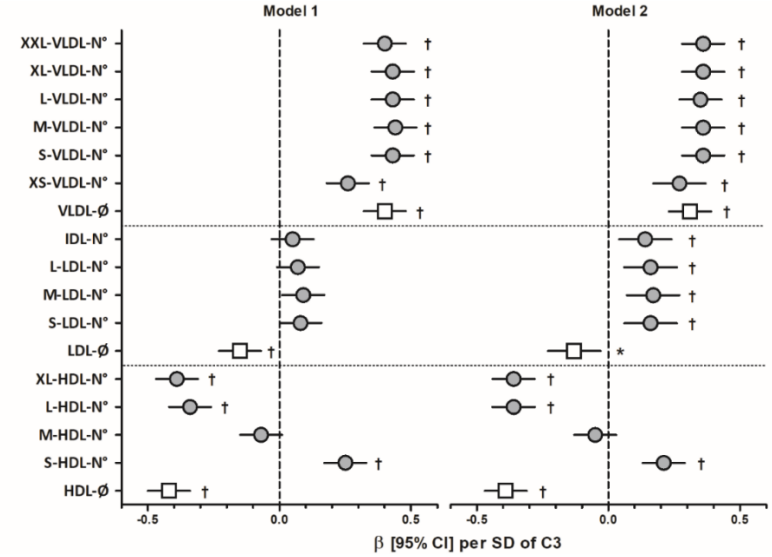

Figure 1. Associations of C3 with lipoprotein concentrations and sizes. C3 concentration was standardized. All data on lipoprotein measures were LN-transformed and standardized. **Model 1** is adjusted for age and sex. **Model 2** is additionally adjusted for glucose metabolism status, medication use (lipid-modifying, antihypertensive, and/or glucose-lowering), smoking status, alcohol consumption, physical activity, and energy intake. ***** FDR q-value<0.05, **†** FDR q-value<0.01. VLDL, very-low-density lipoproteins; IDL, intermediate-density lipoprotein; LDL, low-density lipoproteins; HDL, high-density lipoproteins; XXL, XL, L, M, S, XS refers to the size of the lipoproteins; N° or solid circle, particle concentration; Ø or open square, mean particle size.

When associations between complement C3 and lipoprotein concentrations and sizes were additionally adjusted for obesity (BMI and waist), the associations largely remained (**Figure 2, panel A** compared to **Figure 1, model 2**). The strongest attenuation by additional adjustment for obesity was observed for the positive association between C3 and particle concentration of S-HDL, which was attenuated by 38% (β changed from 0.21 to 0.13, FDR q value remained <0.05). The inverse association between C3 and mean LDL size was attenuated by 15% and became non-significant. The associations of C3 with the other lipoprotein classes remained similar to those observed in the main analyses, with <12% change in the strength of the associations. Results for other complement components were consistent with those for C3, with a few weak associations becoming non-significant after adjustment for obesity (**Figure S3**, **panel A** compared to **Figure S2**, **model 2**). Additional adjustment for low-grade inflammation somewhat strengthened the associations of C3 with concentrations of VLDL subclasses, S-LDL, and L-, S-HDL, as well as with the mean particle size of VLDL, LDL and HDL lipoproteins, by on average, 26% (range 10% to 77%, **Figure 2, panel B** compared to **Figure 1, model 2**). Additional adjustment for low-grade inflammation did not materially affect the associations for the other complement components (**Figure S3, panel B** compared to **Figure S2, model 2**). Additional adjustment for insulin resistance (HOMA2-IR) had a modest attenuating effect on the results obtained in the main analyses. The original associations of C3 with particle concentrations of VLDLs (except for XS-VLDL) and HDL (except for M-HDL) were attenuated by 22%, on average (range 14%-29%). The positive association with VLDL-size and the inverse association with HDL-size were attenuated by 32% and 21%. In contrast, the associations of C3 with IDL/LDL particle concentrations increased by 24%, on average (range 18%-29%, **Figure 2, panel C** compared to **Figure 1, model 2).** All associations that were significant in the main analyses (except for LDL size) remained so after adjustment for insulin resistance. The results for the other complement components were generally consistent with those for C3, although for FH and MASP-3, attenuations in the strength of the associations with the VLDL subclasses resulted in non-significant associations **(Figure S3, panel C** compared to **Figure S2, model 2)**. The associations for factor Bb were not affected by additional adjustment for insulin resistance. The effects of additional adjustment for liver enzymes were very similar to those

of adjustment for insulin resistance, although the associations with IDL/LDL, which strengthened when adjusted for insulin resistance, were attenuated when adjusted for liver enzymes (**Figure 2, panel D and Figure S3, panel D)**. When the associations between complement factors and lipoprotein concentrations and sizes were additionally adjusted for obesity, insulin resistance, low-grade inflammation and liver enzymes simultaneously, the associations, although attenuated, were largely comparable to the results of our main analyses (data not shown). In addition, properdin, FH, FD, and MASP-3 showed associations with lipoprotein characteristics that resembled those of C3. Therefore, we additionally adjusted these associations for C3 concentration, to evaluate if they were due to correlation(s) of properdin, FH, FD, and MASP-3 with C3. The associations of properdin, FD, and MASP-3 with lipoproteins largely remained, although somewhat attenuated, while the associations for FH disappeared after the additional adjustment for C3 (**Figure S4**).

There were no significant interactions of sex or glucose metabolism status with complement components on the associations with lipoprotein characteristics (data not shown). When the main analyses were repeated after excluding participants who use lipid-lowering medication (n = 99), the results were not materially changed (data not shown).

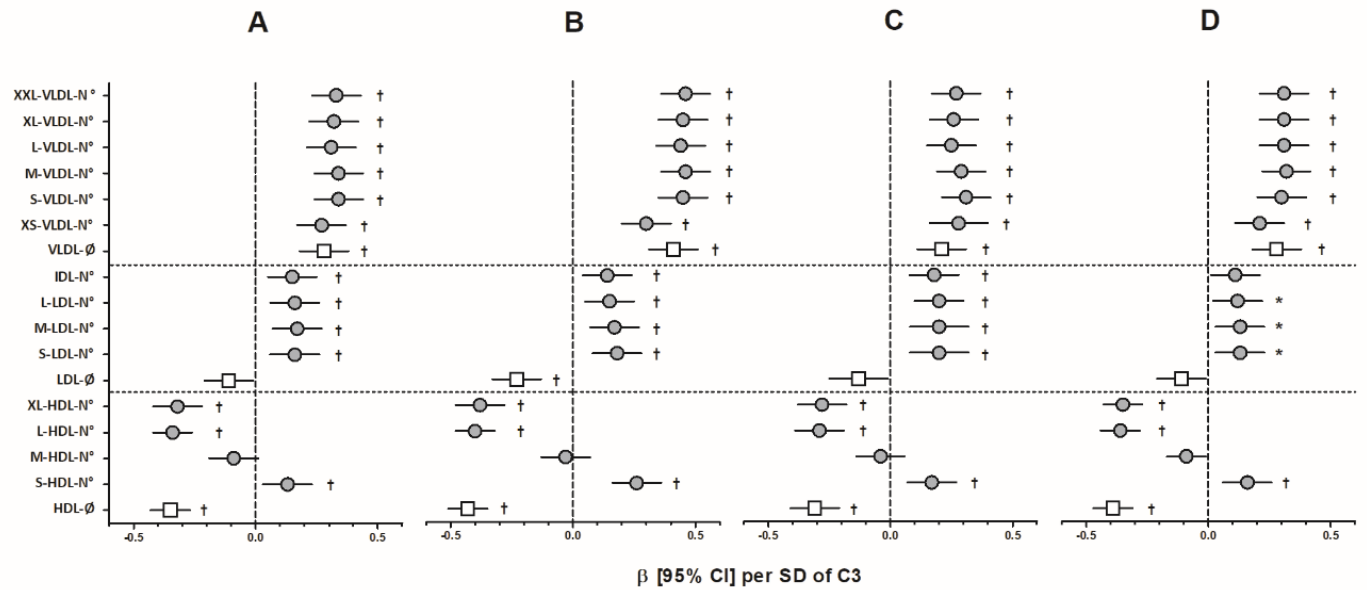

Figure 2. Associations of C3 with lipoprotein concentrations and sizes – Additional adjustment for markers of obesity, low-grade inflammation, insulin-resistance, and liver enzymes. C3 concentration was standardized. All data on lipoprotein measures were LN-transformed and standardized. All analyses were adjusted for age, sex, glucose metabolism status, medication use (lipid-modifying, antihypertensive, and/or glucose-lowering), smoking status, alcohol consumption, physical activity, and energy intake. Panel A: additionally adjusted for obesity (BMI and waist). Panel B: additionally adjusted for a low-grade inflammation score that included haptoglobin, ceruloplasmin, C-reactive protein, serum amyloid A, interleukin-6, interleukin-8, tumour necrosis factor-a, and soluble intercellular adhesion molecule-1 levels, compiled into an average Z score (refer to the methods section for the calculation). Panel C: additionally adjusted for insulin resistance (HOMA2-IR). Panel D: additionally adjusted for a liver enzymes score that included alanine aminotransferase, aspartate aminotransferase, and gamma–glutamyl transferase, compiled into an average Z score (refer to the methods section for the calculation). * FDR q-value<0.05, † FDR q-value<0.01. Individuals with missing data on obesity (n = 2), markers of low-grade inflammation (n = 5), insulin resistance (n = 12), or liver enzymes (n = 7) were additionally excluded in the respective analyses. These analyses were performed in 521, 518, 511, and 516 individuals, respectively. VLDL, very-low-density lipoproteins; IDL, intermediate-density lipoprotein; LDL, low-density lipoproteins; HDL, high-density lipoproteins; XXL, XL, L, M, S, XS refers to the size of the lipoproteins; N° or solid circle, particle concentration; Ø or open square, mean particle size.

3.3 Associations of C3, properdin, FH, FD, and MASP-3 with lipoprotein composition

For the complement components that showed consistent significant associations with lipoprotein concentrations and/or sizes, i.e. C3, properdin, FH, FD, and MASP-3, we further investigated their associations with the lipoprotein compositions in the 14 lipoprotein subclasses. Higher plasma concentrations of C3 were associated with higher absolute concentrations of PL, FC, CE, and TG in VLDL and IDL/LDL lipoproteins and, in contrast, with lower absolute concentrations of PL, FC, CE in larger (XL- and L-) HDL lipoproteins **(Figure 3, panel A).** This was consistent with the main analyses presented above. Despite the fact that C3 was not associated with the overall particle concentration of M-HDL, there was a strong and positive association between C3 and the TG concentration in this HDL class, while there was an inverse association between C3 with FC and CE. A similar positive association was observed between C3 and the amount of TG in S-HDL. The association between C3 and TG content was also positive, but weaker in the XL-HDL subclass and, notably, inverse in L-HDL **(Figure 3, panel A)**.

Next, we evaluated the associations of C3 with the relative amount, i.e. percentage, of individual lipids (PL%, FC%, CE%, and TG%) in each of the 14 lipoprotein classes. The most striking observation was that C3 was associated with a higher TG% in virtually all 14 lipoprotein classes **(Figure 3, panel B)**. This result indicated that a higher plasma concentration of C3 was not only associated with more lipoproteins, but that those lipoproteins were enriched in TG. Conversely, higher concentrations of C3 were associated with a lower CE% in the VLDLs. In the IDL and LDL lipoproteins, the TG enrichment was rather accompanied by a decrease in FC%. Also for the composition of HDL, the associations for C3 differed for the large as compared to the smaller subclasses: in XL-HDL, C3 was associated with a relative enrichment for TG as well as cholesterol (CE and FC) and a relative depletion of PL, while in the smaller subclasses there appeared to be a relative depletion of cholesterol (FC and/or CE) with enrichment in (or no relative effect on) PL and TG **(Figure 3, panel B)**. Again, similar but weaker associations with absolute and relative lipid composition were observed for properdin, FH, FD, and MASP-3 **(Figure S5)**.

A

	Absolute lipid composition							
	PL		FC		CE		TG	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
XXL-VLDL	**0.41**	**0.37**	**0.42**	**0.37**	**0.34**	**0.34**	**0.41**	**0.36**
XL-VLDL	**0.42**	**0.37**	**0.41**	**0.38**	**0.40**	**0.37**	**0.43**	**0.36**
L-VLDL	**0.42**	**0.35**	**0.43**	**0.36**	**0.41**	**0.36**	**0.42**	**0.34**
M-VLDL	**0.44**	**0.37**	**0.43**	**0.36**	**0.34**	**0.36**	**0.44**	**0.35**
S-VLDL	**0.43**	**0.35**	**0.41**	**0.34**	**0.24**	**0.31**	**0.44**	**0.34**
XS-VLDL	**0.15**	**0.19**	**0.11**	**0.14**	0.03	**0.16**	**0.42**	**0.31**
IDL	-0.01	0.09	-0.04	0.06	0.00	**0.13**	**0.33**	**0.24**
L-LDL	0.05	**0.15**	-0.04	0.08	0.04	**0.15**	**0.27**	**0.18**
M-LDL	**0.15**	**0.24**	0.02	**0.15**	0.04	**0.13**	**0.21**	**0.11**
S-LDL	**0.12**	**0.21**	0.01	**0.13**	0.02	**0.12**	**0.29**	**0.20**
XL-HDL	**-0.39**	**-0.34**	**-0.40**	**-0.32**	**-0.34**	**-0.35**	**0.14**	**0.11**
L-HDL	**-0.33**	**-0.33**	**-0.38**	**-0.38**	**-0.35**	**-0.38**	**-0.11**	**-0.21**
M-HDL	-0.06	-0.05	**-0.16**	**-0.12**	**-0.18**	**-0.14**	**0.34**	**0.29**
S-HDL	**0.16**	0.09	0.06	0.07	0.00	0.09	**0.41**	**0.29**

B

	Relative lipid composition							
	PL%		FC%		CE%		TG%	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
XXL-VLDL	**0.32**	**0.25**	**0.37**	**0.30**	**-0.38**	**-0.25**	**0.29**	**0.18**
XL-VLDL	**-0.27**	**-0.13**	**-0.41**	**-0.28**	**-0.43**	**-0.32**	**0.38**	**0.28**
L-VLDL	**0.28**	**0.25**	**-0.13**	-0.03	**-0.41**	**-0.30**	**0.30**	**0.22**
M-VLDL	**0.29**	**0.19**	**0.32**	**0.24**	**-0.40**	**-0.25**	**0.36**	**0.23**
S-VLDL	-0.08	**-0.16**	**-0.30**	**-0.29**	**-0.36**	**-0.19**	**0.39**	**0.26**
XS-VLDL	-0.08	-0.03	**-0.27**	**-0.27**	**-0.30**	**-0.13**	**0.39**	**0.23**
IDL	**-0.24**	**-0.22**	**-0.18**	**-0.13**	**-0.14**	0.02	**0.33**	**0.17**
L-LDL	-0.06	**-0.11**	**-0.31**	**-0.23**	0.00	**0.13**	**0.26**	**0.10**
M-LDL	0.05	-0.01	**-0.16**	**-0.16**	-0.03	0.05	**0.21**	0.08
S-LDL	0.04	-0.03	**-0.15**	**-0.16**	-0.03	0.06	**0.30**	**0.17**
XL-HDL	**-0.34**	**-0.28**	**0.16**	**0.28**	**0.32**	**0.24**	**0.40**	**0.36**
L-HDL	**0.18**	**0.29**	**-0.19**	**-0.13**	**-0.24**	**-0.29**	**0.17**	0.06
M-HDL	**0.21**	**0.13**	**-0.34**	**-0.25**	**-0.32**	**-0.24**	**0.40**	**0.34**
S-HDL	0.00	-0.08	**-0.24**	**-0.19**	-0.09	0.01	**0.38**	**0.26**

Figure 3. Associations of C3 with lipid composition of the individual lipoprotein subclasses. These heat maps represents the β values (standardized) for the associations of C3 with lipid composition of the individual lipoprotein subclasses. C3 concentration was standardized. All data on lipoprotein measures were LN-transformed and standardized. Bold numbers indicate an FDR q-value <0.05. Model 1 is adjusted for age and sex. Model 2 is additionally adjusted for glucose metabolism status, medication use (lipid-modifying, antihypertensive, and/or glucose-lowering), smoking status, alcohol consumption, physical activity, and energy intake. Panel A: the results for the association of C3 with absolute lipid content in lipoprotein classes. Panel B: the results for the association of C3 with relative lipid content in lipoprotein classes. PL, phospholipids; FC, free cholesterol; CE, cholesterol ester; TG, triglyceride; PL%, total PL to total lipids ratio; FC%, total FC to total lipids ratio; CE%, total CE to total lipids ratio; TG%, total TG to total lipids ratio; VLDL, very-low-density lipoproteins; IDL, intermediate-density lipoprotein; LDL, low-density lipoproteins; HDL, high-density lipoproteins; XXL, XL, L, M, S, XS refers to the size of the lipoproteins.

4 Discussion

We investigated the associations of complement C3 and other components of the alternative complement pathway with concentration, size and composition of 14 lipoprotein subclasses. Higher plasma C3 was associated with an adverse lipoprotein subclass profile: more VLDL of all subclasses, with a larger mean particle size; more IDL/LDL of all subclasses, but with a smaller mean particle size; lower concentrations of large HDLs and higher concentrations of small HDL, with a smaller mean particle size for HDL. Moreover, C3 was associated with enrichment in TG for all lipoprotein subclasses. For other components of the alternative complement pathway, i.e. properdin, FH, FD, and MASP-3, these associations were similar but weaker. Strikingly, plasma concentration of C3a and Bb did not show a similar pattern of associations: higher C3a was only associated with lower number of large HDL; higher Bb was modestly associated with lower numbers of large VLDLs and smaller VLDL mean particle size. The observed relationships for C3 and the other components of the alternative pathway were largely independent of obesity, insulin resistance, low-grade inflammation, and liver enzymes, and for this reason we conclude that these conditions are not of major importance for the currently-observed associations.

Lipid and lipoprotein metabolism is a complex physiological process. Not only the major classes (i.e. total TG, cholesterol, HDL-C, LDL-C), but also the distribution and composition of specific lipoprotein subclasses are important in metabolic disorders (23). The complement system is increasingly implicated in metabolism and homeostasis, including obesity-associated dyslipidaemia (4). To the best of our knowledge, this is the first study that comprehensively evaluates the associations of C3 and components of the alternative complement pathway, with detailed characteristics of distinct lipoprotein subclasses. We confirmed that a higher C3 concentration was associated with more atherogenic lipoproteins, e.g. the VLDL and IDL/LDL subclasses (23). We additionally showed that C3 was positively associated with the mean particle size of the VLDL and inversely with that of LDL. Although not yet conclusive, this may indicate that higher C3 is involved in a redistribution of lipoproteins towards higher concentrations of larger VLDL and smaller LDL, hence promoting a lipoprotein profile that is closely related to risk of cardiovascular disease

(24, 25). Given that C3 was reported to be present on VLDL and LDL lipoproteins (1-3), we speculate that C3 on the surface of lipoproteins may affect their metabolism, e.g. via steric hindering the binding of lipoprotein lipase, which would hamper lipolysis and result in TG-rich, large size lipoproteins. In line with this, higher C3 was associated with higher TG content in all lipoprotein subclasses. The idea of a direct, physical effect of complement on lipoprotein metabolism is also supported by the observation that complement C1r, a component of the classical complement pathway, was indeed shown to bind phospholipid transfer protein *in vitro* and to partially stabilize its lipid transfer function (26). This suggests that complement components can directly interfere with the remodelling of lipoproteins.

Within the HDL subclasses, we observed distinct associations: C3 was inversely associated with particle concentrations of the larger HDL lipoproteins, while it was positively associated with the concentration of small HDL lipoproteins. Overall, C3 was inversely associated with mean HDL size, which may indicate that a higher C3 level was associated with a redistribution of HDL subclasses towards smaller size. Similar distribution of HDL lipoprotein subclass was demonstrated in various cardiometabolic disorders (27, 28). Increased level of small HDL may be a consequence of disturbed reverse cholesterol transport function of HDL, which process converts small lipid-poor HDL into mature large-size HDL lipoproteins (29). Given the presence of C3 on HDL lipoproteins, the observed associations with HDL subclasses suggest that the binding of C3 may affect the interaction of HDL with proteins that involved in the reverse cholesterol transport process. Future *in vitro* and *in vivo* research is needed to evaluate whether the physical presence of C3, and possibly also other components of the alternative pathway, on lipoproteins can indeed affect their remodelling and metabolism. On the other hand, HDLs are highly heterogeneous lipoproteins, during pathological processes such as inflammation, HDL can be modified into dysfunctional particles that contain more pro-oxidant and pro-inflammatory proteins and are less atheroprotective (30). Therefore, our observation that C3 is associated with the amount and distribution of specific HDL subclasses may indicate a variation in the composition of the HDL subclasses. In line with this, previous finding demonstrated that, in patients with CVD, more C3 was detected in small-sized HDL, compared to healthy subjects (2).

At first sight, it may appear surprising that, contrary to the strong associations for C3, virtually no associations were observed for its activation product, C3a. Distinct effects of C3 and C3a have, however, been reported before. For instance, we showed that C3 and C3a show distinct associations with CVD (31). In addition, C3 was demonstrated to have an anti-fibrinolytic effect, which was primarily caused by its physical effect via binding to fibrins within the clot, independent of complement activation (32). Moreover, C3 is a rather large protein and presence of C3 on the surface of lipoproteins may directly affect their metabolism, e.g. via interfering with the binding of enzymes that are relevant for their metabolism. In contrast, previous experimental data suggest that the effects of C3a on lipid and/or lipoprotein metabolism are generally considered to occur via the generation of C3desarg, which subsequently activates its receptor, C5L2 (33). In line with this, our previous data showed that the association of C3a with liver fat and liver enzymes was independent of C3 (34). Given the observation that enzymes involved in HDL maturation and metabolism may be suppressed in liver disease (35), the association of C3a with XL-HDL concentration in the present study may indicated an independent association between C3a and HDL metabolism via the liver. Indeed, the modest but significant association of C3a with XL-HDL concentration became non-significant after the additional adjustment for liver enzymes, which is not the case for C3.

The observed associations for most alternative pathway components (i.e. properdin, FH, FD, and MASP-3) were largely consistent with those of C3, although the strength of the associations was generally smaller. To date, C3 is the only alternative pathway component that was shown to be physically and abundantly present on lipoprotein particles (1-3). It is conceivable that the currently observed associations for the other alternative pathway components are partly due to the mutual associations between the complement components. This is, however, not a likely explanation given that the associations of most alternative pathway components (i.e. properdin, FD, and MASP-3) with lipoprotein characteristics remained after additional adjustment for C3, although the associations for FH disappeared. Also, the correlations of plasma C3 with e.g. C3a (Pearson' s r for LN-transformed C3a = 0.35) and properdin (Pearson's r = 0.37) are very similar while their respective associations with plasma lipoproteins were completely different. Moreover,

there are *in vitro* data that FH binds to apoE on HDL and thereby affects complement activation on HDL particles (36).

We showed that higher C3 concentrations were associated with enrichment in TG in all lipoproteins and a reduction of CE in VLDL and large HDL. This resembles the lipoprotein composition that is observed in individuals with cardiometabolic diseases (37-39). Also, besides the well-accepted atherogenic function of LDL-C, emerging evidence suggests that LDL-TG is an independent risk factor for CVD (40). Although less pronounced, the associations of other components of the alternative pathway with particle composition were similar to those of C3. Overall, these results, again, suggest that the complement pathway, and particularly C3, is associated with an adverse lipoprotein profile. For future investigations, it will be interesting to examine the presence of alternative pathway components on lipoproteins of patients with cardiometabolic diseases in more detail, and to evaluate the pathophysiological effect of these complement components on the metabolism and function of the lipoproteins.

The key strength of our present study is the availability of plasma concentrations of multiple complement components, as well as the H^1-NMR measures of lipoprotein characteristics within one cohort. Another strength is the detailed phenotyping of the study participants, which allowed us to thoroughly adjust for potential confounders, and to explore some potential explanatory mechanisms. Nevertheless, limitations of this cohort study also need to be acknowledged. First, our study population is characterized by moderately increased risk for cardiometabolic diseases, which may limit the generalizability of our findings. Second, although we performed comprehensive adjustment for a range of potential confounders, we cannot exclude residual confounding. Third, given our cross-sectional study design, we cannot draw causal conclusions on the relationship between complement and the lipoproteins. In theory, reverse causation cannot be fully excluded. However, although some lipoproteins, e.g. chylomicrons or modified LDL, may induce complement expression *in vitro* (41, 42), our current findings are supported by suggestive biological evidence, such as the altered lipid profile in mouse models deficient in certain complement components, as well as the effect of complement on lipid metabolism in cell lines (11-13). These data suggest that there may indeed be a relevant role of complement

in lipid and lipoprotein metabolism. Lastly, theoretically the detectability of complement components by antibodies might be influenced by their binding to lipoproteins. It is highly unlikely that such a disturbance may have influenced the measurement of our primary determinant, since C3 is a large protein and was detected by a polyclonal antibody in a nephelometric assay. For other complement proteins, especially those detected by monoclonal antibodies, we cannot fully exclude the possibility that their detection, when bound to lipoproteins, might have been hampered to some extent. Suboptimal detection of complement on lipoproteins might result in underestimation of their concentrations.

In conclusion, this is the first comprehensive investigation of the association between the alternative complement pathway and ^{1}H-NMR measures of lipoprotein characteristics in a human cohort. Complement C3 and other alternative pathway components (i.e. properdin, FH, FD, and MASP-3) were consistently associated with an adverse lipoprotein distribution and composition. Our findings highlight the intricate interplay between the complement system and lipoprotein metabolism. Promotion of a pro-atherogenic lipid profile may be another way via which the alternative complement pathway, and particularly C3, may affect cardiometabolic risk. Future studies need to assess the presence of complement on the various lipoprotein subclasses in more detail, as well as the direct and indirect effects of lipoprotein-associated C3 on lipoprotein metabolism *in vitro* and *in vivo*. Such studies will further add to our understanding of how the complement system contributes to the risk of cardiometabolic diseases such as CVD and T2DM.

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Supplementary data

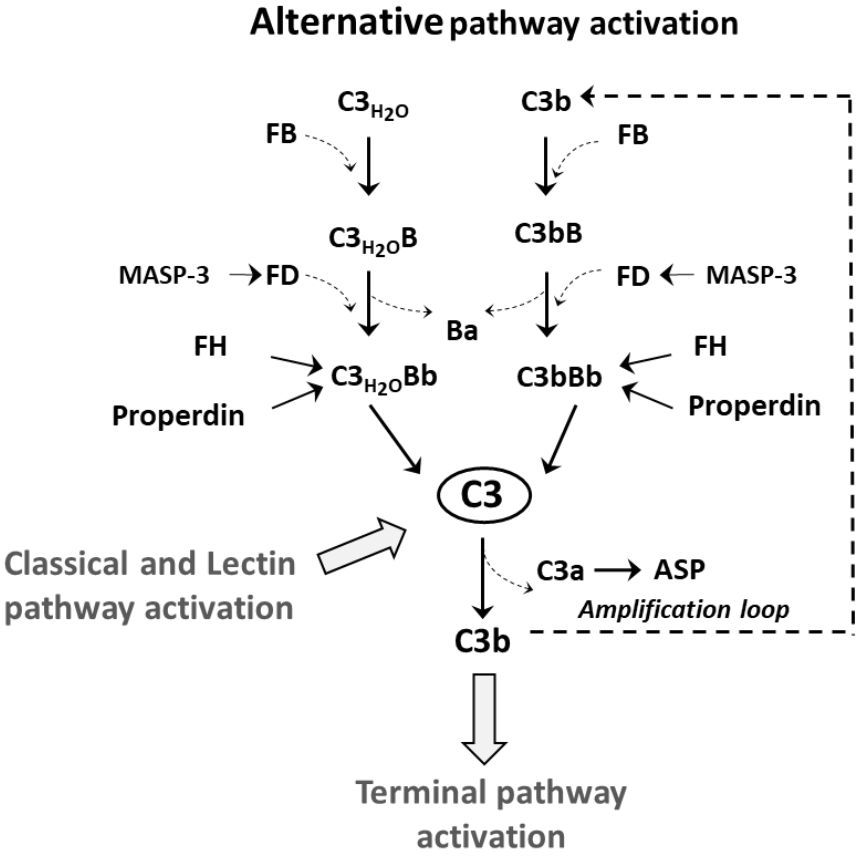

Figure S1. Activation of the complement system with focus on components that are most relevant for the current study. Complement activation can be initiated via the classical, lectin, or alternative pathway. The alternative pathway can be initiated via two routes. Activation can start either with spontaneous hydrolysis of C3, which generates C3(H_2O), or with C3b that is generated by the cleavage of C3 by convertases derived from different complement pathways. C3b and C3(H_2O) make factor B available for cleavage by factor D. This yields Bb which combines with C3b or C3(H_2O) to generate the alternative pathway C3 convertase (C3bBb or C3(H_2O)Bb). Therefore, the alternative pathway functions as an amplification loop for all activation pathways. Activation of the alternative pathway is under strict control of several regulatory proteins, including factor H, which acts as a destabilizer of the C3 convertase, and properdin, which is a C3 convertase stabilizer. Mannan-binding lectin-associated serine proteases 3 (MASP-3) is an activator of factor D. C3 convertases generated via either pathway cleave C3 into C3a and C3b. The anaphylatoxin C3a is rapidly degraded to C3a-desarg, also called acylation stimulating protein (ASP). C3b can contribute to the amplification loop to produce more C3 convertases, and can also lead to the subsequent activation of the terminal pathway.

	C3		Properdin		Factor H		Factor D		MASP-3		C3a		Bb	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
XXL-VLDL-N°	**0.40**	**0.36**	**0.36**	**0.33**	**0.23**	**0.17**	**0.12**	**0.13**	**0.16**	**0.13**	0.02	-0.02	**-0.13**	**-0.12**
XL-VLDL-N°	**0.43**	**0.36**	**0.34**	**0.31**	**0.25**	**0.16**	**0.12**	**0.14**	**0.18**	**0.14**	0.02	-0.03	**-0.14**	**-0.12**
L-VLDL-N°	**0.43**	**0.35**	**0.34**	**0.30**	**0.24**	**0.15**	**0.13**	**0.15**	**0.18**	**0.14**	0.02	-0.03	**-0.14**	**-0.12**
M-VLDL-N°	**0.44**	**0.36**	**0.36**	**0.32**	**0.25**	**0.15**	**0.14**	**0.15**	**0.18**	**0.14**	0.02	-0.03	**-0.12**	**-0.10**
S-VLDL-N°	**0.43**	**0.36**	**0.36**	**0.32**	**0.24**	**0.15**	**0.14**	**0.15**	**0.17**	**0.13**	0.04	-0.01	-0.09	-0.07
XS-VLDL-N°	**0.26**	**0.27**	**0.22**	**0.22**	**0.15**	**0.14**	0.05	0.06	0.07	0.07	0.05	0.03	-0.02	-0.03
VLDL-Ø	**0.40**	**0.31**	**0.32**	**0.28**	**0.23**	**0.12**	**0.14**	**0.15**	**0.18**	**0.13**	0.00	-0.05	**-0.14**	**-0.11**
IDL-N°	0.05	**0.14**	0.09	**0.11**	0.05	**0.11**	-0.05	-0.05	-0.03	0.01	0.00	0.01	0.00	-0.03
L-LDL-N°	0.07	**0.16**	0.09	**0.10**	0.06	**0.12**	-0.07	-0.06	-0.03	0.01	-0.01	0.01	0.00	-0.03
M-LDL-N°	0.09	**0.17**	**0.11**	**0.12**	0.08	**0.13**	-0.07	-0.06	-0.02	0.01	-0.01	0.00	-0.01	-0.03
S-LDL-N°	0.08	**0.16**	**0.11**	**0.12**	0.07	**0.12**	-0.07	-0.06	-0.02	0.01	-0.02	-0.01	-0.01	-0.04
LDL-Ø	**-0.15**	**-0.13**	**-0.14**	**-0.13**	**-0.12**	-0.10	0.08	0.06	-0.03	-0.01	0.08	0.09	0.06	0.07
XL-HDL-N°	**-0.39**	**-0.36**	**-0.28**	**-0.24**	**-0.22**	**-0.17**	**-0.20**	**-0.18**	**-0.11**	**-0.11**	**-0.14**	**-0.10**	-0.01	-0.04
L-HDL-N°	**-0.34**	**-0.36**	**-0.37**	**-0.32**	**-0.23**	**-0.22**	**-0.16**	**-0.14**	-0.03	-0.07	-0.07	-0.04	0.02	-0.01
M-HDL-N°	-0.07	-0.05	**-0.22**	**-0.16**	**-0.12**	**-0.10**	**-0.11**	-0.07	0.05	0.02	-0.04	-0.01	-0.04	-0.08
S-HDL-N°	**0.25**	**0.21**	0.00	0.02	0.09	0.04	-0.02	0.02	**0.15**	0.08	0.04	0.03	-0.04	-0.05
HDL-Ø	**-0.42**	**-0.39**	**-0.35**	**-0.30**	**-0.26**	**-0.21**	**-0.18**	**-0.17**	**-0.10**	**-0.10**	**-0.10**	-0.07	0.01	-0.02

Figure S2. Associations of C3, properdin, factor H, factor D, MASP-3, C3a, and Bb with lipoprotein concentrations and sizes. This heat map represents the β values for the associations of complement components with lipoprotein concentrations and sizes. Associations of C3 with lipoprotein concentrations and sizes are also presented in main text in Figure 1. Complement concentrations were standardized. C3a concentration and all lipoprotein measures were LN-transformed and standardized. Model 1 is adjusted for age and sex. Model 2 is additionally adjusted for glucose metabolism status, medication use (lipid-modifying, antihypertensive, and/or glucose-lowering), smoking status, alcohol consumption, physical activity, and energy intake. Bold numbers indicate an FDR q-value <0.05. VLDL, very-low-density lipoproteins; IDL, intermediate density lipoprotein; LDL, low-density lipoproteins; HDL, high-density lipoproteins; XXL, XL, L, M, S, XS refers to the size of the lipoproteins; N°, particle concentration; Ø, mean particle size.

A

Main analyses additionally adjusted for obesity							
	C3	Properdin	Factor H	Factor D	MASP-3	C3a	Bb
XXL-VLDL-N°	**0.33**	**0.31**	**0.12**	0.09	**0.11**	-0.06	**-0.11**
XL-VLDL-N°	**0.32**	**0.28**	0.10	0.09	**0.11**	-0.06	**-0.11**
L-VLDL-N°	**0.31**	**0.27**	0.09	**0.10**	**0.11**	-0.07	**-0.11**
M-VLDL-N°	**0.34**	**0.30**	**0.10**	**0.11**	**0.12**	-0.07	**-0.10**
S-VLDL-N°	**0.34**	**0.30**	**0.11**	**0.12**	**0.11**	-0.04	-0.06
XS-VLDL-N°	**0.27**	**0.20**	**0.12**	0.04	0.06	0.01	-0.02
VLDL-Ø	**0.28**	**0.25**	0.07	**0.11**	**0.11**	-0.08	**-0.11**
IDL-N°	**0.15**	**0.10**	0.10	-0.05	0.01	0.01	-0.03
L-LDL-N°	**0.16**	0.10	**0.11**	-0.06	0.01	0.00	-0.03
M-LDL-N°	**0.17**	**0.11**	**0.12**	-0.06	0.01	-0.01	-0.03
S-LDL-N°	**0.16**	**0.12**	**0.12**	-0.07	0.01	-0.02	-0.04
LDL-Ø	-0.11	**-0.12**	-0.08	0.08	0.00	**0.10**	0.06
XL-HDL-N°	**-0.32**	**-0.21**	**-0.11**	**-0.14**	-0.09	-0.07	-0.05
L-HDL-N°	**-0.34**	**-0.30**	**-0.19**	**-0.12**	-0.06	-0.01	-0.01
M-HDL-N°	-0.09	**-0.17**	**-0.14**	**-0.10**	0.01	-0.02	-0.08
S-HDL-N°	**0.13**	-0.01	-0.04	-0.03	0.06	-0.01	-0.05
HDL-Ø	**-0.35**	**-0.27**	**-0.15**	**-0.13**	-0.08	-0.03	-0.03

B

Main analyses additionally adjusted for low-grade inflammation							
	C3	Properdin	Factor H	Factor D	MASP-3	C3a	Bb
XXL-VLDL-N°	**0.46**	**0.32**	**0.18**	**0.12**	**0.12**	-0.04	**-0.15**
XL-VLDL-N°	**0.45**	**0.30**	**0.17**	**0.13**	**0.13**	-0.04	**-0.15**
L-VLDL-N°	**0.44**	**0.29**	**0.16**	**0.14**	**0.13**	-0.05	**-0.14**
M-VLDL-N°	**0.46**	**0.31**	**0.16**	**0.14**	**0.13**	-0.05	**-0.13**
S-VLDL-N°	**0.45**	**0.31**	**0.16**	**0.14**	**0.12**	-0.03	-0.09
XS-VLDL-N°	**0.30**	**0.23**	**0.13**	0.05	0.08	0.00	-0.05
VLDL-Ø	**0.41**	**0.26**	**0.14**	**0.14**	**0.12**	-0.05	**-0.14**
IDL-N°	**0.14**	**0.14**	0.10	-0.05	0.03	-0.02	-0.04
L-LDL-N°	**0.15**	**0.14**	**0.11**	-0.06	0.03	-0.03	-0.04
M-LDL-N°	**0.17**	**0.15**	**0.12**	-0.06	0.03	-0.03	-0.04
S-LDL-N°	**0.18**	**0.15**	**0.13**	-0.06	0.02	-0.04	-0.05
LDL-Ø	**-0.23**	**-0.13**	**-0.13**	0.05	0.00	0.07	0.06
XL-HDL-N°	**-0.38**	**-0.22**	**-0.14**	**-0.16**	**-0.10**	-0.07	0.00
L-HDL-N°	**-0.40**	**-0.31**	**-0.21**	**-0.12**	-0.07	0.00	0.02
M-HDL-N°	-0.03	**-0.15**	**-0.09**	-0.06	0.03	0.00	-0.06
S-HDL-N°	**0.26**	0.03	0.04	0.02	**0.09**	0.03	-0.06
HDL-Ø	**-0.43**	**-0.29**	**-0.19**	**-0.15**	**-0.10**	-0.03	0.02

C

Main analyses additionally adjusted for insulin resistance							
	C3	Properdin	Factor H	Factor D	MASP-3	C3a	Bb
XXL-VLDL-N°	**0.27**	**0.28**	0.07	0.09	0.08	-0.06	**-0.11**
XL-VLDL-N°	**0.26**	**0.25**	0.05	0.10	0.08	-0.07	**-0.11**
L-VLDL-N°	**0.25**	**0.24**	0.04	**0.10**	0.08	-0.07	**-0.10**
M-VLDL-N°	**0.29**	**0.27**	0.06	**0.11**	0.09	-0.07	-0.09
S-VLDL-N°	**0.31**	**0.28**	0.08	**0.11**	0.09	-0.04	-0.06
XS-VLDL-N°	**0.28**	**0.20**	**0.12**	0.04	0.06	0.02	-0.02
VLDL-Ø	**0.21**	**0.22**	0.02	**0.11**	0.08	-0.09	**-0.10**
IDL-N°	**0.18**	**0.11**	**0.13**	-0.05	0.02	0.02	-0.03
L-LDL-N°	**0.20**	0.10	**0.13**	-0.06	0.02	0.01	-0.03
M-LDL-N°	**0.20**	**0.12**	**0.14**	-0.06	0.02	0.00	-0.03
S-LDL-N°	**0.20**	**0.12**	**0.14**	-0.07	0.02	-0.01	-0.04
LDL-Ø	-0.13	**-0.12**	-0.09	0.07	-0.02	0.09	0.06
XL-HDL-N°	**-0.28**	**-0.18**	-0.07	**-0.15**	-0.05	-0.07	-0.05
L-HDL-N°	**-0.29**	**-0.27**	**-0.14**	**-0.11**	-0.02	0.00	-0.01
M-HDL-N°	-0.04	**-0.16**	**-0.10**	-0.06	0.03	-0.01	-0.07
S-HDL-N°	**0.17**	-0.02	-0.02	0.00	0.06	0.01	-0.04
HDL-Ø	**-0.31**	**-0.24**	**-0.11**	**-0.13**	-0.04	-0.03	-0.03

D

Main analyses additionally adjusted for liver enzymes							
	C3	Properdin	Factor H	Factor D	MASP-3	C3a	Bb
XXL-VLDL-N°	**0.31**	**0.30**	**0.12**	0.10	0.08	-0.05	**-0.13**
XL-VLDL-N°	**0.31**	**0.28**	**0.11**	**0.11**	0.09	-0.05	**-0.13**
L-VLDL-N°	**0.31**	**0.27**	**0.10**	**0.12**	0.09	-0.06	**-0.13**
M-VLDL-N°	**0.32**	**0.29**	**0.10**	**0.12**	0.10	-0.06	**-0.11**
S-VLDL-N°	**0.30**	**0.29**	0.09	**0.12**	0.08	-0.04	-0.08
XS-VLDL-N°	**0.21**	**0.19**	0.08	0.02	0.02	0.01	-0.04
VLDL-Ø	**0.28**	**0.26**	0.09	**0.13**	**0.10**	-0.07	**-0.12**
IDL-N°	0.11	0.09	0.07	-0.08	-0.02	0.00	-0.04
L-LDL-N°	**0.12**	0.08	0.08	-0.09	-0.03	-0.01	-0.04
M-LDL-N°	**0.13**	0.10	0.09	-0.09	-0.03	-0.02	-0.04
S-LDL-N°	**0.13**	0.10	0.08	-0.10	-0.03	-0.03	-0.05
LDL-Ø	-0.11	**-0.12**	-0.07	0.08	0.02	0.10	0.07
XL-HDL-N°	**-0.35**	**-0.23**	**-0.15**	**-0.17**	-0.08	-0.09	-0.03
L-HDL-N°	**-0.36**	**-0.31**	**-0.21**	**-0.13**	-0.05	-0.03	0.00
M-HDL-N°	-0.09	**-0.18**	**-0.13**	-0.09	0.00	-0.02	-0.08
S-HDL-N°	**0.16**	0.00	-0.01	-0.01	0.05	0.01	-0.06
HDL-Ø	**-0.39**	**-0.28**	**-0.19**	**-0.15**	-0.07	-0.05	-0.01

Figure S3. Associations of C3, properdin, factor H, factor D, MASP-3, C3a, and Bb with lipoprotein concentrations and sizes – Additional adjustment for obesity, low-grade inflammation, insulin resistance, or liver enzymes. These heat maps represent the β values for the associations of complement components with lipoprotein concentrations and sizes with additional adjustments. Results for C3 are also presented in main text in Figure 2. Complement concentrations were standardized. C3a concentration and all lipoprotein measures were LN-transformed and standardized. Bold numbers indicate an FDR q-value <0.05. All analyses were adjusted for age, sex, glucose metabolism status, medication use (lipid-modifying, antihypertensive, and/or glucose-lowering), smoking status, alcohol consumption, physical activity, and energy intake. **Panel A**: additionally adjusted for obesity (BMI and waist). **Panel B**: additionally adjusted for a low-grade inflammation score that included haptoglobin, ceruloplasmin, C-reactive protein, serum amyloid A, interleukin-6, interleukin-8, tumour necrosis factor-a, and soluble intercellular adhesion molecule-1 levels, compiled into an average Z score (refer to the methods section for the calculation). **Panel C**: additionally adjusted for insulin resistance (HOMA2-IR). **Panel D**: additionally adjusted for a liver enzyme score that included alanine aminotransferase, aspartate aminotransferase, and gamma–glutamyl transferase, compiled into an average Z score (refer to the methods section for the calculation). Individuals with missing data on obesity (n = 2), markers of low-grade inflammation (n = 5), insulin resistance (n = 12), or liver enzymes (n = 7) were additionally excluded in the respective analyses. These analyses were performed in 521, 518, 511, and 516 individuals, respectively. VLDL, very-low-density lipoproteins; IDL, intermediate density lipoprotein; LDL, low-density lipoproteins; HDL, high-density lipoproteins; XXL, XL, L, M, S, XS refers to the size of the lipoproteins; N°, particle concentration; Ø, mean particle size.

	Properdin	Factor H	Factor D	MASP-3
XXL-VLDL-N°	**0.24**	0.01	0.10	0.09
XL-VLDL-N°	**0.22**	0.00	**0.11**	**0.10**
L-VLDL-N°	**0.21**	-0.01	**0.11**	**0.10**
M-VLDL-N°	**0.23**	-0.01	**0.12**	**0.10**
S-VLDL-N°	**0.23**	-0.01	**0.11**	0.09
XS-VLDL-N°	**0.15**	0.03	0.03	0.04
VLDL-Ø	**0.20**	-0.01	**0.12**	**0.10**
IDL-N°	0.07	0.06	-0.06	0.00
L-LDL-N°	0.06	0.06	-0.07	-0.01
M-LDL-N°	0.07	0.07	-0.07	-0.01
S-LDL-N°	0.08	0.07	-0.08	-0.01
LDL-Ø	-0.10	-0.05	0.08	0.01
XL-HDL-N°	**-0.14**	-0.01	**-0.15**	-0.07
L-HDL-N°	**-0.23**	-0.08	**-0.11**	-0.03
M-HDL-N°	**-0.16**	-0.11	-0.07	0.02
S-HDL-N°	-0.05	-0.07	0.00	0.06
HDL-Ø	**-0.19**	-0.05	**-0.13**	-0.06

Figure S4. Associations of properdin, factor H, factor D, and MASP-3 with lipoprotein concentrations and sizes – Additional adjustment for C3 concentration. This heat map represents the β values for the associations of complement components with lipoprotein concentrations and sizes with additional adjustment for C3. Complement concentrations were standardized. All lipoprotein measures were LN-transformed and standardized. Bold numbers indicate an FDR q-value <0.05. All analyses were adjusted for age, sex, glucose metabolism status, medication use (lipid-modifying, antihypertensive, and/or glucose-lowering), smoking status, alcohol consumption, physical activity, energy intake, and 3 concentration. VLDL, very-low-density lipoproteins; IDL, intermediate density lipoprotein; LDL, low-density lipoproteins; HDL, high-density lipoproteins; XXL, XL, L, M, S, XS refers to the size of the lipoproteins; N°, particle concentration; Ø, mean particle size.

A

Properdin	Absolute lipid composition				Relative lipid composition			
	PL	FC	CE	TG	PL%	FC%	CE%	TG%
XXL-VLDL	**0.32**	**0.32**	**0.34**	**0.33**	0.04	**0.13**	**-0.16**	**0.15**
XL-VLDL	**0.31**	**0.33**	**0.34**	**0.30**	**-0.17**	**-0.22**	**-0.21**	**0.20**
L-VLDL	**0.28**	**0.30**	**0.33**	**0.29**	**0.12**	-0.06	**-0.22**	**0.16**
M-VLDL	**0.32**	**0.32**	**0.33**	**0.31**	0.06	**0.18**	**-0.20**	**0.18**
S-VLDL	**0.30**	**0.30**	**0.27**	**0.30**	**-0.23**	**-0.27**	**-0.18**	**0.21**
XS-VLDL	**0.12**	0.09	0.08	**0.26**	**-0.10**	**-0.27**	**-0.17**	**0.20**
IDL	0.05	0.00	0.08	**0.19**	**-0.28**	**-0.22**	-0.05	**0.14**
L-LDL	0.09	0.01	0.08	**0.14**	**-0.10**	**-0.29**	0.01	**0.10**
M-LDL	**0.17**	**0.09**	0.05	0.08	0.01	**-0.13**	-0.05	0.05
S-LDL	**0.16**	**0.09**	0.02	**0.14**	-0.01	**-0.12**	**-0.10**	**0.12**
XL-HDL	**-0.28**	**-0.19**	**-0.17**	**0.20**	**-0.28**	**0.22**	**0.25**	**0.34**
L-HDL	**-0.31**	**-0.33**	**-0.33**	**-0.12**	**0.15**	**-0.11**	**-0.22**	**0.13**
M-HDL	**-0.15**	**-0.21**	**-0.24**	**0.21**	**0.19**	**-0.26**	**-0.28**	**0.32**
S-HDL	-0.04	-0.07	-0.06	**0.26**	-0.06	**-0.13**	-0.06	**0.27**

B

Factor H	Absolute lipid composition				Relative lipid composition			
	PL	FC	CE	TG	PL%	FC%	CE%	TG%
XXL-VLDL	**0.17**	**0.17**	**0.17**	**0.17**	0.06	0.06	**-0.11**	**0.11**
XL-VLDL	**0.17**	**0.17**	**0.16**	**0.16**	-0.05	**-0.12**	**-0.14**	**0.12**
L-VLDL	**0.15**	**0.16**	**0.15**	**0.15**	**0.10**	0.03	**-0.13**	0.09
M-VLDL	**0.16**	**0.15**	**0.15**	**0.15**	**0.11**	**0.10**	**-0.11**	**0.10**
S-VLDL	**0.15**	**0.15**	**0.16**	**0.14**	**-0.12**	**-0.11**	-0.05	**0.08**
XS-VLDL	**0.12**	0.08	**0.10**	**0.13**	0.03	**-0.13**	-0.05	0.07
IDL	0.09	0.06	**0.10**	**0.10**	-0.08	-0.05	0.02	0.04
L-LDL	**0.11**	0.08	**0.11**	**0.09**	**-0.11**	**-0.12**	**0.10**	0.03
M-LDL	**0.14**	**0.12**	**0.11**	0.08	-0.07	**-0.12**	0.06	0.05
S-LDL	**0.13**	**0.11**	**0.10**	**0.10**	-0.08	**-0.12**	0.05	0.07
XL-HDL	**-0.19**	**-0.14**	**-0.14**	0.05	**-0.18**	**0.14**	**0.15**	**0.16**
L-HDL	**-0.20**	**-0.22**	**-0.23**	**-0.14**	**0.17**	-0.01	**-0.16**	0.02
M-HDL	**-0.10**	**-0.13**	**-0.13**	0.08	**0.10**	**-0.16**	**-0.12**	**0.15**
S-HDL	-0.04	-0.03	0.08	**0.12**	**-0.11**	**-0.12**	0.06	**0.12**

C

Factor D	Absolute lipid composition				Relative lipid composition			
	PL	FC	CE	TG	PL%	FC%	CE%	TG%
XXL-VLDL	**0.13**	**0.14**	**0.14**	**0.13**	0.07	**0.15**	-0.05	0.00
XL-VLDL	**0.14**	**0.14**	**0.15**	**0.14**	**-0.11**	**-0.12**	**-0.10**	**0.11**
L-VLDL	**0.15**	**0.14**	**0.15**	**0.15**	**0.10**	-0.08	**-0.12**	**0.11**
M-VLDL	**0.15**	**0.15**	**0.13**	**0.15**	0.03	**0.11**	**-0.12**	**0.12**
S-VLDL	**0.15**	**0.13**	0.08	**0.16**	-0.04	**-0.15**	**-0.13**	**0.15**
XS-VLDL	-0.02	-0.02	-0.01	**0.15**	**-0.15**	**-0.15**	**-0.08**	**0.16**
IDL	-0.08	-0.09	-0.07	**0.11**	**-0.18**	**-0.14**	-0.06	**0.15**
L-LDL	-0.07	**-0.09**	-0.07	0.07	0.04	**-0.11**	**-0.10**	**0.12**
M-LDL	-0.04	-0.07	-0.08	0.02	**0.10**	0.03	**-0.11**	0.04
S-LDL	-0.06	-0.08	-0.09	0.06	0.08	0.04	**-0.10**	**0.09**
XL-HDL	**-0.17**	**-0.19**	**-0.20**	0.06	**-0.13**	**0.08**	**0.08**	**0.19**
L-HDL	**-0.14**	**-0.16**	**-0.14**	-0.03	0.06	**-0.11**	-0.09	**0.09**
M-HDL	-0.06	**-0.11**	**-0.13**	**0.14**	**0.16**	**-0.15**	**-0.17**	**0.19**
S-HDL	0.03	-0.01	-0.09	**0.18**	0.04	-0.04	-0.09	**0.19**

D

MASP-3	Absolute lipid composition				Relative lipid composition			
	PL	FC	CE	TG	PL%	FC%	CE%	TG%
XXL-VLDL	**0.13**	**0.13**	**0.12**	**0.13**	**0.10**	**0.12**	**-0.09**	0.06
XL-VLDL	**0.14**	**0.14**	**0.14**	**0.13**	-0.05	**-0.11**	**-0.12**	**0.10**
L-VLDL	**0.13**	**0.14**	**0.14**	**0.13**	0.08	-0.01	**-0.11**	0.09
M-VLDL	**0.14**	**0.14**	**0.13**	**0.14**	0.05	**0.09**	**-0.10**	**0.09**
S-VLDL	**0.12**	**0.12**	0.08	**0.13**	-0.06	**-0.12**	**-0.11**	**0.11**
XS-VLDL	0.02	-0.02	0.00	**0.11**	-0.06	**-0.19**	**-0.09**	**0.11**
IDL	-0.01	-0.05	-0.01	0.08	**-0.10**	**-0.16**	-0.06	**0.08**
L-LDL	0.01	-0.04	-0.01	0.06	0.02	**-0.15**	-0.07	0.07
M-LDL	0.04	0.00	-0.04	-0.01	0.06	-0.02	**-0.10**	-0.01
S-LDL	0.04	-0.01	-0.05	0.06	0.05	-0.03	**-0.11**	0.06
XL-HDL	-0.08	**-0.10**	**-0.14**	0.07	-0.04	**0.09**	0.02	**0.14**
L-HDL	-0.06	**-0.08**	**-0.08**	-0.05	**0.11**	-0.07	**-0.10**	0.00
M-HDL	0.01	0.01	-0.01	**0.13**	0.00	-0.01	-0.08	**0.12**
S-HDL	0.07	0.07	-0.07	**0.12**	0.03	0.00	**-0.10**	**0.11**

Figure S5. Associations of properdin, factor H, factor D, and MASP-3 with lipid composition of the individual lipoprotein subclasses. These heat maps represent the β values for the associations of complement components with lipid composition of the individual lipoprotein subclasses. Complement concentrations were standardized. All lipoprotein measures were LN-transformed and standardized. Bold numbers indicate an FDR q-value <0.05. All analyses were adjusted for age, sex, glucose metabolism status, medication use (lipid-modifying, antihypertensive, and/or glucose-lowering), smoking status, alcohol consumption, physical activity, and energy intake. **Panel A**: associations of properdin with absolute and relative lipid content in lipoprotein classes. **Panel B**: associations of factor H with absolute and relative lipid content in lipoprotein classes. **Panel C**: associations of factor D with absolute and relative lipid content in lipoprotein classes. **Panel D**: associations of MASP-3 with absolute and relative lipid content in lipoprotein classes. PL, phospholipids; FC, free cholesterol; CE, cholesterol ester; TG, triglyceride; PL%, total PL to total lipids ratio; FC%, total FC to total lipids ratio; CE%, total CE to total lipids ratio; TG%, total TG to total lipids ratio; VLDL, very-low-density lipoproteins; IDL, intermediate density lipoprotein; LDL, low-density lipoproteins; HDL, high-density lipoproteins; XXL, XL, L, M, S, XS refers to the size of the lipoproteins

Table S1. Characteristics of lipoproteins measured by ^{1}H NMR spectroscopy

	Mean particle size (nm)	*Particle concentration (nmol/l)*	*Total PL concentration (mmol/l)*	*Total FC concentration (mmol/l)*	*Total CE concentration (mmol/l)*	*Total TG concentration (mmol/l)*	*Total PL to total lipids ratio (%)*	*Total FC to total lipids ratio (%)*	*Total CE to total lipids ratio (%)*	*Total TG to total lipids ratio (%)*
					median [inter quartile range]					
VLDL particles	36.73 (36.0-37.5)									
XXL-VLDL		0.25 (0.19-0.34)	0.01 (0.00-0.01)	0.00 (0.00-0.01)	0.01 (0.01-0.01)	0.04 (0.03-0.05)	11.5 (11.2-11.7)	7.8 (7.5-8.1)	13.0 (11.7-14.2)	67.8 (67.0-68.8)
XL-VLDL		1.05 (0.65-1.59)	0.02 (0.01-0.03)	0.01 (0.01-0.02)	0.02 (0.01-0.02)	0.06 (0.03-0.09)	17.5 (17.0-18.3)	13.2 (11.8-15.3)	16.2 (14.3-19.1)	53.0 (47.4-56.9)
L-VLDL		5.4 (3.2-8.3)	0.05 (0.03-0.08)	0.04 (0.02-0.06)	0.06 (0.04-0.08)	0.17 (0.10-0.27)	16.6 (15.5-17.1)	11.9 (11.6-12.3)	18.1 (15.4-22.1)	53.6 (49.9-55.9)
M-VLDL		20.6 (15.0-27.5)	0.13 (0.10-0.18)	0.08 (0.06-0.11)	0.16 (0.14-0.20)	0.32 (0.22-0.45)	18.8 (18.5-19.1)	11.7 (11.3-12.0)	23.7 (20.4-27.8)	45.9 (42.1-48.9)
S-VLDL		38.5 (31.78-45.8)	0.17 (0.15-0.20)	0.12 (0.10-0.14)	0.25 (0.22-0.28)	0.24 (0.18-0.31)	22.0 (21.5-22.6)	15.1 (14.7-15.6)	32.6 (29.0-35.6)	30.3 (26.7-33.9)
XS-VLDL		53.0 (47.5-59.5)	0.18 (0.16-0.21)	0.12 (0.10-0.13)	0.29 (0.26-0.31)	0.11 (0.08-0.13)	26.6 (25.5-27.5)	16.9 (16.3-17.3)	41.9 (39.3-43.8)	14.7 (12.5-17.7)
IDL particles	23.7 (23.6-23.7)	131 (116-151)	0.33 (0.29-0.38)	0.25 (0.22-0.29)	0.68 (0.60-0.78)	0.09 (0.07-0.11)	24.4 (24.0-24.8)	18.5 (17.8-18.9)	50.8 (49.2-51.7)	6.6 (5.3-8.0)
LDL particles										
L-LDL		207 (180-242)	0.37 (0.33-0.42)	0.30 (0.27-0.34)	0.75 (0.65-0.89)	0.07 (0.06-0.09)	24.8 (24.2-25.5)	20.1 (19.5-20.7)	50.3 (49.0-51.2)	4.6 (3.8-5.9)
M-LDL		159 (136-189)	0.24 (0.22-0.27)	0.18 (0.17-0.20)	0.39 (0.31-0.48)	0.02 (0.01-0.03)	28.7 (27.3-30.5)	22.1 (20.8-23.6)	46.7 (43.6-48.9)	2.4 (1.6-3.6)
S-LDL		185 (160-217)	0.16 (0.15-0.18)	0.12 (0.11-0.13)	0.24 (0.19-0.29)	0.02 (0.01-0.02)	30.4 (28.9-32.3)	21.9 (20.6-23.3)	44.6 (41.3-47.1)	3.1 (2.1-4.4)
HDL particles	9.9 (9.8-10.1)									
XL-HDL		406 (343-500)	0.14 (0.09-0.19)	0.07 (0.06-0.08)	0.20 (0.18-0.23)	0.02 (0.02-0.02)	32.2 (26.3-37.4)	16.4 (15.3-17.7)	46.6 (43.0-51.1)	4.4 (3.3-5.8)
L-HDL		708 (493-1020)	0.21 (0.15-0.31)	0.06 (0.04-0.08)	0.16 (0.11-0.24)	0.02 (0.01-0.02)	47.6 (46.0-50.1)	12.5 (12.0-13.0)	36.5 (34.3-38.4)	3.1 (2.1-4.3)
M-HDL		1690 (1510-1916)	0.34 (0.30-0.39)	0.07 (0.06-0.08)	0.26 (0.22-0.30)	0.04 (0.04-0.05)	47.5 (46.8-48.5)	9.6 (9.2-10.0)	36.8 (34.9-38.5)	6.0 (4.9-7.3)
S-HDL		4160 (3936-4415)	0.52 (0.48-0.57)	0.11 (0.11-0.12)	0.26 (0.23-0.29)	0.03 (0.03-0.04)	56.2 (53.6-59.1)	12.1 (11.7-12.6)	28.0 (24.9-30.8)	3.7 (2.9-4.8)

PL, phospholipids; FC, free cholesterol; CE, cholesterol ester; TG, triglyceride; VLDL, very-low-density lipoproteins; IDL, intermediate density lipoprotein; LDL, low-density lipoproteins; HDL, high-density lipoproteins; XXL, XL, L, M, S, XS refers to the size of the lipoproteins

Chapter 4

Complement C3 and C4, but not their regulators or activated products, are associated with incident metabolic syndrome: The CODAM study

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Abstract

Purpose: We investigated the associations of components of the alternative (C3, C3a, Bb, factor D [FD], factor H [FH], properdin, and the classical complement pathway (C4, C1q, C1-inhibitor [C1-INH]) with prevalent and incident metabolic syndrome in a cohort with a moderately increased risk of cardiometabolic disease.

Methods: The study cohort was comprised of 574 participants (61% men, age 59.6±7.0 years) at baseline and 489 participants after 7-year follow-up. Multiple logistic regression analyses were done to investigate the associations of concentrations of baseline plasma complement (standardized values) with prevalent and incident (in those without metabolic syndrome at baseline, n = 189) metabolic syndrome.

Results: C3 (odds ratio (OR) = 1.48 [95% confidence interval: 1.02; 2.14]) and C4 (OR = 1.95 [1.32; 2.88]), but none of the other complement components were associated with *incident* metabolic syndrome (n = 40 cases). Notably, in the cross-sectional analyses, we did observe higher levels of C3a (OR = 1.25 [1.03; 1.52]), FH (OR = 2.93 [2.24; 3.83]), and properdin (OR = 1.88 [1.50; 2.34]), in addition to C3 (OR = 3.60 [2.73; 4.75]) and C4 (OR = 1.39 [1.13; 1.69]), in those with the metabolic syndrome compared to those without, while no association was observed for FD, Bb, C1q or C1-INH.

Conclusions: In the cross-sectional analyses, the effects sizes (standardized regression coefficients) for C3 and C4 were similar to those of (some of) the regulators and activators, yet only C3 and C4 were associated with incident disease. These findings suggest a role for C3 and C4, but not their regulators or activated products, in the development of the metabolic syndrome.

1 Introduction

The metabolic syndrome is a cluster of cardiovascular risk factors that is characterized by central obesity, dyslipidemia, hypertension, and insulin resistance. Obesity is one of the initial events in the pathological processes that define the metabolic syndrome (1). Dysfunctional adipose tissue is considered an important driver in the development of the adverse metabolic profiles in people with obesity (1).

The complement system is an essential part of the innate immune system. It is widely expressed in adipose tissue, and its expression is increased in adipose tissue of obese individuals. The complement system has three main activation pathways: the alternative, the classical, and the lectin pathway (as reviewed in (2), **Supplementary Figure 1 [Figure S1]**). Previous research has shown that the expression of some complement components, especially of the alternative and classical pathways, is higher in adipose tissue of people with obesity (as reviewed in (3)). Several, mainly cross-sectional, studies have addressed the associations of individual complement factors with adverse metabolic profiles (as reviewed in (4)). However, these studies often focused on one or a few complement components and on a specific aspect of metabolism. At the same time, studies on the associations of complement with metabolic diseases mostly focused on advanced disease states such as cardiovascular disease (CVD, as reviewed in (5)) or type 2 diabetes mellitus (T2DM) (6, 7). As it stands, the association of complement with the metabolic syndrome, which is the main comorbidity of obesity and predisposes to both CVD and T2DM (8) has received much less attention.

Most available studies on complement and the metabolic syndrome are cross-sectional and mainly focused on C3 (9-11), the central component of the alternative pathway, and its degradation product C3a-desarg (also known as acylation stimulating protein, ASP) (12, 13). To the best of our knowledge, the prospective association of C3 with the metabolic syndrome was only reported in 2 human observational studies. One demonstrated that Caucasian individuals in highest C3 quartile had a higher risk to develop the metabolic syndrome during 3 years of follow-up (14). The other study was performed in Chinese men and reported a similar positive association for C3. In this latter study, C4,

the downstream component of the classical (and lectin) pathway, was also reported to be positively associated with the development of the metabolic syndrome (15). The information on the relationships, especially the prospective ones, of other complement proteins, regulators, and activated products with the metabolic syndrome is even more limited, particularly in humans. Factor D [FD], also known as adipsin, is the rate-limiting enzyme of alternative pathway and is mainly produced by adipocytes. Although previous data revealed a possible role for FD in lipid metabolism and β cell function (16, 17), little is known about its association with the metabolic syndrome. Factor B [FB], factor H [FH], and properdin are important components and regulators of the alternative complement pathway. Thus far, only one cross-sectional study in Caucasian men reported positive associations of FH and FB with several aspects of metabolism (18). Although a possible role of properdin in lipid metabolism was recently demonstrated in a properdin-deficient mouse model (19), human data are still lacking. In addition, Hillian et al. revealed a protective effect of C1q, the initiator of classical pathway activation, on high-fat-diet-induced hepatic insulin resistance and impaired glucose homeostasis in a C1q-knockout mouse model (44). And apolipoprotein E-deficient mice on an atherogenic diet that were treated with C1-inhibitor [C1-INH] showed a decrease in serum triglyceride (20). Yet again, except for some information on C4, human data on the association of the classical pathway with the metabolic syndrome are largely lacking.

In the present study, we hypothesized that, activation of the alternative and the classical complement pathways, represented by higher circulating levels of their components, is associated with the metabolic syndrome. Therefore, we investigated in a Caucasian cohort with moderately increased risk of cardiometabolic disease, the associations of these components, especially those of the alternative (i.e. C3, C3a, Bb, FD, FH, and properdin) and classical (i.e. C1q, C1-INH, and C4) pathways with the prevalence of the metabolic syndrome, as well as its incidence during a 7-year follow-up period.

2 Material and Methods

2.1 Participants

The Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) is a prospective observational cohort. Participants were selected from a large population-based study as described previously (21). CODAM participants are of Caucasian descent and > 40 years of age with one or more of the following characteristics: body mass index (BMI) > 25 kg/m^2; use of antihypertensive medication; positive family history of T2DM; postprandial blood glucose level > 6.0 mmol/L; history of gestational diabetes and/or glycosuria. Data were collected for 574 participants at baseline and for 495 participants at follow-up. The median follow-up time was 7 years (interquartile range (IQR) 6.9–7.1). After exclusion of participants who had missing data (n = 37, 6%), 537 individuals with complete data were included in the cross-sectional analyses. Similarly, of the 209 participants who did not have the metabolic syndrome at baseline, 20 (10%) had either missing information or were lost to follow-up and were therefore not included in the prospective analyses. A flowchart for the inclusion of the participants is shown in **Figure S2.** This study was approved by the medical ethics committee of Maastricht University. All participants gave written informed consent.

2.2 Measurements

2.2.1 Complement measurements

Participants were asked to stop their lipid-modifying medication 14 days and any other medication 1 day prior to the measurements. Blood samples were collected after an overnight fast and stored at –80 °C until use. FH and C1-INH were measured in EDTA plasma using commercially available ELISA kits (FH: DuoSet, R&D Systems, Minneapolis, MN, USA; C1-INH: MicroVue C1-INH EIA kit, Quidel, Catalogue NO. A037, San Diego, USA) according to the manufacturer's instructions, inter-assay variation was 13.5 % and 4.4%, respectively.

C4 was measured in serum by auto-analyzer (Hitachi) using a Roche kit assay (Roche diagnostics Netherland BV, Almere, The Netherlands). The inter-assay variation was 2.0%. Measurements of other complement factors (i.e. C3, C3a, Bb, FD, properdin, and C1q) were performed as previously described (22-24).

2.2.2 Definition of the metabolic syndrome

The metabolic syndrome was defined according to the AHA/NHLBI & IDF 2009 harmonized criteria (25). Participants who met three or more of the following criteria were classified as having the metabolic syndrome: (1) waist circumference (waist) ≥88 cm in women and ≥102 cm in men; (2) triglycerides (TG) ≥ 1.7 mmol/L, and/or use of medication for elevated TG; (3) high density lipoprotein–cholesterol (HDL) < 1.0 mmol/L in men, and < 1.3 mmol/L in women, and/or use of medication for reduced HDL; (4) Systolic blood pressure (SBP) ≥ 130 mmHg and/or diastolic blood pressure (DBP) ≥ 85 mmHg, and/or use of antihypertensive medication; (5) fasting glucose ≥ 100 mg/dl , and/or use of glucose-lowering medication.

2.2.3 Other variables

Anthropometric measurements, plasma biochemistry, lifestyle factors, and medication use were obtained exactly as described in our previous publications. (21, 22, 26, 27).

2.3 Statistical analyses

Variables are presented as mean (SD) or percentages. Those with skewed distribution are presented as median (IQR). Independent sample t-test, Mann-Whitney U test, and chi-square test were used to compare two groups, as indicated.

Variables with skewed distribution (C3a, TG, fasting plasma glucose, HOMA2-IR, aspartate aminotransferase [AST], alanine aminotransferase [ALT], gamma-glutamyl transferase [GGT], C-reactive protein [CRP], serum amyloid A [SAA], interleukin-6 [IL-6], interleukin-8 [IL-8], tumor necrosis factor-a [TNF-a], and soluble intercellular adhesion molecule-1 [sICAM-1]) were $\log_2$ transformed prior further analyses. For the various

complement components standardized values ([individual's observed values − population mean]/standard deviation of the population) were calculated to allow direct comparison of their effect sizes in the regression analyses. All analyses were performed using IBM SPSS statistics version 22 and a 2-tailed *P*-value of <0.05 was considered significant.

2.3.1 Cross-sectional analyses

At baseline, multiple logistic regression analyses were done to investigate the associations of plasma complement concentrations (standardized values, main independent variable) with the prevalence of the metabolic syndrome (main outcome). Subsequently, multiple linear regression analyses were performed to investigate the associations between complement concentrations and individual components of the metabolic syndrome (i.e. TG, HDL, fasting plasma glucose, SBP, DBP, and waist circumference). All analyses were initially adjusted for age (years) and sex (male/female) (Model 1), then additionally for medication use (lipid-modifying, antihypertensive, and/or glucose-lowering, each yes/no), smoking status (current or previous tobacco smoking, yes/no), alcohol consumption (g/d), physical activity (METs/week) and energy intake (kJ/d) to control for potential confounding (Model 2).

2.3.2 Prospective analyses

In those who did not have the metabolic syndrome at baseline, multiple logistic regression analyses were done to evaluate the associations between baseline complement concentrations and the incident metabolic syndrome (main outcome). Models and adjustments were as mentioned for the cross-sectional analyses.

2.3.3 Sensitivity analyses

Use of medication to control blood pressure, plasma glucose and dyslipidemia could affect an individual's risk to develop the metabolic syndrome during follow-up. For this reason, we repeated the prospective analyses while excluding all participants who used lipid-modifying,

antihypertensive, and/or glucose-lowering medication at baseline and/or at follow-up. Besides, plasma complement concentrations may be affected by certain disease conditions. The prospective analyses were therefore also repeated after excluding participants who, at baseline had (1) acute or chronic infections (CRP>10 mg/L), (2) a (suspected) history of autoimmune disease (defined as self-reported current chronic joint inflammation/ rheumatoid arthritis or a severe intestinal disorder that lasted for the past 3 months or longer), (3) a self-reported current malignant condition or cancer, or, (4) self-reported liver disease.

2.3.4 Additional analyses

Several additional analyses were done to explore the possible pathways that may contribute to the associations between complement and incidence of the metabolic syndrome. Firstly, participants who developed the metabolic syndrome during follow-up may at baseline, even though they did not meet the criteria of having the metabolic syndrome, have already had a somewhat worse metabolic status. Thus, to find out via which of the metabolic syndrome components the complement system may contribute to development of the metabolic syndrome, we additionally adjusted the prospective analyses for the baseline levels of the individual components of the metabolic syndrome. Besides, higher systemic complement levels may reflect ongoing liver dysfunction (27), which may accelerate the development of the metabolic syndrome (28). Therefore, additional adjustments were done for liver function markers (i.e. AST, ALT, and GGT). Also, the metabolic syndrome may coincide with an enhanced acute phase response (APR) (29) and complement C3 and C4 are known as acute phase proteins (30). Prolonged APR results in a state of chronic low-grade-inflammation (LGI) which may contribute to the pathogenesis of metabolic syndrome (as reviewed in (31)). Therefore, the analyses were adjusted for several markers of LGI, i.e. CRP, SAA, IL-6, IL-8, TNF-a, and sICAM1. Lastly, since complement activation may induce insulin resistance (32), which is an important contributor to the metabolic syndrome, the prospective regression analyses were additionally adjusted for baseline levels of HOMA2-IR.

3 Results

3.1 Baseline characteristics of the study population

At baseline, complete data were available for 537 participants of whom 328 (61%) had the metabolic syndrome (**Table 1**). Participants with the metabolic syndrome were more often men, were slightly older, had lower insulin sensitivity and worse low-grade inflammation, had higher prevalence of CVD, were more likely to use medication and reported lower physical activity. In addition, the plasma concentrations of several complement factors (i.e. C3, C3a, FD, FH, properdin, C4) were higher in participants with the metabolic syndrome.

Of the 189 participants who did not have the metabolic syndrome at baseline, 40 (21%) developed the metabolic syndrome during the 7-year follow-up period. The baseline characteristics of those who did and did not develop the metabolic syndrome during follow-up are also presented separately in **Table 1**. Already at baseline, those who developed the metabolic syndrome were more obese, had higher TG and lower HDL, higher SBP, higher prevalence of T2DM, and were more likely to use antihypertensive medication. They also had higher concentrations of plasma C3 and C4.

Table 1. Baseline characteristics of the study population according to the presence or absence of the metabolic syndrome at baseline or at follow-up

	Prevalent MetS		***Incident MetS***	
	MetS at baseline	**No MetS at baseline[a]**	**No MetS at baseline[a]**	
			MetS at follow-up	**No MetS at follow-up**
	N=328 (61%)	**N=209**	**N=40** (21%)	**N=149**
Age (years)	60.0 ± 6.6	58.4 ± 7.4 *	58.2 ± 7.3	58.4 ± 7.4
Sex (% men)	65.5	54.5 *	55.0	53.7
BMI (kg/m^2)	30.0±4.3	26.4±3.6 *	27.5 ± 3.6	26.1 ± 3.3 †
Waist (cm)	104.0±10.8	92.2±10.4 *	95.0 ± 9.6	91.3 ± 10.2 †
Triglycerides (mmol/L)	1.80 (1.30-2.20)	1.00 (0.80-1.40) *	1.35 (1.03-1.50)	1.00 (0.80-1.20)†
HDL-cholesterol (mmol/L)	1.05 ± 0.27	1.41 ± 0.34 *	1.29 ± 0.27	1.45 ± 0.36 †
Systolic BP (mmHg)	144.8 ± 17.0	131.8 ± 18.4 *	136.7 ± 20.1	129.8 ± 16.6 †
Diastolic BP (mmHg)	83.9 ± 8.9	78.0 ± 8.2*	79.4 ± 8.6	77.4 ± 7.9
Fasting plasma glucose (mmol/L)	6.03 (5.59-7.19)	5.20 (4.97-5.45) *	5.29 (4.94-5.44)	5.19 (4.98-5.45)
Type 2 Diabetes (%)	38.7	4.3 *	12.5	1.3 †
Cardiovascular Disease (%)	34.5	18.2 *	22.5	17.4
HOMA2-IR [b]	2.15 (1.46-3.29)	1.12 (0.84-1.51) *	1.16 (0.88; 1.61)	1.07 (0.85; 1.44)
Inflammation score [c]	0.20 ± 0.96	-0.32 ± 0.98 *	-0.19 ± 1.02	-0.47 ± 0.88
Ever smoking (%)	23.5	21.1	10.0	21.5

Table 1. Baseline characteristics of the study population according to the presence or absence of the metabolic syndrome at baseline or at follow-up (continued)

	Prevalent MetS		*Incident MetS*	
	MetS at baseline	**No MetS at baseline[a]**	**No MetS at baseline[a]**	
			MetS at follow-up	**No MetS at follow-up**
	N=328 (61%)	**N=209**	**N=40** (21%)	**N=149**
Alcohol intake (g/d)	7.85 (0.64-21.4)	9.92 (2.30-23.91)	11.23 (2.46-22.51)	8.29 (2.30-23.15)
Physical activity (10^3·METs/week)	6.37 ± 3.88	7.13 ± 4.49 *	7.18 ± 4.99	7.20 ± 4.39
Energy intake (10^3·kj/day)	9.27 ± 2.76	9.29 ± 2.80	9.43 ± 2.95	9.29 ± 2.81
Glucose-lowering medication (%)	21.3	1.4 *	5.0	0.7
Lipid-modifying medication (%)	23.5	13.4 *	22.5	12.1
Antihypertensive medication (%)	48.8	23.4 *	35.0	20.1 †
C3 (g/l)	1.07 ± 0.15	0.92±0.13*	0.96 ± 0.12	0.91 ± 0.13†
C3a (µg/l)	61.1 (51.1-75.4)	56.3 (47.4-67.6) *	58.5 (47.4-74.2)	55.1 (46.6-67.0)
Bb (mg/l)	0.71 ± 0.18	0.73±0.20	0.71 ± 0.19	0.72 ± 0.20
Factor D (mg/l)	1.02 ± 0.24	0.97 ± 0.23*	0.96 ± 0.26	0.97 ± 0.22
Factor H (mg/l)	350.2 ± 77.7	285.8 ± 60.9*	280.8 ± 52.6	284.3 ± 61.7
Properdin (mg/l)	6.30 ± 1.29	5.63 ± 1.14*	5.70 ± 0.90	5.65 ± 1.18
C1q (mg/l)	73.1 ± 15.6	70.8 ± 14.8	71.5 ± 14.7	71.2 ± 14.8
C1-INH (mg/l)	170.2 ± 12.3	168.6 ± 12.4	170.4 ± 12.0	167.2 ± 12.4
C4(g/l)	0.30 ± 0.07	0.28 ± 0.07*	0.31 ± 0.07	0.26 ± 0.06†

MetS, metabolic syndrome; BP blood pressure.

[a] Of those who did not have the metabolic syndrome at baseline (n=209), n=20 were lost to follow up or had missing information on one or more components of the metabolic syndrome; the total number of participants available for analyses on incident metabolic syndrome is n=189.

[b] HOMA2-IR: n=12 missing at baseline, n=1 missing at follow-up.

[c] higher value means more low-grade inflammation.

*p<0.05 between participants with and without metabolic syndrome at baseline.

† p<0.05 between participants who did or not develop metabolic syndrome during the 7-year follow-up period.

3.2 Cross-sectional associations of proteins, regulators and activated products of the complement system with prevalence of the metabolic syndrome

All participants who had complete data at baseline (n = 537) were included in the cross-sectional analyses. Several components of the alternative pathway (i.e. C3, C3a, FH, and properdin) as well as for one component of the classical pathway activation (C4) were significantly and positively associated with the metabolic syndrome. These associations remained significant in the fully adjusted model (**Table 2,** model 2, C3, odds ratio (OR) = 3.60 [95% confidence interval (CI): 2.73; 4.75]; C3a, OR = 1.25 [1.03; 1.52]); FH, OR = 2.93

[2.24; 3.83]; properdin, OR = 1.88 [1.50; 2.34]; C4, OR = 1.39 [95% CI 1.13; 1.69]). The OR of 3.6 for C3 implies that, after adjustment for confounders, those participants who had a 1 SD higher C3 concentration were 3.6 times more likely to have metabolic syndrome. Factor Bb, FD, C1q, and C1-INH were not significantly associated with the metabolic syndrome (**Table 2,** model 2, Bb, OR = 0.90 [0.74; 1.09]; FD, OR = 1.06 [0.87; 1.31]; C1q, OR = 1.10 [1.10 [0.91; 1.34]; C1-INH OR = 1.12 [0.91; 1.36]). In line with this, most complement factors (except for factor Bb) were significantly, and in an adverse direction, associated with one or more individual components of the metabolic syndrome (**Supplementary Table1 [Table S1]**).

Table 2. Cross-sectional associations of complement proteins, regulators and activated products with prevalence of the metabolic syndrome

	Metabolic syndrome N=537; 328 prevalent cases (61 %)			
	Model 1		**Model 2**	
	OR [95% CI]	*P* value	OR [95% CI]	*P* value
C3 (SD)	**3.71 [2.86; 4.82]**	**<0.001**	**3.60 [2.73; 4.75]**	**<0.001**
C3a (SD)	**1.31 [1.09; 1.58]**	**0.004**	**1.25 [1.03; 1.52]**	**0.023**
Bb (SD)	0.89 [0.75; 1.07]	0.209	0.90 [0.74; 1.09]	0.279
Factor D (SD)	1.11 [0.92; 1.33]	0.293	1.06 [0.87; 1.31]	0.558
Factor H (SD)	**3.00 [2.34; 3.86]**	**<0.001**	**2.93 [2.24; 3.83]**	**<0.001**
Properdin (SD)	**1.79 [1.46; 2.19]**	**<0.001**	**1.88 [1.50; 2.34]**	**<0.001**
C1q (SD)	1.17 [0.98; 1.40]	0.083	1.10 [0.91; 1.34]	0.316
C1-INH (SD)	1.14 [0.95; 1.36]	0.172	1.12 [0.91; 1.36]	0.282
C4 (SD)	**1.43 [1.18; 1.72]**	**<0.001**	**1.39 [1.13; 1.69]**	**0.002**

All complement components were standardized, C3a was $\log_2$-transformed prior to standardization.
Model 1 (M1) is adjusted for age and sex.
Model 2: M1 + medication use (lipid-modifying, antihypertensive, and/or glucose lowering), smoking status, alcohol consumption, physical activity, and energy intake.

3.3 Prospective associations of proteins, regulators and activated products of the complement system with incidence of the metabolic syndrome

In contrast to the strong associations that were observed in the cross-sectional analyses, only a limited number of complement components were associated with incidence of the metabolic syndrome (**Table 3**). In the fully adjusted models (model 2), significant associations were observed for C3 (OR = 1.48 [1.02; 2.14]) and C4 (OR = 1.95 [1.32; 2.88]).

Thus, participants with 1 SD higher baseline concentration of C3 or C4 were approximately 1.5 and 2.0 times more likely to develop the metabolic syndrome during the 7 year follow-up period. Positive associations were also observed for C3a and C1-INH, but these did not reach statistical significance (C3a, OR = 1.37 [0.96; 1.96]; C1-INH, OR = 1.34 [0.89; 2.02]). The associations of the other complement factors were weaker and non-significant, with ORs around 1 (Bb, OR = 0.94 [0.64; 1.38]; FD, OR = 0.91 [0.62; 1.34]; FH, OR = 0.87 [0.59; 1.27]; properdin, OR = 1.09 [0.75; 1.58]; C1q, OR = 1.01 [0.69; 1.46], **Table 3**).

Table 3. Prospective associations of baseline concentrations of complement proteins, regulators and activated products with incidence of the metabolic syndrome

	Incident metabolic syndrome in those who did not have the metabolic syndrome at baseline N=189, 40 incident cases (21%)			
	Model 1		Model 2	
	OR [95% CI]	*P* value	OR [95% CI]	*P* value
C3 (SD)	**1.55 [1.09; 2.21]**	**0.016**	**1.48 [1.02; 2.14]**	**0.038**
C3a (SD)	1.36 [0.97; 1.91]	0.075	1.37 [0.96; 1.96]	0.087
Bb (SD)	0.94 [0.66; 1.34]	0.731	0.94 [0.64; 1.38]	0.749
Factor D (SD)	0.93 [0.64; 1.36]	0.715	0.91 [0.62; 1.34]	0.632
factor H (SD)	0.95 [0.66; 1.35]	0.760	0.87 [0.59; 1.27]	0.462
Properdin (SD)	1.04 [0.73; 1.47]	0.849	1.09 [0.75; 1.58]	0.662
C1q (SD)	1.02 [0.72; 1.45]	0.907	1.01 [0.69; 1.46]	0.980
C1-INH (SD)	1.36 [0.92; 2.00]	0.122	1.34 [0.89; 2.02]	0.158
C4 (SD)	**1.94 [1.34; 2.82]**	**<0.001**	**1.95 [1.32; 2.88]**	**0.001**

All complement components were standardized, C3a was $\log_2$ –transformed prior to standardization.
Model 1 (M1) is adjusted for age and sex.
Model 2: M1 + medication use (lipid-modifying, antihypertensive, and/or glucose-lowering, smoking status, alcohol consumption, physical activity, and energy intake.

We evaluated the robustness of the associations of C3 and C4 with development of the metabolic syndrome in several sensitivity analyses. Exclusion of participants who used medication at baseline and/or at follow-up resulted in a substantial decrease in the number of events (n = 12), but the results remained largely consistent with the main analyses (**Table S2**). Likewise, when patients with acute or chronic infections, with a (suspected) history of autoimmune disease, with a current malignant condition/cancer or with self-reported liver disease were excluded from the analyses, the results remained similar to what was observed in the main analyses (**Table S2**).

Several additional analyses were done to evaluate how complement may contribute to the development of the metabolic syndrome. For this, firstly we adjusted the prospective

regression analyses for baseline levels of the individual components of the metabolic syndrome. This substantially attenuated the association of baseline C3, but not C4, with incident metabolic syndrome (C3, OR = 1.04 [0.66; 1.64], C4, OR = 1.73 [1.14; 2.64] **Table 4**). Secondly, the development of the metabolic syndrome may also be accelerated by more generalized underlying pathologies such as fatty liver disease, LGI or insulin resistance. Therefore, we also performed additional adjustments with measurements that reflect fatty liver disease, LGI and insulin resistance, respectively. Additional adjustment for baseline levels of ALT, AST, and GGT resulted in a slight attenuation of the association of baseline C3 with incidence of the metabolic syndrome, which lost significance (OR = 1.30 [0.87; 1.93]), while for C4 the association remained significant (OR = 2.01 [1.34; 3.00], **Table 5**). Additional adjustment for LGI had only minor effects on the association of baseline C3 and C4 with incident metabolic syndrome (C3, OR = 1.36 [0.90; 2.05]); C4, OR = 1.91 [1.28; 2.83]), although the association for C3 was, again, no longer significant (**Table 5)**. Also, when HOMA2-IR was included, the associations with incident metabolic syndrome were largely consistent with the main results (C3, OR = 1.46 [0.97; 2.21]); C4, OR = 1.94 [1.31; 2.86], **Table 5**).

Table 4. Prospective associations of the baseline concentrations of complement C3 and C4 with incident metabolic syndrome: Additional adjustment for baseline levels of the individual components of the metabolic syndrome

	Outcome: incident metabolic syndrome, N=189, 40 cases (21%)			
	C3		**C4**	
Model	OR [95% CI]	*P* value	OR [95% CI]	*P* value
1	**1.48 [1.02; 2.14]**	**0.038**	**1.95 [1.32; 2.88]**	**0.001**
2a	1.34 [0.90; 2.01]	0.151	**1.95 [1.31; 2.89]**	**0.001**
2b	1.27 [0.86; 1.89]	0.244	**1.82 [1.23; 2.70]**	**0.003**
2c	1.33 [0.90; 1.95]	0.156	**1.72 [1.16; 2.56]**	**0.007**
2d	1.45 [0.99; 2.11]	0.056	**1.95 [1.31; 2.90]**	**0.001**
2e	**1.48 [1.02; 2.14]**	**0.038**	**1.97 [1.33; 2.93]**	**0.001**
2f	1.04 [0.66; 1.64]	0.860	**1.73 [1.14; 2.64]**	**0.011**

Model 1 (M1) is adjusted for age, sex, medication use (lipid-modifying, antihypertensive, and/or glucose-lowering), smoking status, alcohol consumption, physical activity, and energy intake.
Model 2a: M1+waist.
Model 2b: M1+triglyceride levels.
Model 2c: M1+HDL-cholesterol level.
Model 2d: M1+systolic and diastolic blood pressure levels.
Model 2e: M1+ fasting plasma glucose level.
Model 2f: M1+ waist, triglyceride, HDL-cholesterol, systolic and diastolic blood pressure, and fasting plasma glucose.

Table 5. Additional analyses: Prospective associations of the baseline concentrations of complement C3 and C4 with incident metabolic syndrome adjusted for liver function markers, markers of low-grade inflammation or insulin-resistance (HOMA2-IR)

	Outcome: incident metabolic syndrome N = 186, 39 incident cases (22%)			
	C3		**C4**	
Model	OR [95% CI]	*P* value	OR [95% CI]	*P* value
1	**1.48 [1.02; 2.15]**	**0.039**	**1.96 [1.33; 2.90]**	**0.001**
2a	1.30 [0.87; 1.94]	0.196	**2.01 [1.34; 3.00]**	**0.001**
2b	1.36 [0.90; 2.05]	0.139	**1.91 [1.28; 2.83]**	**0.001**
2c	1.45 [0.95; 2.20]	0.084	**1.93 [1.30; 2.85]**	**0.001**

Model 1 (M1) is adjusted for age, sex, medication use (lipid-modifying, antihypertensive, and/or glucose-lowering), smoking status, alcohol consumption, physical activity, and energy intake.
Model 2a: M1 + aspartate aminotransferase, alanine aminotransferase, and gamma-glutamyl transferase levels at baseline, compiled into an average Z score*.
Model 2b: M1 + C-reactive protein, serum amyloid A, interleukin-6, interleukin-8, tumor necrosis factor-a, and soluble intercellular adhesion molecule-1 levels at baseline, compiled into an average Z score*.
Model 2c: M1 + HOMA2-IR at baseline.
* Calculation of average Z score: standardized values for each marker were calculated ([individual's observed values - population mean]/standard deviation of the population) within each participant, the standardized values of the individual markers were summed, then averaged and the standardized values of this averaged score was included in the regression models.

4 Discussion

We here show that, at baseline, C3 and C4, but also systemic concentrations of C3a, factor H, and properdin, were higher in individuals with metabolic syndrome compared to those without. We also show that baseline concentrations of complement C3 and C4 were positively and significantly associated with development of the metabolic syndrome during the 7-year follow-up period. In contrast, this was not the case for the other complement components (i.e. C3a, Bb, FD, FH, properdin, C1q, and C1-INH).

Cross-sectional associations of complement with components of the metabolic syndrome, such as obesity (33) and insulin resistance (34), were described more than 10 years ago. Cross-sectional associations of complement components, mainly C3 and its downstream product C3adesarg/ASP, with the metabolic syndrome were also reported previously (9-13). In line with this, we confirm the positive associations of C3 and C3a (the precursor of ASP) with prevalence of the metabolic syndrome. Also, we extend previous findings by reporting the associations of factor Bb, FD, FH, and properdin (components and regulators of the alternative pathway), with presence of the metabolic syndrome (significant only for FH and properdin). In our current evaluations, the associations of the

proximal factors of the classical pathway, (C1q and C1-INH), with the metabolic syndrome were modest. Only C4 was significantly associated with the presence of the metabolic syndrome. Positive associations between C4 and prevalence of the metabolic syndrome were reported previously (12, 15), although not consistently (11). Thus, our cross-sectional observations indeed suggested that activation of the complement system, especially the alternative pathway, is related to the metabolic syndrome.

We also evaluated to what extent the components of the complement system were associated with incidence of the metabolic syndrome over a 7-year follow-up period. Positive associations were observed for C3 and C3a, but only reached statistical significance for C3. Notably, the associations of all the other components of the alternative pathway, including FH and properdin, were non-significant with ORs close to 1. This strongly suggests that, despite the observed strong cross-sectional associations, FH and properdin are not risk factors for the development of metabolic syndrome. Two previous human studies have reported on the role of C3 in the development of the metabolic syndrome (14, 15), and their results are in line with our current observation. We additionally observed a very robust association of C4, but not the proximal components of the classical pathway, with development of the metabolic syndrome. To the best of our knowledge, only one previous study reported on the association between C4 and incident metabolic syndrome (15). In that study, a positive association with incidence of the metabolic syndrome was shown but no adjustments were made for components of the metabolic syndrome. So taken together, in our prospective cohort, systemic concentration of the major complement components C3 and C4, but not their activators or activated products, were associated with incident metabolic syndrome.

The association with incidence of the metabolic syndrome was stronger for C4 than C3. Moreover, for C4, but not for C3, this association was independent of baseline levels of the individual components of the metabolic syndrome and its main underlying metabolic aberrancies. This suggests that the effects of C3 and C4 on the development of the metabolic syndrome are distinct. This is for instance illustrated by the observation that the association of complement C3, but not of C4, with incident metabolic syndrome was partly explained by liver function. Also, activation of the C3-C3a-C3adesArg/ASP axis may provide

a partial explanation for the observation that C3 is a risk factor for the development of the metabolic syndrome. Experimental data showed that C3adesArg/ASP stimulates triglyceride synthesis and glucose uptake and inhibits hormone-sensitive lipase in several cell types (as reviewed in (4)). These known effects of C3 and its activation products may affect lipid metabolism and thus contribute to the development of the metabolic syndrome. An exciting novel mechanism of complement activation is the so-called intracellular complement system. Intracellular complement activation was first identified in T-cells and has been implicated in the regulation of increased glycolysis and oxidative phosphorylation in Th1 cells (35). In the circulation, C3 can be spontaneously activated and transformed into C3(H_2O), the hydrolytic product of C3, via the so-called tick-over mechanism. And recently it was established that many cell types can take up C3(H_2O) from plasma, and part of this intracellular C3(H_2O) provides an intracellular source of C3a via a process that is independent of C3-convertase (36). This latter finding is particularly interesting given our current observation that C3, but not FH or properdin, was associated with the development of the metabolic syndrome. The effects of intracellular C3 activation have not yet been established. However, given the effects of intracellular complement activation on TH1 metabolism, intracellular C3 activation will likely affect metabolism in many cell types and, as such, potentially affect the metabolic dysregulation that characterizes the metabolic syndrome. Notably, C4 cannot be spontaneously activated via the tick-over mechanism, which illustrates that the above-mentioned intracellular route may be particularly relevant for C3. This may add to the possibility that C3 and C4 contribute to the metabolic syndrome via distinct mechanisms.

The available information on C4 in relation to metabolism is limited. Genetic variation in the C4 binding protein (C4BP), which is an inhibitor of the classical and the lectin pathway, has been related to higher blood pressure and higher fasting blood glucose (37). C4BP may also have a protective effect on β-cell function (38). In addition, recent developments showed that C4a, which is generated upon activation of C4, can bind to and activate the G-coupled protein receptor (GCPR) Protease-Activated Receptor (PAR)1 and PAR4 (39). Notably, this receptor cannot be activated by C3a (39). Activation of PAR1 and 4 by C4a leads to cellular activation and enhanced endothelial permeability (39), and PAR1 has been

previously implicated in e.g. cardiac remodeling (40) and hepatic injury (41). Notably, C4a cannot signal via the C3aR (42). These data, again, support the notion that in addition to their well-known conjunction on the terminal complement pathway, activation of C3 and C4 may indeed have distinct effects on metabolism. This also opens the path towards the investigation of a possible relation of C4, activated via the classical or the lectin pathway, with platelet activation and/or endothelial dysfunction, irrespective of the extent of terminal pathway activation.

The major strength of our study is that it provides information on several components and regulators of the alternative and the classical complement pathways, measured within one cohort, in the relation to the metabolic syndrome. Another key strength is that it provides prospective information on the development of the metabolic syndrome. Nevertheless, some limitations need to be acknowledged. The number of cases in the prospective analyses was relatively small, which may have limited our power to detect associations when effect sizes were smaller than those of C3 and C4. This may have been the case for C3a and perhaps also C1-INH. With respect to the interpretation of the data, we are limited by the fact that all complement measures were obtained in plasma which does not provide insight in local activation of these complement components relevant metabolic organs. Also, our cohort consists of individuals with a moderately increased risk to develop cardiometabolic diseases, which limits the generalizability of our findings. In addition, our study may be limited by the missing of information for several participants in the cross-sectional (6%) and the prospective (10%) analyses. However, our current observations are substantiated by their consistency with previous reports. Moreover, given our observational study design, we cannot draw causal conclusions on the relationship between complement and the metabolic syndrome. However, our findings are consistent with previous reports and are also supported by suggestive biological evidence, such as the altered metabolic profiles in several complement gene-deficient mouse models (17, 19, 43), which suggests that complement may play a role in the development of metabolic syndrome.

In conclusion, we herein report that complement C3 and C4 are positively associated with incidence of the metabolic syndrome. For C3, but not for C4, these effects appear to

be related to the metabolic profile at baseline. Importantly, properdin and FH, which showed strong associations in the cross-sectional analyses, were not related to incident metabolic syndrome. In the discussion, we refer to novel developments in our understanding of activation and signaling of complement in order to provide some explanation for these divergent observations (see graphical summary in **Figure S3**), but further work is needed to better understand the etiological role of complement activation in the cellular and metabolic pathways that underlie the development of the metabolic syndrome and related cardiometabolic diseases. Future work should include direct metabolic effects of C3 and C4 as well as effects of their activation products, C3a/C3a-desarg and C4a.

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Supplementary data

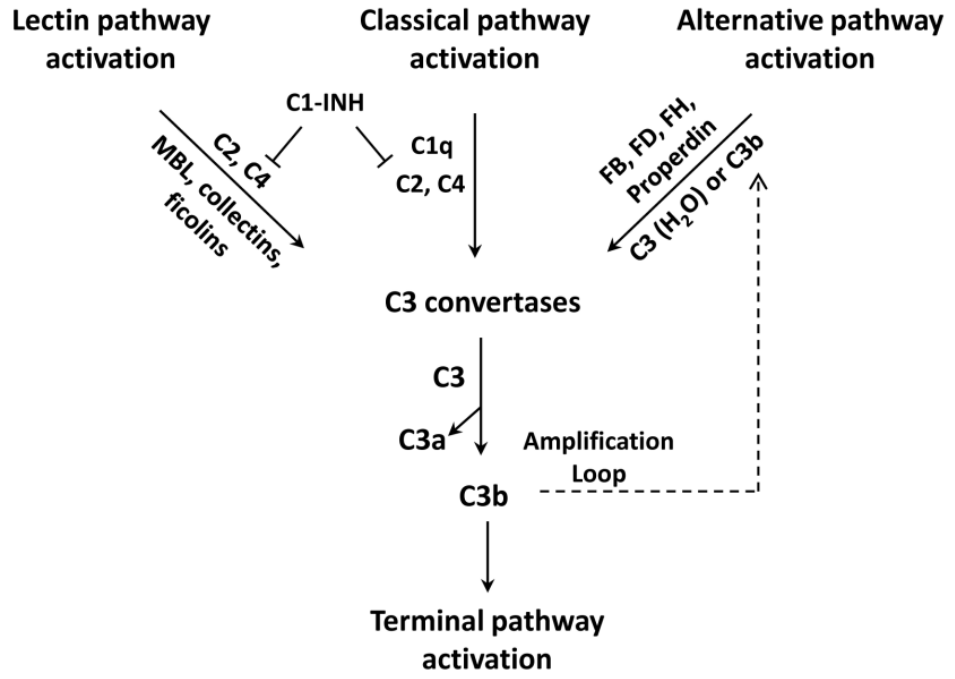

Figure S1. Activation of the complement system with focus on components that are most relevant for the current study. Complement activation can be initiated via the classical, lectin, or alternative pathway. Classical pathway activation starts with interaction between C1q and its ligands, lectin pathway activation starts with the recognition of its ligands by mannose binding lectin (MBL), collectins, or ficolins. Both can result in the cleavage of C2 and C4 to form C3 convertase (C4b2a). Activation of these two pathways is controlled by the regulator C1-inhibitor (C1INH). The alternative pathway can be initiated via two routes. Activation can start with spontaneous hydrolysis of C3 which generates C3(H_2O) or with C3b that is generated by the classical or lectin pathways. C3b and C3(H_2O) make factor B (FB) available for cleavage by factor D (FD). This yields Bb which combines with C3b and C3(H_2O) to generate the alternative pathway C3 convertase (C3bBb and C3(H_2O)Bb). The alternative pathway functions as an amplification loop for all activation pathways. Activation of the alternative pathway is under strict control of several regulatory proteins, including factor H (FH), which acts as a destabilizer of the C3 convertase, and properdin which is a C3 convertase stabilizer. C3 convertases generated via either pathway cleave C3 into C3a and C3b. The anaphylatoxin C3a is rapidly degraded to C3a-desarg, also called acylation stimulating protein (ASP). C3b can contribute to the amplification loop to produce more C3 convertases and, by combining with the C3 convertase, generate a C5 convertase and lead to the activation of the terminal pathway.

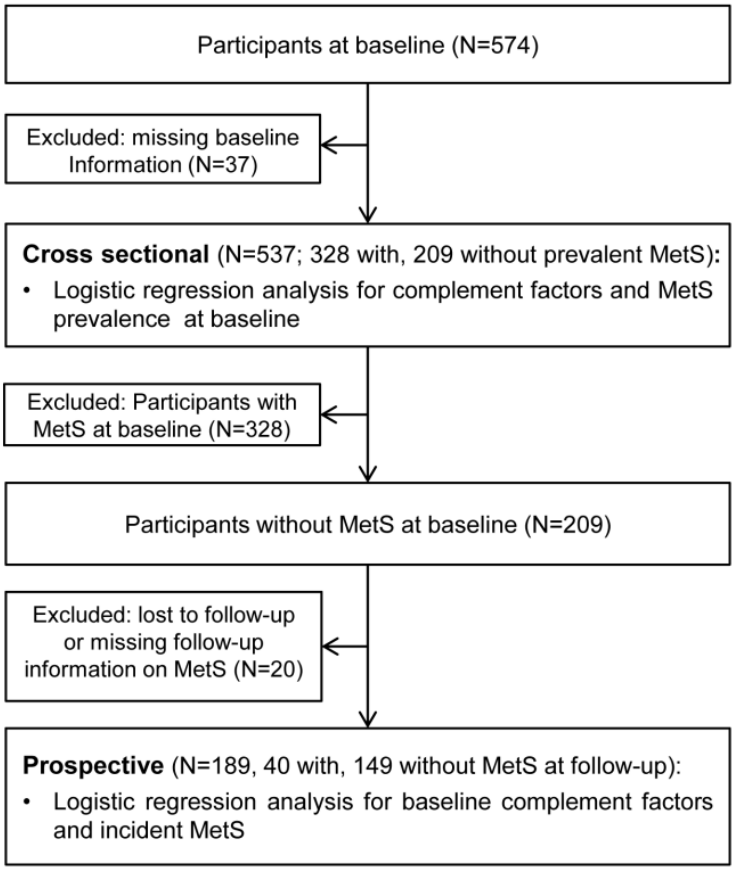

Figure S2. Flowchart of the individuals included in the main analyses in this study. Abbreviations: MetS, metabolic syndrome.

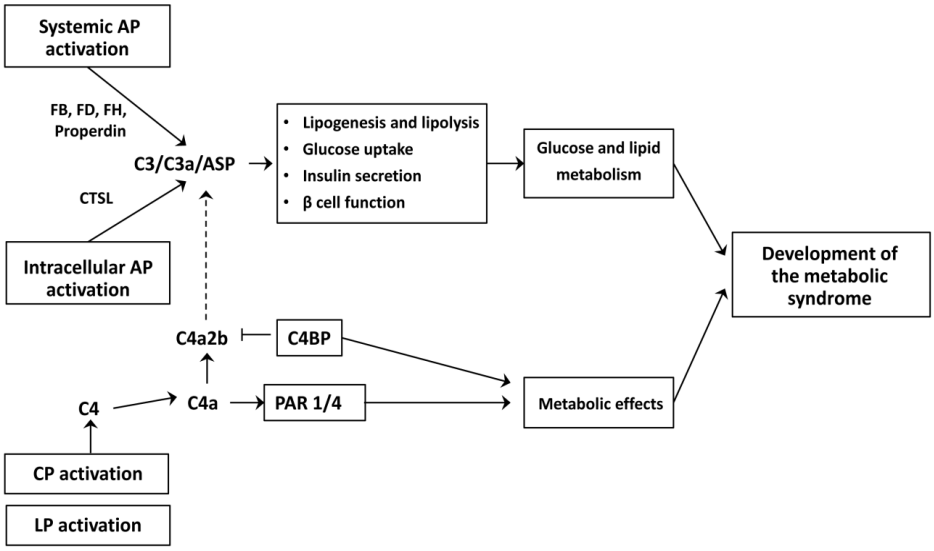

Figure S3. Graphic summary of the possible explanations for the main findings. Abbreviations: AP, alternative pathway; CP, classical pathway; TP, terminal pathway; FB, factor B; FD, factor D; FH, factor H; ASP, Acylation stimulating protein; CTSL, cathepsin L; C4BP, C4 binding protein; PAR 1/4, protease-activated receptor 1 and 4.

Table S1. Cross-sectional associations of complement with components of the metabolic syndrome (N=537)

	Waist (cm)	TG (mmol/L)[a]	HDL (mmol/L)	SP (mmHg)	DP (mmHg)	FPG (mmol/L)[a]
	β [95% CI]	β [95% CI]	β [95% CI]	β [95% CI]	β [95% CI]	β [95% CI]
C3 (SD)	**5.47 [4.59; 6.35]**	**0.33 [0.27; 0.38]**	**-0.13 [-0.15; -0.10]**	**3.22 [1.71; 4.73]**	**1.96 [1.18; 2.73]**	**0.05 [0.03; 0.07]**
C3a (SD)	**1.94 [0.99; 2.89]**	0.01 [-0.05; 0.07]	-0.02 [-0.04; 0.01]	**1.97 [0.50; 3.45]**	0.15 [-0.62; 0.91]	0.01 [-0.01; 0.03]
Bb (SD)	-0.53 [-1.51; 0.44]	**-0.09 [-0.15; -0.03]**	-0.01 [-0.04; 0.02]	-0.56 [-2.05;0.94]	**-0.92 [-1.69; -0.16]**	-0.02 [-0.04; 0.00]
Factor D (SD)	**2.06 [1.07; 3.04]**	**0.08 [0.02; 0.14]**	**-0.06 [-0.09; -0.03]**	-1.17 [-.70; 0.36]	-0.24 [-1.03; 0.55]	-0.02 [-0.04; 0.00]
factor H (SD)	**3.94 [3.03; 4.84]**	**0.15 [0.10; 0.21]**	**-0.08 [-0.11; -0.06]**	**1.68 [0.20; 3.16]**	**1.32 [0.56; 2.07]**	**0.04 [0.02; 0.06]**
Properdin (SD)	**2.01 [1.06; 2.95]**	**0.23 [0.18; 0.29]**	**-0.10 [-0.13; -0.07]**	**2.06 [0.60; 3.52]**	**1.50 [0.75; 2.25]**	**0.02 [0.00; 0.04]**
C1q (SD)	0.21 [-0.74; 1.17]	**0.08 [0.02; 0.14]**	**-0.06 [-0.08; -0.03]**	0.11 [-0.13; 1.57]	0.26 [-0.49; 1.01]	**0.02 [0.00; 0.04]**
C1-INH (SD)	0.57 [-0.41; 1.54]	0.05 [-0.01; 0.11]	0.01 [-0.01; 0.04]	1.28 [-0.21; 2.77]	**0.90 [0.14; 1.67]**	0.01 [-0.01; 0.03]
C4 (SD)	**2.30 [1.37; 3.24]**	**0.09 [0.03; 0.15]**	**-0.05 [-0.08; -0.02]**	**1.53 [0.07; 2.99]**	**0.92 [0.16; 0.67]**	-0.00 [-0.02; 0.02]

Abbreviations: TG, triglycerides; HDL, HDL-cholesterol; SP, systolic blood pressure; DP, diastolic blood pressure; FPG, fasting plasma glucose.

[a] TG and FPG were $\log_2$-transformed prior to the analyses.

All analyses were adjusted for age, sex, medication use (lipid-modifying, antihypertensive, and/or glucose-lowering), smoking status, alcohol consumption, physical activity, and energy intake.

Table S2. Sensitivity analyses: Prospective associations of baseline concentrations of complement proteins, regulators and activated products with incident metabolic syndrome

	Outcome: incident metabolic syndrome			
	Model 1		Model 2	
	OR 95% CI	*P* value	OR 95% CI	*P* value
No use of lipid-modifying, antihypertensive, and/or glucose-lowering medication; N=97 [a], 12 incident cases (12%)				
C3 (SD)	1.27 [0.68; 2.38]	0.460	1.22 [0.65; 2.31]	0.538
C4 (SD)	**2.63 [1.32; 5.23]**	**0.006**	**2.55 [1.27; 5.09]**	**0.008**
No chronic or acute infections; N=183 [b], 39 incident cases (21%)				
C3 (SD)	**1.63 [1.13; 2.34]**	**0.009**	**1.53 [1.05; 2.23]**	**0.029**
C4 (SD)	**2.05 [1.40; 3.02]**	**<0.001**	**2.09 [1.39; 3.15]**	**<0.001**
No autoimmune disease; N=164 [c], 35 incident cases (21%)				
C3 (SD)	**1.50 [1.03; 2.19]**	**0.034**	1.36 [0.91; 2.01]	0.132
C4 (SD)	**1.98 [1.32; 2.99]**	**0.001**	**1.91 [1.24; 2.93]**	**0.003**
No malignant disease and/or cancer; N=180 [d], 37 incident cases (21%)				
C3 (SD)	**1.63 [1.13; 2.35]**	**0.009**	**1.55 [1.06; 2. 26]**	**0.024**
C4 (SD)	**2.07 [1.40; 3.06]**	**<0.001**	**2.07 [1. 37; 3.13]**	**0.001**
No liver disease; N=186 [e], 38 incident cases (20%)				
C3 (SD)	**1.62 [1.13; 2.32]**	**0.009**	**1.54 [1.06; 2.25]**	**0.025**
C4 (SD)	**1.99 [1.37; 2.91]**	**<0.001**	**2.05 [1.37; 3.08]**	**0.001**

Model 1 (M1) is adjusted for age and sex.

Model 2: M1 + medication use (lipid-modifying, antihypertensive, and/or glucose-lowering, if applicable), smoking status, alcohol consumption, physical activity, and energy intake.

[a] 92 individuals who used lipid-modifying, antihypertensive, and/or glucose-lowering medication at baseline and/or at follow-up were excluded.

[b] 6 individuals with chronic or acute infections at baseline were excluded.

[c] 25 individuals with a (suspected) history of autoimmune disease at baseline were excluded.

[d] 9 individuals have malignant condition or cancer at baseline were excluded.

[e] 3 individuals have liver disease at baseline were excluded.

Chapter 5

The alternative pathway of complement is a determinant of insulin resistance, rather than β-cell function in humans: The CODAM study

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Submitted

ABSTRACT

Objective: Experimental data suggest that activation of the alternative pathway of complement results in worse insulin resistance but at the same time better insulin secretion by the β-cells. To investigate which effect prevails in humans we performed a comprehensive evaluation of the alternative complement pathway in relation to insulin resistance and β-cell function.

***Research Design and Methods*:** Plasma concentrations of the alternative pathway components C3, factor D, MASP-3, C3a, Bb, properdin and factor H were measured in a prospective, observational cohort (n=574 at baseline, 495 at 7 years follow-up). Insulin resistance and β-cell function were derived from standard oral glucose tolerance tests. The overall associations of alternative pathway components with insulin resistance, β-cell function and incident type 2 diabetes were determined using logistic regression and generalized estimating equations with adjustment for potential confounders, including age, sex, lifestyle and medication.

Results: Over the 7-year period, C3, MASP-3, properdin, and factor H were associated with worse insulin resistance (standardized (std)βs for Matsuda index [95%CI]: -0.33[-0.40;-0.25], -0.14[-0.20;-0.07], -0.20[-0.26;-0.13], -0.19[-0.26;-0.12], respectively), while Bb was associated with slightly less insulin resistance (stdβ= 0.09[0.03;0.15]), all adjusted for potential confounders and β-cell function. After taking into account insulin resistance and confounders, most alternative pathway components were not significantly associated with markers of β-cell function such as insulin secretion rate, C-peptidogenic index, β-cell glucose sensitivity or the β-cell potentiation factor. Factor D, but no other alternative pathway component, was associated with worse β-cell rate-sensitivity, independent of insulin resistance ($OR_{rate\text{-}sensitivity>median}$= 0.81[0.66;0.98]. Only C3 was significantly associated with incident type 2 diabetes (OR=1.58 [1.08; 2.30], and this association was driven by insulin resistance, not β-cell function.

Conclusions: In middle-aged Caucasian individuals with a moderately increased cardiometabolic risk, the alternative pathway of complement is a determinant of insulin resistance, rather than β-cell function.

1 INTRODUCTION

Development of type 2 diabetes mellitus (T2DM) is preceded by a prolonged period of insulin resistance and relative normoglycaemia. This prediabetic state is maintained as long as insulin secretion by the β-cells is sufficient to keep the circulating glucose concentration in the non-diabetic range. However, when the amount and/or dynamics of insulin secretion are not sufficiently aligned with the prevailing insulin resistance, circulating glucose concentrations increase and T2DM can develop. This makes insulin resistance and β-cell function key processes in the development of T2DM. For this reason, regulatory pathways that contribute to either comprise important targets for prevention of T2DM.

The alternative pathway of complement activation, particularly the central complement component C3, has been implicated in (obesity-associated) insulin resistance (as reviewed in (1)), and we (2) and others (3) have identified C3 as an independent risk factor for T2DM. C3a, an activation product of C3, is well-known for its pro-inflammatory properties. A higher circulating concentration of C3a is generally associated with more low-grade inflammation ((4), and reviewed in (5)), which is known to induce insulin resistance (6, 7). Also, higher circulating levels of C3a and/or its derived peptide C3a-desarg were shown to be associated with metabolic states that are characterized by insulin resistance, such as fatty liver disease (8) and obesity (9, 10). Moreover, in a genetically modified mouse model, it was shown that disruption of signaling via the C3a-C3aR1 axis ameliorated high-fat-diet induced macrophage infiltration and insulin resistance (11).

In the past few years it was shown that the alternative complement pathway may not only induce obesity-associated insulin resistance but may also be involved in the regulation of insulin secretion, and therefore β-cell function. In mouse models of obesity-induced diabetes, the rate limiting enzyme in activation of the alternative complement pathway, factor D (FD) (12), was proposed to induce insulin secretion via generation of the alternative pathway anaphylatoxin C3a, which was in turn identified as an *ex vivo* islet insulin secretagogue (13). In line with this, the receptor for C3a (C3aR1) on β-cells has been implicated in potentiation of insulin secretion in response to glucose (14). While FD is decreased in several mouse models of obesity-associated diabetes (13, 15), it is generally

increased in human obesity (16-18), so the relevant of pathways in human needs to be established. Notwithstanding, in one study in individuals without diabetes, circulating concentrations of C3 were indeed positively associated with insulin secretion, independent of adiposity and insulin resistance (19). These data thus suggest a potential positive effect of the alternative complement pathway on β-cell function.

Taken together, experimental data in the literature suggest that activation of the alternative pathway of complement results in worse insulin resistance but at the same time in better insulin secretion by the β-cells. We herein investigated which of these effects prevails in the human situation. Human data on alternative pathway activation in relation to insulin secretion are scarce, and data on insulin resistance primarily focus on C3. We therefore performed, in a prospective cohort study, a comprehensive evaluation of the alternative pathway (see also **supplemental figure 1**), as represented by C3, FD, MASP-3, C3a, Bb, properdin and factor H (FH) in relation to insulin resistance and β-cell function. C3 and FD are proximal factors of the alternative pathway, Bb is generated upon alternative pathway activation and is part of the C3 convertase that converts C3 into C3a and C3b (20) MASP-3 can activate FD (21) while properdin and FH are regulators of alternative pathway activation (22, 23). In addition, we investigated in what direction (detrimental or protective) the baseline concentrations of these complement factors were associated with the most relevant clinical outcome, i.e. presence of T2DM at follow-up.

2 METHODS

2.1 Study design and participants

The Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) study is a prospective observational study on, among others, the natural progression of glucose tolerance (24). At baseline, 574 individuals were selected from a large population-based cohort (25, 26) and included on the basis of an elevated risk of T2DM and cardiovascular disease (27). After a median follow-up period of 7.0 years (interquartile range [IQR] 6.9–7.1 years), 495 individuals participated in the follow-up measurements. CODAM was approved by the

Medical Ethical Committee of the Maastricht University Medical Center, and all participants gave written informed consent.

2.2 Complement measurements

FD, C3, and FH were measured as reported previously (2, 28-30) and were available at baseline and at follow-up. MASP-3, C3a, Bb, properdin were measured only at baseline, as reported earlier (4, 28). C3 was measured using a nephelometric assay, all other components of the alternative pathway were obtained using ELISA techniques. The inter-assay coefficients of variation were 7%, 4.2%, 13%, , 6.6% , 5.6% , 7.7%, 13.5% for C3, FD, MASP-3, C3a, Bb, and properdin, and FH, respectively.

2.3 Insulin resistance and β-cell function

Plasma glucose, insulin and C-peptide were measured in fasting plasma (2, 31) as well as in EDTA plasma samples that were obtained during a 75 grams oral glucose tolerance test ((31), at baseline time points were 0-30-60-120 minutes, at follow-up 0-15-30-60-120 min). OGTT data were used to calculate the Matsuda index (32) and the modified Stumvoll insulin sensitivity index (33) as alternative measures of insulin resistance/sensitivity. The OGTT-data were also used to derive the C-peptidogenic index t0-t30, which is the best simple OGTT-derived predictor of incident T2DM (31), as well as multiple functional aspects of insulin secretion by the β-cell using the mathematical model of Mari et al (34). Basal and total insulin secretion rates represent the rate of insulin secretion in the fasting state and during the OGTT, respectively. β-cell glucose sensitivity reflects responsiveness of the β-cell to absolute blood glucose levels. β-cell glucose sensitivity is modulated by the β-cell potentiation factor. β-cell rate sensitivity reflects the early-phase insulin release. For completeness, the HOMA2 calculator (35), was used to estimate insulin resistance from fasting glucose and insulin, as reported before (2, 31).

2.4 Other covariates

Body mass index (BMI), waist circumference, blood pressure, fasting cholesterol, HDL-cholesterol, triglycerides, HbA1c, kidney function (estimated glomerular filtration rate [eGFR]), smoking behavior, caloric intake, physical activity, use of antihypertensive, glucose-lowering, and/or lipid-modifying medication, and glucose metabolism status (i.e. normal glucose metabolism, impaired glucose metabolism, or T2DM) were all determined as previously described (8, 25, 26, 36-38). Six inflammatory markers (i.e. C-reactive protein [CRP], interleukin-6 [IL-6], interleukin-8 [IL-8], tumour necrosis factor-a [TNF-α], serum amyloid A [SAA], intercellular cell adhesion molecule [ICAM]) were measured in plasma and combined into an overall z-score for low-grade inflammation, as reported (26).

2.5 Statistical analyses

Variables with normal distribution are presented as mean ± standard deviation (SD), those with non-normal distribution (C3a, HOMA2IR, Matsuda index, Stumvoll index, basal and total insulin secretion rate, β-cell glucose sensitivity, the β-cell potentiation factor, β-cell rate sensitivity) are presented as median (IQR). T-test, Mann-Whitney U-test, and Chi-square test were used to compare two groups, as indicated.

Except for C3, and to a lesser extent C3a/C3adesarg, the relations between components of the alternative complement pathway investigated here, and general aspects of metabolism are not well-known in humans. Therefore, we first performed cross-sectional associations between complement components and well-known characteristics of T2DM, i.e. adiposity (BMI and waist) and low-grade inflammation. For this we used linear regression analyses with adjustment for age and sex.

In the main analyses, linear generalized estimating equations (GEE) with an exchangeable correlation structure were used to evaluate the overall associations between components of the alternative complement pathway and insulin sensitivity (Matsuda index) or β-cell function (basal and overall insulin secretion rate, C-peptidogenic index, β-cell glucose sensitivity, β-cell potentiation factor, or β-cell rate sensitivity), over the 7 years

follow-up period. The use of GEE allowed optimal use of the all the information that was available for each participant. The main independent variables were the components of the alternative complement pathways (at baseline), the main dependent variables were insulin resistance/sensitivity and indices of β-cell function (at baseline and at follow-up). All main variables were standardized ([individual's observed value - population mean]/standard deviation of the population) prior to further analyses, to allow direct comparison of the effect sizes. C3a, Matsuda index, basal and overall insulin secretion rate, C-peptidogenic index, β-cell glucose sensitivity and the β-cell potentiation factor were ln-transformed prior to standardization. The β-cell rate sensitivity could not be normalized via transformation and was therefore evaluated as a dichotomous variable (i.e. higher than vs. equal to or lower than the median). The GEE analyses were first adjusted for age, sex, follow-up time (years) and time-point (baseline/follow-up) [Model 1], then additionally for potential confounders including waist circumference, eGFR, lifestyle factors (smoking, physical activity, caloric intake) and use of medication (i.e. antihypertensive and/or lipid-modifying medication) [Model 2], then additionally for diabetes characteristics, i.e. presence of T2DM, diabetes duration and use of glucose-lowering medication [model 3]. Lastly, when insulin resistance was the outcome additional adjustments were done for β-cell function [model 4], and when β-cell function was the outcome, the analyses were adjusted for insulin resistance [model 4]. This latter adjustment provides information on which hypothesized effect of alternative pathway activation, worse insulin resistance of better β-cell function, prevails. Since it may be argued that part of the assumptions in the calculations of OGTT-derived measures might differ between individuals with and without T2DM, we repeated these analyses while excluding T2DM patients and interaction with presence of T2DM was evaluated using products of the complement factor*T2DM (yes/no). In addition, potential sex differences were evaluated by adding the products of the complement factor*sex. We also repeated the main analyses using the Stumvoll index as measure insulin sensitivity.

Logistic GEE was done to evaluate the associations between components of the alternative complement pathway and the main clinical outcome, T2DM. Models 1 and 2 were evaluated as described above. Then, model 2 was additionally adjusted for the Matsuda index [model 3a] or the C-peptidogenic index [model 3b]. In logistic GEE, both

prevalent and incident cases of T2DM are included and the regression coefficient reflects the association between the complement factors and T2DM over the 7-year period, reflecting both between subjects (differences during the 7 years) and within subjects (changes through the 7 years) associations. To specifically address the prospective associations between baseline concentrations of the complement components and incident T2DM, we additionally performed logistic regression with incident T2DM as outcome, including only those who did not have diabetes at baseline.

All analyses were performed using IBM SPSS statistics version 23. As shown in **supplementary table S1**, the main independent (**S1A**) and dependent (**S1B**) variables that were used in the analyses were correlated amongst one another, which can be expected since these sets of variables each represent (parts of) a defined biological construct (i.e. the alternative complement pathway, insulin sensitivity/resistance, and β cell function, respectively). Thus, we perform multiple tests, but these tests evaluate a limited number of biological constructs. To acknowledge this, we considered a nominal 2-tailed P-value <0.05 a significant result, but additionally indicated in the full models of the main analyses presented in **Table 3**, **Table 4A** and **Table 4B** whether the false discovery rate (FDR) adjusted q-value, which takes into account the number of individual determinants and outcomes, was <0.05. **Supplementary Figure 2** provides a flowchart with the numbers included in the analyses.

3 RESULTS

3.1 Study population

Characteristics of the study population are provided in **Table 1**. Compared to those who remained free of T2DM, those who developed diabetes were, at baseline, slightly more obese, had higher blood pressure, worse lipid profiles, and already showed signs of a disturbed glucose metabolism and worse insulin resistance. Also, most measures of β-cell function, except the β-cell potentiation factor, differed between those who did and did not develop T2DM. In addition, those who developed T2DM had higher plasma concentrations of C3 and C3a.

Table 1. ***Baseline characteristics*** **of whole study population and of those who did/did not develop type 2 diabetes**

	All participants at baseline	***Participants without T2DM at baseline***	
	N=518	***Free of T2DM at follow-up N=339***	***Progressed to T2DM N=58***
Age (years)	59.4 ± 7.0	58.8 ± 7.3	59.6 ± 6.1
Sex (men)	317 (61%)	207 (61%)	28 (48%)
Body mass index (kg/m^2)	28.5 ± 4.3	**27.7 ± 3.8**	**29.7 ± 5.3***
Waist circumference (cm)	99.3 ± 12.0	**96.9 ±11.2**	**101.1 ± 13.1***
Systolic blood pressure (mm/Hg)	139.9 ± 18.7	**136.3 ± 17.7**	**145.0 ± 19.3***
Diastolic blood pressure (mm/Hg)	81.6 ± 9.0	**80.4 ± 8.5**	**83.1 ± 9.4***
Total cholesterol (mmol/L)	5.2 ± 1.0	5.3 ± 0.9	5.1 ± 0.9
Triglycerides (mmol/L)	1.4 [1.0; 2.0]	**1.3 [0.9; 1.8]**	**1.6 [1.1; 2.1]***
HDL-cholesterol (mmol/L)	1.2 ± 0.4	**1.3 ± 0.4**	**1.2 ± 0.3***
Glucose (mmol/L)	6.0 ± 1.3	**5.4 ± 0.5**	**5.8 ± 0.5**†
HbA1c (%) [A]	6.3 ± 0.7	**6.0 [5.8; 6.3]**	**6.6 [6.2; 6.9]**†
Type 2 diabetes (T2DM, yes)	121 (23%)	n.a.	n.a.
HOMA2IR	1.6 [1.1; 2.6]	**1.4 [1.0; 2.0]**	**2.0 [1.2; 3.6]**†
Matsuda index [B]	2.9 [1.7; 4.5]	**3.3 [2.2; 4.9]**	**2.2 [1.2; 3.5]**†
Stumvoll index [B]	0.07 [0.03; 0.09]	**0.08 [0.06; 0.10]**	**0.05 [0.00; 0.07]**†
HOMA2B%	103 [82; 127]	109 [91; 130]	113 [91; 141]
C-peptidogenic index [B]	371 [230; 562]	**458 [321; 650]**	**257 [188; 358]**†
Basal insulin secretion rate	30.2 [23.0; 41.9]	**26.6 [21.6; 37.2]**	**35.0 [26.0; 47.7]**†
Total insulin secretion	17.7 [13.7; 22.3]	**17.3 [13.7; 22.2]**	**19.9 [14.8; 25.8]***
β-cell glucose sensitivity [B]	35 [23; 50]	**41.7 [30.0; 57.3]**	**25.7 [18.9; 34.2]**†
Potentiation factor [B]	1.12 [1.02; 1.46]	1.14 [1.03; 1.58]	1.11 [1.03; 1.41]
β-cell rate sensitivity [B]	79 [00; 261]	**89 [0; 350]**	**14 [0; 190]***
eGFR	91 [79; 101]	89 [79; 99]	92 [76; 102]
Low grade inflammation (SD) [C]	0.00 ± 1.00	**-0.16 ± 1.02**	**0.08 ± 0.7***
Smoking behavior, current smoker (yes)	114 (22%)	74 (22%)	13 (22%)
Physically active (yes)	327 (63%)	214 (63%)	35 (60%)
Energy intake (10^3 kJ/d)	9.3 ± 2.8	9.5 ± 2.8	9.0 ± 2.9
Glucose-lowering medication (yes)	59 (11%)	n.a.	n.a.
Lipid-modifying medication (yes)	95 (18%)	54 (16%)	13 (22%)
Antihypertensive medication (yes)	191 (38%)	**98 (29%)**	**29 (50%)**†
Factor C3 (g/L)	1.02 ± 0.16	**0.98 ± 0.15**	**1.07 ± 0.14**†
Factor D (ng/ml)	1.00 ± 0.24	1.00 ± 0.24	1.05 ± 0.24
MASP-3 (µg/ml)	7.10 ± 2.31	6.83± 2.26	7.12 ± 1.86
Factor C3a (ng/ml)	59 [50; 72]	**58.6 [49.0; 70.3]**	**64.5 [56.0; 77.0]***
Factor Bb (µg/ml)	0.72 ± 0.19	0.73 ± 0.19	0.74 ± 0.20
Properdin (µg/ml)	6.04± 1.28	5.96 ± 1.31	6.15 ± 1.05
Factor H (mg/L)	326 ± 79	313 ± 74	330 ± 66

* P<0.05, †P<0.001 in the comparison of baseline characteristic of those who did vs. did not develop T2DM (t-test for mean ±SD, Mann-Whitney U test for Median [IQR], Chi^2 for percentages). [A] available for n=442 in all participants at baseline, n=291 in those who did not progress to T2DM, and n=58 in those who progressed to T2DM. [B] available for n=476-486 in all participants at baseline, n=330-338 in those who did not progress to T2DM, and n=58 in those who progressed to T2DM. [C] the combined score of CRP, IL6, IL8, TNF-α,SAA, ICAM.

Table 2. Age- and sex-adjusted ross-sectional associations between complement factors and several characteristics of diabetes at baseline

	n	C3 β [95% CI]	Factor D β [95% CI]	MASP3. β [95% CI]	C3a β [95% CI]	Bb β [95% CI]	Properdin β [95% CI]	Factor H β [95% CI]
BMI (kg/m^2)	517	**0.11 [0.09; 0.12]**	**0.05 [0.03; 0.07]**	**0.03 [0.01; 0.05]**	**0.04 [0.02; 0.06]**	-0.01 [-0.03; 0.01]	**0.04 [0.02; 0.06]**	**0.08 [0.06; 0.10]**
Waist (cm)	518	**0.04 [0.04; 0.05]**	**0.02 [0.01; 0.02]**	**0.01 [0.01; 0.02]**	**0.02 [0.01; 0.02]**	-0.01[-0.01; 0.00]	**0.02 [0.01; 0.02]**	**0.03 [0.03; 0.04]**
Inflammation (SD) [A]	518	**0.46 [0.38; 0.54]**	**0.25 [0.16; 0.33]**	0.09 [-0.00; 0.18]	**0.31[0.23; 0.39]**	**0.22 [0.13; 0.31]**	**0.15 [0.06; 0.24]**	**0.34 [0.26; 0.43]**

All complement factors were standardized, C3a was also ln-transformed. [A] a combined score of CRP, IL6, IL8, TNF-α, SAA, ICAM. As an example for interpretation, 1 SD higher concentration of plasma C3 was associated with 0.11 kg/m^2 greater BMI, 0.04 cm larger waist and 0.46 SD greater low-grade inflammation, while controlling for age and sex.

Except for Bb, all alternative pathway components were significantly associated with BMI and waist with the strongest association for C3 and in descending order FH, FD, properdin, C3a, and MASP-3 (**Table 2**). Moreover, all were associated with inflammation with the strongest association again for C3 and then in descending order FH, C3a, FD, Bb, properdin and MASP-3 (borderline significant for MASP-3).

3.2 Longitudinal associations of the alternative complement pathway with insulin resistance and β-cell function

Next, we evaluated the overall association of the baseline concentrations of the components of the alternative pathway with insulin resistance and markers of β-cell function, over the 7-year follow-up period. Of the seven complement components included in the analyses, six were significantly associated with worse insulin resistance, as reflected by a lower Matsuda index, only Bb was associated with less insulin resistance (**Table 3, column 1, model 1**). After full adjustments, the association for C3, MASP-3, properdin, and FH remained significant, for C3a and FD it was no longer significant, and factor Bb remained associated with less insulin resistance, although this latter association did not reach a FDR q-value < 0.05 (**Table 3, column 1, model 4**). Positive age- and sex-adjusted associations were observed for C3, FD, MASP-3, C3a, properdin, and FH with basal and overall insulin secretion (**Table 3, column 2, model 1**). After adjustments for potential confounders, C3, MASP-3, properdin, and FH remained significant, the association with FD and C3a was attenuated and became non-significant, while the Bb was associated with a lower overall insulin secretion rate (**Table 3, column 2, model 3**) When additionally adjusted for the Matsuda index, most complement components were not associated with the insulin secretion rate, except for FD and FH, which became significantly, although only weakly, associated with the basal insulin secretion (**Table 3, column 2 and 3, model 4**). C3, MASP-3, and FH were inversely and significantly associated with the C-peptidogenic index and β-cell glucose sensitivity, but only in the age and sex adjusted analyses (**Table 3, column 3 and 4, model 1**), while C3, properdin and FH were inversely and significantly associated with the β-cell potentiation factor (**Table 3, column 5, model 1**). None of these associations

remained significant after adjustments for potential confounders, diabetes characteristics and insulin resistance (**Table 3, column 3-5, model 2-4**). FD was consistently inversely associated with β-cell rate sensitivity, a marker of the first phase insulin response, while C3 was positively associated with β-cell rate sensitivity but not after adjustment for the Matsuda index. Upon adjustments for potential confounders, diabetes characteristics and the Matsuda index, the inverse association of FD with β-cell rate sensitivity remained significant at the nominal P-value, but did not meet the FDR q-value of <0.05 (**Table 3, column 6, models 1-4**).

For most of these associations, no interaction with sex was observed in the full model (model 3, not adjusted for insulin resistance). For a few associations (i.e. C3 with basal insulin secretion rate and with C-peptidogenic index; properdin with C-peptidogenic index and with the β-cell potentiation factor; MASP-3 with the β-cell potentiation factor) the interaction terms (complement*sex) were significant ($p<0.05$). When these analyses were repeated in men and women separately, the association of C3 with the basal secretion rate was slightly stronger in men but positive and significant in both sexes (men 0.30 [0.21; 0.40], women (0.25 [0.15; 0.34], both $p<0.001$), the associations of C3 and properdin with C-peptidogenic index were positive for men and inverse for women, while associations of properdin and MASP-3 with the β-cell potentiation factor were inverse for men and positive for women. However, these were significant only for C3 with the C-peptidogenic index and for properdin with the β-cell potentiation factor in men, and disappeared when the Matsuda index was added to the model.

Table 3. Longitudinal associations of baseline plasma complement levels with insulin resistance and indices of β-cell function (baseline and follow-up) over a 7-year period

		Matsuda index	Basal insulin secretion rate	Overall insulin secretion rate	C-peptidogenic index	β-cell glucose sensitivity	β-cell potentiation	β-cell rate sensitivity, median
		β [95% CI]	β [95% CI]	β [95% CI]	β [95% CI]	β [95% CI]	β [95% CI]	OR [95% CI]
C3	**M1**	**-0.56 [-0.63; -0.50]** [A]	**0.53 [0.46; 0.59]** [A]	**0.33 [0.25; 0.42]** [A]	**-0.15 [-0.23; -0.07]** [A]	**-0.19[-0.32; -0.06]** [A]	**-0.16 [-0.24; -0.07]** [A]	1.03 [0.88; 1.21]
	M2	**-0.34 [-0.41; -0.27]** [A]	**0.31 [0.24; 0.38]** [A]	**0.26 [0.17; 0.35]** [A]	-0.08 [-0.17; 0.02]	-0.13 [-0.30; 0.04]	-0.08 [-0.19; 0.02]	1.09 [0.90; 1.32]
	M3	**-0.31 [-0.38; -0.23]** [A]	**0.27 [0.20; 0.34]** [A]	**0.30 [0.21; 0.38]** [A]	0.04 [-0.05; 0.12]	-0.03 [-0.17; 0.12]	-0.06 [-0.17; 0.04]	**1.23 [1.01; 1.50]**
	M4	**-0.33 [-0.40; -0.25]** [A]	0.05 [0.00; 0.10]	0.05 [0.02; 0.11]	0.04 [-0.04; 0.13]	-0.04 [-0.20; 0.12]	-0.01 [-0.12; 0.10]	1.15 [0.93; 1.42]
Factor D	**M1**	**-0.11 [-0.19; -0.03]** [A]	**0.19 [0.11; 0.28]** [A]	**0.13 [0.05; 0.21]** [A]	0.01 [-0.07; 0.09]	0.06 [-0.02; 0.13]	-0.01 [-0.08; 0.07]	**0.83 [0.71; 0.98]**
	M2	0.02 [-0.05; 0.09]	0.04 [-0.04; 0.11]	0.01 [-0.08; 0.09]	-0.01 [-0.10; 0.08]	0.01 [-0.07; 0.09]	0.04 [-0.40; 0.11]	**0.83 [0.70; 1.00]**
	M3	0.01 [-0.06; 0.08]	0.05 [-0.03; 0.12]	0.01 [-0.08; 0.09]	-0.03 [-0.10; 0.04]	-0.01 [-0.08; 0.06]	0.03 [-0.04; 0.10]	**0.81 [0.66; 0.99]**
	M4	0.01 [-0.06; 0.08]	**0.05 [0.01; 010]**	-0.01 [-0.05; 0.06]	-0.03 [-0.10; 0.04]	-0.01 [-0.08; 0.06]	0.03 [-0.05; 0.10]	**0.81 [0.66; 0.98]**
MASP3	**M1**	**-0.24 [-.0.35; -0.12]** [A]	**0.21 [0.11; 0.31]** [A]	**0.10 [0.20; 0.19]** [A]	**-0.08 [-0.15; -0.01]**	**-0.10 [-0.19; -0.00]**	-0.07 [-0.14; 0.01]	0.98 [0.86; 0.12]
	M2	**-0.16 [-0.23; -0.09]** [A]	**0.14 [0.07; 0.21]** [A]	**0.07 [0.00; 0.14]**	-0.06 [-0.13; 0.01]	-0.08 [-0.17; 0.01]	-0.04 [-0.12; 0.03]	0.98 [0.86; 1.11]
	M3	**-0.13 [-0.20; -0.06]** [A]	**0.12 [0.05; 0.18]** [A]	**0.10 [0.02; 0.17]** [A]	0.02 [-0.04; 0.09]	0.00 [-0.07; 0.07]	-0.03 [-0.10; 0.04]	1.05 [0.91; 1.21]
	M4	**-0.14 [-0.20; -0.07]** [A]	0.02 [-0.02; 0.05]	0.03 -0.03; 0.09]	0.01 [-0.06; 0.07]	-0.01 [-0.08; 0.06]	-0.01 [-0.08; 0.06]	1.02 [0.88; 1.17]
C3a	**M1**	**-0.14 [-0.23; -0.04]** [A]	**0.16 [0.07; 0.25]** [A]	**0.12 [0.03; 0.21]** [A]	-0.04 [-0.12; 0.04]	-0.01 [-0.09 ;0.06]	-0.06 [-0.14; 0.02]	0.94 [0.80; 1.11]
	M2	-0.02 [-0.09; 0.05]	0.04 [-0.03; 0.10]	0.06 [-0.03; 0.15]	-0.00 [-0.09; 0.08]	0.02 [-0.05; 0.09]	-0.02 [-0.10; 0.06]	0.96 [0.81; 1.13]
	M3	-0.02 [-0.08; 0.05]	0.04 [-0.03; 0.10]	0.04 [-0.04; 0.13]	-0.02 [-0.04; 0.09]	-0.00 [-0.06; 0.06]	-0.02 [-0.10; 0.06]	0.92 [0.78; 1.08]
	M4	-0.02 [-0.09; 0.05]	0.03 [-0.02; 0.07]	0.02 [-0.03; 0.08]	-0.03 [-0.04; 0.09]	-0.01 [-0.06; 0.05]	-0.02 [-010; 0.06]	0.92 [0.78; 1.08]
Bb	**M1**	**0.12 [0.04; 0.20]** [A]	-0.08 [-0.16; 0.00]	-0.07 [-0.15; 0.01]	0.00 [-0.08; 0.08]	0.02 [-0.05; 0.09]	0.02 [-0.06; 0.09]	0.89 [0.75; 1.04]
	M2	**0.09 [0.03; 0.15]**	**-0.07 [-0.16; -0.01]**	**-0.08 [-0.16; -0.01]**	-0.01 [-0.09; 0.07]	-0.00 [-0.07; 0.06]	0.01 [-0.06; 0.09]	0.89 [0.76; 1.05]
	M3	**0.08 [0.02; 0.15]** [A]	0.06 [-0.12; 0.03]	**-0.10 [-0.17; -0.03]** [A]	-0.05 [-0.11; 0.02]	-0.04 [-0.09; 0.01]	0.01 [-0.07; 0.08]	0.85 [0.72; 1.01]
	M4	**0.09 [0.03; 0.15]**	-0.00 [-0.04; 0.04]	-0.04 [-0.9; 0.01]	-0.05 [-0.11; 0.02]	-0.03 [-0.09; 0.02]	-0.01 [-0.08; 0.06]	0.86 [0.73; 1.03]
Properdin	**M1**	**-0.30 [-0.38; -0.22]** [A]	**0.25 [0.18; 0.33]** [A]	**0.19 [0.11; 0.27]** [A]	-0.03 [-0.12; 0.06]	-0.02 [-0.09; 0.06]	**-0.09 [-0.17; -0.02]** [A]	1.10 [0.94; 1.28]
	M2	**-0.20 [-0.26; -0.13]** [A]	**0.15 [0.09; 0.22]** [A]	**0.13 [0.06; 0.21]** [A]	-0.01 [-0.09; 0.08]	0.00 [-0.07; 0.08]	-0.06 [-0.13; 0.01]	1.11 [0.95; 1.29]
	M3	**-0.19 [-0.25; -0.13]** [A]	**0.14 [0.08; 0.21]** [A]	**0.13 [0.06; 0.20]** [A]	-0.00 [-0.07; 0.07]	0.01 [-0.05; 0.06]	-0.06 [-0.13; 0.01]	1.11 [0.94; 1.29]
	M4	**-0.20 [-0.26; -0.13]**[A]	0.00 [-0.03; 0.04]	-0.03 [-0.08; 0.03]	-0.00 [-0.07; 0.07]	-0.01 [-0.07; 0.05]	-0.03 [-0.09; 0.04]	1.06 [0.89; 1.24]
Factor H	**M1**	**-0.40 [-0.47; -0.33]** [A]	**0.39 [0.32; 0.46]** [A]	**0.39 [0.33; 0.46]** [A]	**-0.11 [-0.19; -0.03]** [A]	**-0.12 [-0.19; -0.05]** [A]	**-0.11 [-0.18; -0.05]** [A]	0.95 [0.81; 1.11]
	M2	**-0.22 [-0.28; -0.15]** [A]	**0.21 [0.14; 0.27]** [A]	**0.11 [0.03; 0.18]** [A]	-0.06 [-0.14; 0.03]	-0.07 [-0.15; 0.01]	-0.05 [-0.12; 0.02]	0.97 [0.82; 1.15]
	M3	**-0.18 [-0.25; -0.12]** [A]	**0.17 [0.11; 0.24]** [A]	**0.12 [0.05; 0.20]** [A]	0.02 [-0.04; 0.09]	0.00 [-0.05; 0.06]	-0.04 [-0.11; 0.03]	1.03 [0.87; 1.24]
	M4	**-0.19 [-0.26; -0.12]** [A]	**0.04 [0.00; 0.08]**	-0.03 [-0.08; 0.03]	0.03 [-0.04; 0.09]	-0.01 [-0.07; 0.04]	0.01 [-0.08; 0.07]	0.99 [0.83; 1.19]

All main independent variables (i.e. the complement factors) were standardized, C3a was also ln-transformed; all outcomes (i.e. measures of insulin resistance and β-cell function were ln-transformed and standardized, except for β-cell rate sensitivity (smaller of equal to vs. larger than median). N=848, max 2 observations per participant; bold is p<0.05 at the nominal p-value, [A] FDR-adjusted q-value <0.05. **M1**: adjusted for age, sex, follow-up time, time-point.**M2**: Model 1 additionally adjusted for waist, kidney function, smoking, physical activity, energy intake, and lipid-modifying and/or anti-hypertensive medication at baseline. **M3**: Model 2 additionally adjusted for diabetes-duration, use of glucose lowering medication, diabetes status at baseline (and when basal insulin secretion rate is the outcome, additionally for fasting plasma glucose concentration). **M4**: Model 3 additionally adjusted for baseline C-peptide index (column 1) or Matsuda index (columns 2-7). βs are standardized regression coefficients and represent the longitudinal associations between plasma complement levels at baseline and outcomes over the whole 7-year follow-up period. GEE-derived β coefficients can be interpreted as a combination of the between-subject effects and within-subject effects over time. Taking the association of C3 with the Matsuda index as an example, the coefficient of -0.33 may, on the one extreme, mean that in those participants who have a 1 SD greater concentration of plasma C3 concentration at baseline, the Matsuda index is on average 0.33 SD smaller during the 7-year period, potentially without any within-subject effect. At the other extreme, the coefficient may reflect that in those participants who have a 1 SD greater baseline C3, the Matsuda index decreases, on average, by 0.33 SD during the 7-year period. In reality, the coefficient represents a combination of these two scenarios, thus representing the longitudinal association between baseline C3 and the Matsuda index over the 7-year period, while controlling for the potential confounders that are mentioned in M4.

When the Matsuda index was exchanged for Stumvoll index, as an alternative measure of insulin sensitivity (**supplementary table S2**), the results remained largely consistent, with three exceptions. The association of Bb with the Stumvoll index was weaker than for the Matsuda index and was not significant after adjustment for diabetes variables and C-peptidogenic index. In addition, the Bb with a lower overall insulin secretion rate of and C3 with better β-cell rate sensitivity remained significant (at the nominal P-value) after adjustment for the Stumvoll, but not after adjustment for the Matsuda index.

When the analyses presented in table 3 were restricted to include only participants without diabetes, the results were not materially altered (**supplementary table S3**), indicating that the observed associations in the main analyses were not driven by the presence of individuals with T2DM. In line with this, we did not observe interactions with presence of T2DM (all interactions terms p>0.05).

3.3 Associations of components of the alternative complement pathway at baseline, with prevalent and incident diabetes

Next, we evaluated the associations of complement components at baseline with presence of T2DM over the follow-up period using logistic GEE. C3, MASP-3, and FH were positively and significantly associated with presence of T2DM, and these associations remained significant after adjustments for relevant potential confounders (**Table 4A, model 2**). For C3 and FH, these associations were largely explained by insulin resistance (**Table 4A, model 3a**), but not by β-cell function (**Table 4A, model 3b**). For MASP-3, insulin resistance only partly explained the association with T2DM since the OR was attenuated but remained significant (**Table 4A, model 3a**) while the OR was not changed when β-cell function was included in the model (**Table 4A, model 3b**). The ORs represent the overall associations with prevalent and incident disease because in logistic GEE analyses, all cases of T2DM (at baseline and at follow-up) are included in the outcome. To further differentiate this, we subsequently performed logistic regression analyses with incident T2DM only, by excluding participants who already had diabetes at baseline. For CODAM we previously reported that baseline C3 is associated with incident T2DM (2), and we now show that this is unique for C3, since none

of the other alternative pathway components were associated with incident T2DM (**Table 4B, model 2**). These associations did not differ between men and women. Moreover, we now also show that the association of C3 with incident T2DM (**Table 4B, model 2**) is largely explained by insulin resistance, as represented by the Matsuda index (**Table 4B, model 3a**) but not by general β-cell (dys)function, as represented by the C-peptidogenic index (**Table 4B, model 3b**). In line with this, the association of C3 with incident T2DM was also substantially attenuated when it was adjusted for other measures insulin sensitivity/resistance but not by additional adjustment for different aspects of β-cell function. When the association between C3 incident and T2DM (OR 1.58 [1.08; 2.30], **Table 4B, model 2**) was additionally adjusted for HOMA2-IR or the Stumvoll index, the OR was attenuated to 1.29 [0.85; 1.95] and 1.21 [0.80; 1.81], respectively (both not significant). When additionally adjusted for basal or total insulin secretion rate, β-cell glucose sensitivity, β-cell potentiation factor of β-cell glucose sensitivity rate, the OR of C3 for incident T2DM was 1.49 [1.00; 2.34], 1.60 [1.08; 2.38], 1.82 [1.19; 2.78], 1.60 [1.09; 2.34], and 1.65 [1.13; 2.43], respectively (all $p<0.05$).

Table 4. Associations between baseline complement factors and prevalent & incident T2DM combined (4A) and development of T2DM in those who did not have T2DM at baseline (4B) over the 7-year period

4A	**Logistic GEE: Prevalent and incident T2DM**						
	C3	**Factor D**	**MASP3**	**C3a**	**Bb**	**Properdin**	**Factor H**
	OR (95% CI)	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**
M1	**2.14 [1.73; 2.64]** [A]	1.06 [0.86; 1.31]	**1.43 [1.12; 1.84]** [A]	1.13 [0.94; 1.36]	0.91 [0.74; 1.10]	1.14 [0.93; 1.39]	**1.64 [1.33; 2.02]** [A]
M2	**1.66 [1.29; 2.13]** [A]	1.05 [0.82; 1.36]	**1.38 [1.10; 1.73]** [A]	0.95 [0.77; 1.17]	0.92 [0.74; 1.15]	1.03 [0.82; 1.28]	**1.35 [1.07; 1.71]** [A]
M3a	1.29 [0.97; 1.71]	1.07 [0.84; 1.38]	**1.25 [1.02; 1.52]**	0.93 [0.75; 1.15]	0.98 [0.78; 1.25]	0.87 [0.70; 1.09]	1.17 [0.92; 1.50]
M3b	**2.03 [1.51; 2.73]** [A]	0.92 [0.66; 1.28]	**1.39 [1.04; 1.86]**	0.93 [0.72; 1.20]	0.90 [0.71; 1.14]	1.15 [0.87; 1.51]	**1.44 [1.14; 1.83]** [A]
4B	**Logistic regression: Incident T2DM mellitus**						
	C3	**Factor D**	**MASP3**	**C3a**	**Bb**	**Properdin**	**Factor H**
	OR (95% CI)	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**
M1	**1.88 [1.37; 2.59]** [A]	1.26 [0.95; 1.66]	1.15 [0.88; 1.51]	1.23 [0.95; 1.60]	1.05 [0.80; 1.37]	1.15 [0.88; 1.50]	1.25 [0.93; 1.66]
M2	**1.58 [1.08; 2.30]**	1.17 [0.84; 1.63]	1.16 [0.87; 1.56]	1.12 [0.84; 1.50]	1.05 [0.79; 1.40]	1.06 [0.79; 1.41]	1.00 [0.73; 1.38]
M3a	1.26 [0.83; 1.90]	1.16 [0.83; 1.60]	1.06 [0.77; 1.46]	1.08 [0.79; 1.46]	1.17 [0.87; 1.58]	0.88 [0.64; 1.20]	0.84 [0.60; 1.18]
M3b	**1.91 [1.22; 3.00]** [c]	1.08 [0.73; 1.61]	1.28 [0.94; 1.74]	1.04 [0.75; 1.45]	0.91 [0.66; 1.27]	1.15 [0.82; 1.61]	1.04 [0.73; 1.48]

All main independent variables (i.e. the complement factors) were standardized, C3a was also ln-transformed. Table 4A, N=848, max 2 observations per participant; Table 4B, M1 and M2, N=397 (58 cases); M3 and M4, N=389 (58 cases); A FDR adjusted q-value <0.05. M1: age, sex (index, follow-up time). M2: Model 1 additionally adjusted for waist, kidney function (MDRD), smoking, physical activity, energy intake, use of lipid-modifying and/or anti-hypertensive medication at baseline. M3a: Model 2 additionally adjusted for Matsuda index at baseline. M3b: Model 2 additionally adjusted for C-peptide index at baseline. The interpretation of the β coefficients in table 4A is similar to the explanation provided in the legend of table 3. As an example for interpretation of the β coefficients in table 4B, 1 SD higher baseline concentration of plasma C3 in those who did not have diabetes was associated 1.58-fold higher odds to develop T2DM over the 7 year follow-up period, while controlling for confounders (model M2) and the strength of this association was substantially attenuated (i.e. explained) by the degree of insulin resistance at baseline (as represented by the Matsuda index) but not by the degree of β-cell function (as represented by the C-peptidogenic index).

4 DISCUSSION

This study presents a comprehensive evaluation of the alternative complement pathway in relation to insulin resistance, β-cell function and incident T2DM. Over the 7-year follow-up period, C3, MASP-3, properdin, and FH were positively associated with worse insulin resistance, while for factor Bb this association may be inverse, all adjusted for potential confounders and β-cell function. In contrast, after taking into account the prevailing level of insulin sensitivity/resistance and potential confounders, the alternative pathway components were not associated with most markers of β-cell function (i.e. basal and overall insulin secretion rate, C-peptidogenic index, β-cell glucose sensitivity or β-cell potentiation). As an exception, FD was associated lower with β-cell rate sensitivity, independent of insulin resistance. Notably, only C3 was significantly associated with incident T2DM. This association was driven by insulin resistance, not β-cell function.

C3, FH and MASP-3 were all positively, and independent of confounders, associated with presence of T2DM in the GEE analyses. Notably, for MASP-3 and FH these associations did not translate into a positive association with *incident* T2DM. C3 was the only complement factor that was significantly associated with incident T2DM. Engstrom et al. also showed that C3, but not C4 or other inflammatory markers, was associated with the development of T2DM, at least in men (3) although they did not yet establish a direct causal relation (39). Supported by this previous independent report (3) we conclude that the association between C3 and incident T2DM represents a solid and relevant result. In mediation analyses we further evaluated whether the path from baseline C3 towards T2DM occurs via insulin resistance, via β-cell (dys)function, or both. Measures of insulin resistance (Matsuda index, Stumvoll index or HOMA2-IR) substantially reduced the association. In contrast, the association was not affected when the mediating effects of measures of β-cell function (basal or total insulin secretion rate, β-cell glucose sensitivity, β-cell potentiation factor or β-cell glucose sensitivity rate) were evaluated. This shows that the association between C3 and incident T2DM is most likely driven by insulin resistance, and not by β-cell (dys)function.

β-cells will adapt their insulin secretion to keep up with the prevailing level of glucose which, in turn, is affected by insulin resistance. Therefore, β-cell function cannot be evaluated without taking insulin resistance into account. On the other hand, the long-term persistence of hyperglycaemia and hyperinsulinaemia in insulin resistance may actually affect β-cell function. This latter phenomenon makes insulin sensitivity/resistance a possible causal factor in the association between complement and β-cell function. Hence, the current adjustment for insulin sensitivity/resistance when β-cell function is the outcome might have resulted in (partial) overadjustment. Until now, only one study reported on the relation between C3 and insulin secretion (19) and no data are available for the other complement components. In that cross-sectional report (19), a positive association was shown for C3 with first and second phase insulin secretion, independent of insulin sensitivity, as represented by $ISI_{0\text{-}120}$ (40). Our current observations are partly in line with these data. We also observe a positive association of C3 with the basal and overall insulin secretion rates. This association disappeared after adjustment for the Matsuda index, but not after adjustment for the Stumvoll index (despite the very strong correlation between these two indices). The associations between C3 and other properties of β-cell function appear more incidental and should be interpreted with caution. These associations mostly pointed towards worse β-cell function and disappeared after adjustment for confounders. An exception is the association of C3 with better β-cell rate sensitivity after adjustment for confounders (although not at FDR $q<0.05$). Similar to what was observed for the insulin secretion rates, this association was attenuated by adjustment for the Matsuda, but not the Stumvoll index.

The associations for MASP-3, properdin, and FH were very similar to those observed for C3. This may be partly, but not fully, attributed to the mutual correlations among the plasma concentrations of these factors. The correlations of properdin and FH with C3 are quite strong ($\rho=0.392$ and 0.514 respectively) but this is not the case for MASP-3 ($\rho=0.139$) for which similar associations with insulin resistance and the insulin secretion rate were seen. The associations of MASP-3, properdin, and FH with dynamic aspects of β-cell function were mostly weak or absent. This again suggests a more pronounced relationship of the alternative pathway with insulin sensitivity/ resistance than with β-cell function

A striking observation in our data is the apparent discordance between the findings for C3 and C3a, despite their rather strong correlation (ρ=0.354) and the experimental data reported in the literature (see below). As discussed above, C3 is strongly associated with worse insulin sensitivity and also with the insulin secretion rate, although the latter may not be independent of the prevailing level of insulin resistance. C3a, on the other hand, is at best weakly associated with those measures and only when adjusted for age and sex, not after further adjustment for confounders. Current knowledge on the potential role of the alternative complement pathway on β-cell function primarily derives from animal and *in vitro* studies models. The data point towards C3a as the main effector since the receptor for C3a, C3aR1, is expressed in mouse pancreatic islets and β-cells (13, 14, 41) and was shown to enhance their glucose-induced insulin secretion. There are however, besides similarities, also clear differences between mouse and human β-cells (reviewed in (42)). For instance, the expression of C3aR1 in mouse islets was 20-fold higher than in human islets (14). Moreover, the expression of the receptor for C5a, which is generated during terminal pathway activation, was very low-to-absent in mouse islets (13, 14) and higher in human islets (14). This suggests that for human β-cell function, signaling by of C5a via its receptor on β-cells, might be more relevant than via C3a. Notably, similar effects on insulin secretion as described for C3aR1 have also been reported for the C5aR1 (14).

It was suggested that complement may serve as a metabolic feedback system to support insulin secretion in a situation of inflammation-induced insulin resistance (43). In obesity-associated insulin resistance, complement components that are produced in e.g. adipose tissue or the liver, or locally in the pancreas, may act as signals to keep β-cell function in balance with the existing level of insulin resistance. The associations of C3, properdin, and FH with low-grade inflammation as well as with worse insulin sensitivity and a higher insulin secretion rate appear to be in line with this view. At the same time, MASP-3 is also associated with insulin sensitivity and secretion, but only weakly with low-grade inflammation. Moreover, the activated products C3a and Bb are clearly associated with low-grade inflammation but not (C3a) or even beneficially (Bb) associated with insulin sensitivity. It is conceivable that C3 is produced as a systemic signal of overall inflammation-induced insulin resistance, together with properdin and FH to control alterative pathway activation,

while activation of C3 occurs locally in the islets and is therefore less reflected in the circulation. At this point we do not have a mechanistic explanation for the apparent beneficial the association of Bb with insulin resistance/sensitivity, but it has been reported before (22).

FD was proposed to be beneficial for β cell function in mouse models of diabetes and insufficient availability of FD, the rate-limiting protease for C3 activation, was suggested to be causal for obesity-associated diabetes in mice (13). Notably, the association between obesity and FD differs substantially between humans and mice. Mouse models of diabetes and obesity are often characterized by (very) low circulating concentrations of FD (13, 15). Obese humans, on the other hand, generally have high circulating levels of FD ((16-18) and current results). In our study, the sex- and age-adjusted associations of FD with worse insulin resistance and a higher secretion rate disappeared upon adjustment for additional confounders and this was largely attributable to the adjustment for obesity (data not shown). The one previous publication on FD and β-cell function suggested that higher baseline FD might protect individuals with T2DM from towards β-cell failure, as represented by initiation of insulin use (13). Our current data focus on earlier stages, i.e. the development of T2DM and show that the association of FD with less insulin sensitivity is explained by confounders (mainly adiposity), that FD is not associated with different aspects of β-cell function human, except for a weak association with worse β-cell rate sensitivity, and is not a risk factor for the development of T2DM.

The main strengths of our study are the comprehensive evaluation of the alternative complement pathway, including activation products and regulators, the availability of longitudinal information, and the availability of OGTT data that allowed us to address several aspects of β-cell function. This study also has several limitations. The fact that we used OGTT data, which include incretin effects, for estimation of β-cell function, instead of e.g. a hyperglycaemic clamp may be regarded a limitation. Other limitations include the relatively small size of the cohort which may have limited the power to detect associations and interactions, particularly if the effect sizes were modest and given the rigorous adjustment for potential confounders, the fact that the complement measurements were done in the circulation, the observational character of our data, which hampers causal

inference, and the fact that our cohort is characterized by an increased risk of cardiometabolic diseases, which may limit the generalizability of our observations.

Taken together, we herein showed that most factors of the alternative complement pathway were longitudinally associated with more insulin resistance. Moreover, higher baseline concentrations of complement C3, but none of the other alternative pathway components, were associated with incident T2DM, and this was mediated by insulin resistance, rather than β-cell function. Thus, our current human observational data suggest that the resultant of activation of the alternative pathway is, at the whole body, more insulin resistance, rather than better β-cell function. Moreover, it remains to be determined in human studies whether manipulation of the terminal, rather than the alternative complement pathway may have better potential for β cell preservation in humans.

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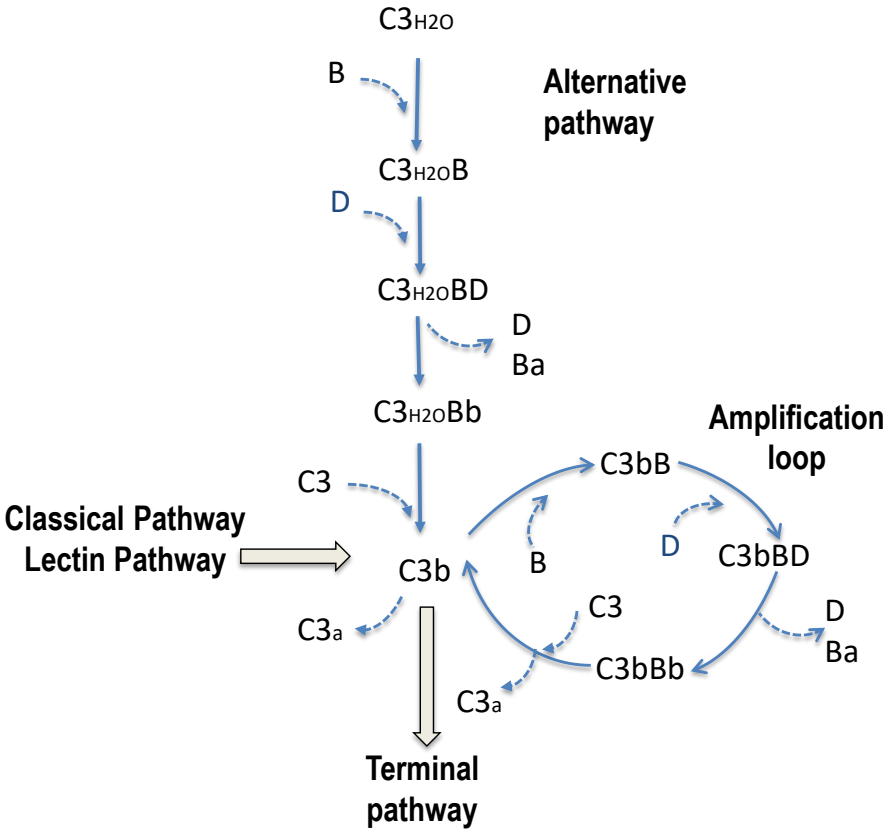
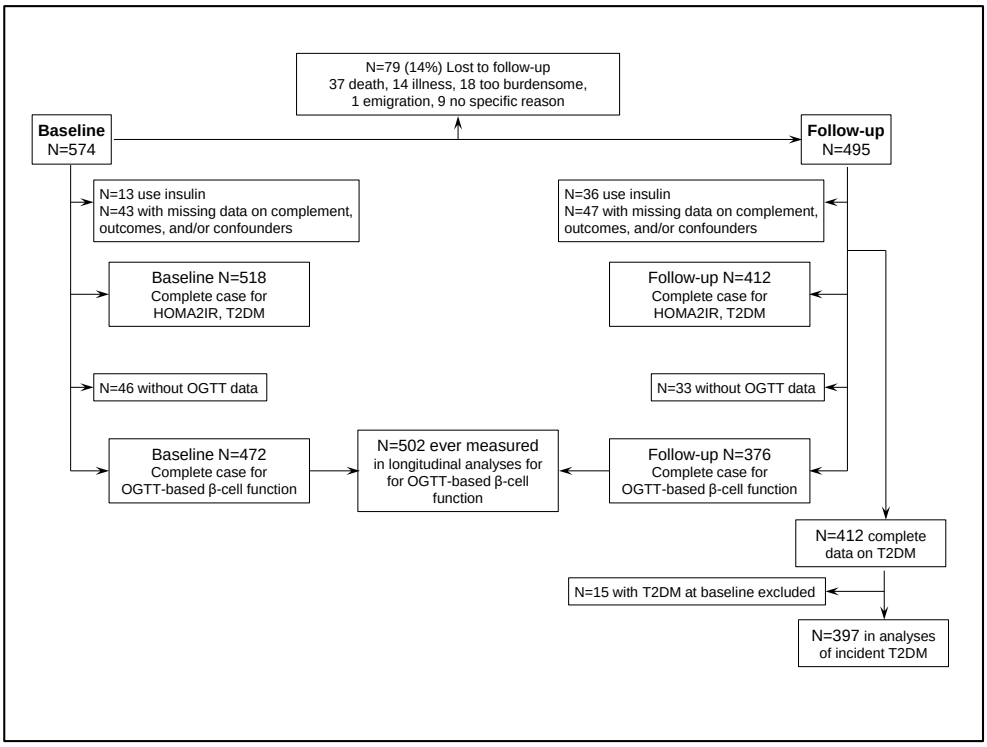
Supplementary data



Supplemental Figure 1: The alternative pathway and amplification loop. The classical, the lectin and the alternative pathway all converge on C3. Complement activation via any of these pathways activates the amplification loop, thereby generating the alternative pathway convertase which activates the common downstream terminal pathway. Activated C3 binds Factor B (B), and the complex of activated C3 and B binds factor D (FD). FD is a constitutively active protease and cleaves B in Ba and Bb. This complex of activated C3 and Bb functions itself as C3 convertase and activates further C3 molecules by cleaving C3 in C3b and C3a.MASP-3 can activate factor D. Properdin stabilizes and FH destabilizes the C3 convertases ($C3H_2OBb$/ C3bBb) (see references 21 and 44 in the main text).



Supplemental Figure 2: Flow-chart of study participants. Of the 574 individuals who were included in the original CODAM cohort, 495 participated in the 7 year follow-up evaluation. For baseline cross-sectional analyses there was complete information on the main variables and potential confounders in N=518. At baseline there was complete information on the main OGTT variables and potential confounders for N=472, at follow up this was N=376. Among these, there were 502 unique individuals and in total there were N=848 measurements available with a maximum of 2 measurements per participant. In addition, at follow up complete information on the main variables and potential confounders was available in 412 participants. After exclusion of 15 participants who already had T2DM at baseline, 397 were available for the logistic regression for incident T2DM.

Table S1A. Cross-sectional associations among the main independent variables used in the analyses

	C3	FD	MASP-3	C3a	Bb	Properdin	FH
C3	1	**0.101 ***	**0.139***	**0.354 †**	-0.039	**0.392 †**	**0.514 †**
FD		1	**0.112***	0.079	**0.201 †**	**0.120***	**0.104 ***
MASP-3			1	0.055	-0.012	**0.165 †**	**0.090 ***
C3a				1	**0.232 †**	-0.024	**0.324 †**
Bb					1	**-0.138 ***	**0.132***
Properdin						1	**0.249 †**
FH							1

Spearman correlations, N=518, * p<0.05, †P<0.001

Table S1B. Cross-sectional associations among the dependent variables used in the analyses

	HOMA2-IR	Matsuda index	Stumvoll index	Basal insulin Secretion rate	Total insulin secretion rate	C-peptidogenic index	β-cell glucose sensitivity	β-cell potentiation	β-cell rate sensitivity
	1	**-0.938 †**	**-0.798 †**	**0.888 †**	**0.552 †**	**-0.116 ***	**-0.124 ***	**-0.251**	0.067
Matsuda index		1	**0.871 †**	**-0.880 †**	**-0.679 †**	**0.183 †**	**0.130 ***	**0.253 †**	-0.020
Stumvoll index			1	**-0.743 †**	**-0.583 †**	**0.335 †**	**0.292 †**	**0.333 †**	0.051
Basal insulin secretion rate				1	**0.664 †**	**-0.091 ***	-0.086	**-0.207 †**	0.071
Total insulin secretion rate					1	**0.148 ***	**0.290 †**	**-0.117 ***	-0.006
C-peptidogenic index						1	**0.781 †**	0.050	**0.423 †**
β-cell gluc. sensitivity							1	0.042	-0.066
β-cell potentiation								1	-0.027
β-cell rate sensitivity									1

Spearman correlations, N=472-477, * p<0.05, †P<0.001

Table S2. Longitudinal associations of baseline plasma complement levels with insulin resistance and indices of β-cell function (baseline and follow-up) over a 7-year period

		Stumvoll index β [95% CI]	Basal insulin secretion rate β [95% CI]	Overall insulin secretion rate β [95% CI]	C-peptidogenic index β [95% CI]	β-cell glucose sensitivity β [95% CI]	β-cell potentiation β [95% CI]	β-cell rate sensitivity, median OR [95% CI]
C3	**M1**	**-0.52 [-0.59; -0.45]**	**0.53 [0.46; 0.59]**	**0.33 [0.25; 0.42]**	**-0.15 [-0.23; -0.07]**	**-0.19 [-0.32; -0.06]**	**-0.16 [-0.24; -0.07]**	1.03 [0.88; 1.21]
	M2	**-0.33 [-0.41; -0.26]**	**0.31 [0.24; 0.38]**	**0.26 [0.17; 0.35]**	-0.08 [-0.17; 0.02]	-0.13 [-0.30; 0.04]	-0.08 [-0.19; 0.02]	1.09 [0.90; 1.32]
	M3	**-0.27 [-0.35; -0.20]**	**0.27 [0.20; 0.34]**	**0.30 [0.21; 0.38]**	0.04 [-0.05; 0.12]	-0.03 [-0.17; 0.12]	-0.06 [-0.17; 0.04]	**1.23 [1.01; 1.50]**
	M4	**-0.29 [-0.36; -0.21]**	**0.14 [0.08; 0.22]**	**0.16 [0.08; 0.24]**	0.07 [-0.02; 0.15]	-0.02 [-0.17; 0.14]	-0.00 [-0.11; 0.11]	**1.25 [1.02; 1.55]**
Factor D	**M1**	**-0.10 [-0.18; -0.10]**	**0.19 [0.11; 0.28]**	**0.13 [0.05; 0.21]**	0.01 [-0.07; 0.09]	0.06 [-0.02; 0.13]	-0.01 [-0.08; 0.07]	**0.83 [0.71; 0.98]**
	M2	0.00 [-0.09; 0.08]	0.04 [-0.04; 0.11]	0.01 [-0.08; 0.09]	-0.01 [-0.10; 0.08]	0.01 [-0.07; 0.09]	0.04 [-0.40; 0.11]	**0.83 [0.70; 1.00]**
	M3	-0.02 [-0.10; 0.06]	0.05 [-0.03; 0.12]	0.01 [-0.08; 0.09]	-0.03 [-0.10; 0.04]	-0.01 [-0.08; 0.06]	0.03 [-0.04; 0.10]	**0.81 [0.66; 0.99]**
	M4	-0.02 [-0.10; 0.06]	0.04 [-0.02; 010]	-0.01 [-0.08; 0.06]	-0.03 [-0.10; 0.05]	-0.01 [-0.08; 0.06]	0.03 [-0.04; 0.11]	**0.81 [0.66; 0.98]**
MASP3	**M1**	**-0.24 [-0.36; -0.13]**	**0.21 [0.11; 0.31]**	**0.10 [0.20; 0.19]**	**-0.08 [-0.15; -0.01]**	**-0.10 [-0.19; -0.00]**	-0.07 [-0.14; 0.01]	0.98 [0.86; 0.12]
	M2	**-0.25 [-0.33; -0.17]**	**0.14 [0.07; 0.21]**	**0.07 [0.00; 0.14]**	-0.06 [-0.13; 0.01]	-0.08 [-0.17; 0.01]	-0.04 [-0.12; 0.03]	0.98 [0.86; 1.11]
	M3	**-0.13 [-0.20; -0.07]**	**0.12 [0.05; 0.18]**	**0.10 [0.02; 0.17]**	0.02 [-0.04; 0.09]	0.00 [-0.07; 0.07]	-0.03 [-0.10; 0.04]	1.05 [0.91; 1.21]
	M4	**-0.13 [-0.20; -0.06]**	0.05 [-0.00; 0.10]	0.03 -0.03; 0.09]	0.04 [-0.03; 0.10]	0.01 [-0.07; 0.08]	-0.00 [-0.07; 0.07]	1.05 [0.91; 1.22]
C3a	**M1**	**-0.13 [-0.22; -0.05]**	**0.16 [0.07; 0.25]**	0.12 [0.03; 0.21]	-0.04 [-0.12; 0.04]	-0.01 [-0.09 ;0.06]	-0.06 [-0.14; 0.02]	0.94 [0.80; 1.11]
	M2	-0.03 [-0.10; 0.04]	0.04 [-0.03; 0.10]	0.06 [-0.03; 0.15]	-0.00 [-0.09; 0.08]	0.02 [-0.05; 0.09]	-0.02 [-0.10; 0.06]	0.96 [0.81; 1.13]
	M3	-0.03 [-0.10; 0.04]	0.04 [-0.03; 0.10]	0.04 [-0.04; 0.13]	-0.02 [-0.04; 0.09]	-0.00 [-0.06; 0.06]	-0.02 [-0.10; 0.06]	0.92 [0.78; 1.08]
	M4	-0.04 [-0.11; 0.03]	0.02 [-0.03; 0.08]	0.02 [-0.05; 0.09]	-0.02 [-0.09; 0.05]	-0.02 [-0.09; 0.06]	-0.02 [-010; 0.06]	0.92 [0.79; 1.08]
Bb	**M1**	**0.09 [0.01; 0.17]**	-0.08 [-0.16; 0.00]	-0.07 [-0.15; 0.01]	0.00 [-0.08; 0.08]	0.02 [-0.05; 0.09]	0.02 [-0.06; 0.09]	0.89 [0.75; 1.04]
	M2	**0.07 [0.01; 0.14]**	**-0.07 [-0.16; -0.01]**	**-0.08 [-0.16; -0.01]**	-0.01 [-0.09; 0.07]	-0.00 [-0.07; 0.06]	0.01 [-0.06; 0.09]	0.89 [0.76; 1.05]
	M3	0.06 [-0.01; 0.12]	0.06 [-0.12; 0.03]	**-0.10 [-0.17; -0.03]**	-0.05 [-0.11; 0.02]	-0.04 [-0.09; 0.01]	0.01 [-0.07; 0.08]	0.85 [0.72; 1.01]
	M4	-0.02 [-0.10; 0.07]	-0.03 [-0.08; 0.02]	**-0.08 [-0.13; -0.02]**	-0.05 [-0.12; 0.01]	-0.01 [-0.08; 0.07]	-0.01 [-0.08; 0.06]	0.85 [0.72; 1.01]
Properdin	**M1**	**-0.25 [-0.35; -0.15]**	**0.25 [0.18; 0.33]**	**0.19 [0.11; 0.27]**	-0.03 [-0.12; 0.06]	-0.02 [-0.09; 0.06]	**-0.09 [-0.17; -0.02]**	1.10 [0.94; 1.28]
	M2	**-0.17 [-0.25; -0.08]**	**0.15 [0.09; 0.22]**	**0.13 [0.06; 0.21]**	-0.01 [-0.09; 0.08]	0.00 [-0.07; 0.08]	-0.06 [-0.13; 0.01]	1.11 [0.95; 1.29]
	M3	**-0.16 [-0.24; -0.08]**	**0.14 [0.08; 0.21]**	**0.13 [0.06; 0.20]**	-0.00 [-0.07; 0.07]	0.01 [-0.05; 0.06]	-0.06 [-0.13; 0.01]	1.11 [0.94; 1.29]
	M4	**-0.16 [-0.24; -0.08]**	**0.07 [0.02; 0.12]**	0.05 [-0.02; 0.11]	0.01 [-0.06; 0.08]	0.01 [-0.04; 0.07]	-0.02 [-0.09; 0.05]	1.11 [0.95; 1.30]
Factor H	**M1**	**-0.39 [-0.47; -0.31]**	**0.39 [0.32; 0.46]**	**0.39 [0.33; 0.46]**	**-0.11 [-0.19; -0.03]**	**-0.11 [-0.18; -0.05]**	**-0.11 [-0.18; -0.05]**	0.95 [0.81; 1.11]
	M2	**-0.23 [-0.31; -0.16]**	**0.21 [0.14; 0.27]**	**0.11 [0.03; 0.18]**	-0.06 [-0.14; 0.03]	-0.07 [-0.15; 0.01]	-0.05 [-0.12; 0.02]	0.97 [0.82; 1.15]
	M3	**-0.18 [-0.25; -0.11]**	**0.17 [0.11; 0.24]**	**0.12 [0.05; 0.20]**	0.02 [-0.04; 0.09]	0.00 [-0.05; 0.06]	-0.04 [-0.11; 0.03]	1.03 [0.87; 1.24]
	M4	**-0.18 [-0.25; -0.11]**	**0.09 [0.03; 0.14]**	0.03 [-0.04; 0.10]	0.04 [-0.03; 0.11]	0.01 [-0.05; 0.07]	0.00 [-0.07; 0.07]	1.04 [0.87; 1.24]

All main independent variables (i.e. the complement factors) were standardized, C3a was also ln-transformed; measures β-cell function were ln-transformed and standardized, except for β-cell rate sensitivity (smaller of equal to vs. larger than the median). N=848, max 2 observations per participant. **M1**: adjusted for age, sex, follow-up time, time-point. **M2**: Model 1 additionally adjusted for waist, kidney function, smoking, physical activity, energy intake, and lipid-modifying and/or anti-hypertensive medication at baseline. **M3**: Model 2 additionally adjusted for diabetes-duration, use of glucose lowering medication, diabetes status at baseline (and when basal insulin secretion rate is the outcome, additionally for fasting plasma glucose concentration). **M4**: Model 3 additionally adjusted for baseline C-peptide index (column 1) or Stumvoll index (columns 2-7).

Table S3: Longitudinal associations of baseline plasma complement levels with insulin resistance and indices of β-cell function (baseline and follow-up) over a 7-year period (non-diabetic participants only)

		Matsuda index β [95% CI]	Basal insulin secretion rate β [95% CI]	Overall insulin secretion rate β [95% CI]	C-peptidogenic index β [95% CI]	β-cell glucose sensitivity β [95% CI]	β-cell potentiation β [95% CI]	β-cell rate sensitivity, median OR [95% CI]
C3	**M1**	**-0.59 [-0.63; -0.48]**	**0.53 [0.45; 0.61]**	**0.44 [034; 0.53]**	-0.02 [-0.10; 0.06]	0.00 [-0.06; 0.06]	**-0.18 [-0.27; -0.08]**	1.17 [0.97; 1.41]
	M2	**-0.34 [-0.43; -0.26]**	**0.29 [0.21; 0.38]**	**0.29 [0.18; 0.39]**	0.00 [-0.09; 0.09]	0.01 [-0.06; 0.09]	**-0.12 [-0.23; -0.01]**	1.15 [0.92; 1.45]
	M3	**-0.35 [-0.44; -0.26]**	0.05 [-0.01; 0.11]	0.01 [-0.07; 0.08]	0.00 [-0.10; 0.10]	-0.02 [-0.10; 0.06]	-0.05 [-0.17; 0.07]	1.05 [0.82; 1.35]
Factor D	**M1**	**-0.13 [-0.22; -0.04]**	**0.22 [0.12; 0.31]**	**0.15 [0.04; 0.25**	-0.01 [-0.08; 0.06]	0.05 [-0.01; 0.11]	-0.01 [-0.10; 0.08]	**0.81 [0.66; 0.98]**
	M2	0.01 [-0.06; 0.09]	0.06 [-0.02; 0.14]	0.01 [-0.08; 0.10]	-0.03 [-0.11; 0.05]	0.01 [-0.06; 0.08]	0.01 [-0.08; 0.10]	**0.80 [0.65; 0.99]**
	M3	0.01 [-0.06; 0.09]	**0.06 [0.00; 0.11]**	0.00 [-0.06; 0.07].	-0.03 [-0.11; 0.05]	0.01 [-0.06; 0.08]	0.01 [-0.08; 0.10]	**0.81 [0.65; 0.99]**
MASP3	**M1**	**-0.14 [-0.26; -0.03]**	**0.12 [0.13; 0.23]**	0.10 [-0.01; 0.21]	0.01 [-0.06; 0.08]	0.03 [-0.04; 0.09]	-0.04 [-0.12; 0.05]	1.03 [0.89; 1.19]
	M2	**-0.11 [-0.19; -0.04]**	**0.09 [0.02; 0.17]**	0.07 [-0.02; 0.16]	0.01 [-0.06; 0.08]	0.02 [-0.04; 0.08]	-0.03 [-0.12; 0.05]	1.02 [0.88; 1.18]
	M3	**-0.11 [-0.19; -0.04]**	0.00 [-0.04; 0.05]	-0.02 [-0.07; 0.04]	0.01 [-0.07; 0.08]	0.01 [-0.02; 0.14]	-0.01 [-0.08; 0.04]	0.99 [0.86; 1.15]
C3a	**M1**	**-0.12 [-0.22; -0.02]**	**0.15 [0.05; 0.24]**	**0.12 [0.01; 0.22]**	-0.05 [-0.13; 0.02]	-0.02 [-0.08 ;0.03]	-0.07 [-0.16; 0.02]	0.95 [0.80; 1.13]
	M2	-0.03 [-0.10; 0.05]	0.05 [-0.02; 0.12]	0.05 [-0.04; 0.14]	-0.04 [-0.12; 0.03]	-0.02 [-0.08; 0.04]	-0.04 [-0.13; 0.05]	0.94 [0.79; 1.12]
	M3	-0.03 [-0.10; 0.04]	0.03 [-0.02; 0.07]	0.02 [-0.03; 0.08]	-0.04 [-0.12; 0.03]	-0.03 [-0.08; 0.03]	-0.04 [-0.12; 0.06]	0.93 [0.78; 1.11]
Bb	**M1**	**0.12 [0.04; 0.21]**	**-0.09 [-0.18; -0.01]**	**-0.11 [-0.19; 0.02]**	-0.05 [-0.12; 0.02]	-0.02 [-0.08; 0.03]	0.01 [-0.07; 0.10]	**0.83 [0.69; 0.99]**
	M2	**0.11 [0.05; 0.18]**	**-0.09 [-0.16; -0.03]**	**-0.12 [-0.20; -0.04]**	-0.06 [-0.13; 0.01]	-0.04 [-0.09; 0.02]	0.01 [-0.08; 0.10]	0.84 [0.70; 1.00]
	M3	**0.12 [0.05; 0.18]**	-0.02 [-0.07; 0.03]	-0.02 [-0.09; 0.02]	-0.06 [-0.13; 0.01]	-0.03 [-0.09; 0.02]	-0.02 [-0.11; 0.07]	0.86 [0.72; 1.04]
Properdin	**M1**	**-0.28 [-0.36; -0.19]**	**0.24 [0.16; 0.33]**	**0.18 [0.08; 0.27]**	-0.00 [-0.08; 0.07]	0.00 [-0.05; 0.06]	**-0.10 [-0.18; -0.02]**	1.15 [0.96; 1.37]
	M2	**-0.19 [-0.26; -0.12]**	**0.15 [0.08; 0.22]**	**0.12 [0.04; 0.20]**	0.00 [-0.07; 0.08]	0.00 [-0.05; 0.06]	-0.08 [-0.16; 0.01]	1.13 [0.95; 1.35]
	M3	**-0.19 [-0.26; -0.12]**	0.01 [-0.03; 0.05]	-0.05 [-0.11; 0.10]	0.00 [-0.08; 0.08]	-0.01 [-0.07; 0.05]	-0.04 [-0.12; 0.04]	1.08 [0.90; 1.30]
Factor H	**M1**	**-0.34 [-0.43; -0.26]**	**0.35 [0.27; 0.44]**	**0.35 [0.27; 0.43]**	-0.01 [-0.08; 0.06]	-0.01 [-0.07; 0.05]	**-0.10 [-0.18; -0.01]**	1.04 [0.87; 1.25]
	M2	**-0.17 [-0.25; -0.10]**	**0.17 [0.09; 0.25]**	0.09 [-0.00; 0.18]	0.00 [-0.07; 0.08]	-0.01 [-0.08; 0.05]	-0.05 [-0.13; 0.04]	1.02 [0.84; 1.23]
	M3	**-0.17 [-0.25; -0.09]**	0.05 [0.00; 0.09]	-0.04 [-0.12; 0.01]	0.00 [-0.07; 0.08]	-0.03 [-0.09; 0.03]	0.01 [-0.09; 0.08]	0.97 [0.80; 1.18]

All main independent variables (i.e. the complement factors) were standardized, C3a was also ln-transformed; all outcomes (i.e. measures of insulin resistance and β-cell function were ln-transformed and standardized, except for β-cell rate sensitivity (smaller of equal to vs. larger than median. N=647, max 2 observations per participant. **M1**: adjusted for age, sex, follow-up time, time-point. **M2**: Model 1 additionally adjusted for waist, kidney function, smoking, physical activity, energy intake, and lipid-modifying and/or anti-hypertensive medication at baseline (when insulin secretion rate is the outcomes also for fasting plasma glucose). **M3**: Model 2 additionally adjusted for baseline C-peptide index (column 1) or Matsuda index (columns 2-7).

Chapter 6

The role of dicarbonyl stress in complement activation: The CODAM study

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Submitted

Abstract

Objective

Glycation of complement regulatory proteins can influence their capacity to control complement activation. Reactive α-dicarbonyl compounds are major precursors in the formation of advanced glycation end products (AGEs) and may lead to glycation of circulating and/or cell-associated complement regulators. We investigated, in a human cohort, whether greater dicarbonyl stress was associated with more complement activation.

Methods

Circulating concentrations of dicarbonyl stress markers, i.e. α-dicarbonyls (methylglyoxal [MGO], glyoxal [GO], and 3-deoxyglucosone [3-DG]), and the free AGEs, (N^{ε}-(carboxymethyl)lysine [CML], N^{ε}-(carboxyethyl)lysine [CEL], and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine [MG-H1]), and protein-bound AGEs (CML, CEL, pentosidine), as well as the complement activation products C3a and sC5b-9, were measured in 530 participants (59.5 ± 7.0 years, 61% men) of the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) study. Multiple linear regression analyses were used to investigate the associations between dicarbonyl stress and complement activation with adjustment of potential confounders, including age, sex, and factors related to obesity, lifestyle, and use of medication. In addition, the associations of two functional polymorphisms of *glyoxalase 1* (*GLO1*), the rate-limiting detoxifying enzyme for MGO, with C3a and sC5b-9 were evaluated.

Results

Plasma concentration of protein-bound CEL was inversely associated with C3a (standardized β: –0.17, 95%CI –0.25; –0.08). GO was inversely associated with sC5b-9 (–0.12 [–0.21; –0.02]), while protein-bound pentosidine was positively associated with sC5b-9 (0.15 [0.05; 024]). No associations were observed for other α-dicarbonyls and AGEs with C3a or sC5b-9. The two *GLO1* polymorphisms were not associated with the plasma concentration of C3a. *GLO1 rs2736654* was also not associated with sC5b-9 concentration.

Individuals with the *AG* and *AA* genotype of *rs1049346* had, on average, 0.32 SD and 0.40 SD lower plasma concentrations of sC5b-9 than those with the *GG* genotype.

Conclusions

Plasma concentrations of dicarbonyl stress markers showed distinct associations with complement C3a and sC5b-9. This suggests different biological effects of individual plasma α-dicarbonyls and AGEs on complement activation.

1 Introduction

Chronic hyperglycaemia, as is present in diabetes, can induce dicarbonyl stress, which is characterised by increased presence of reactive α-dicarbonyl compounds, and advanced glycation end products (AGEs) (1, 2). Prolonged exposure of proteins to these glycating agents, may change their normal function and/or their susceptibility to enzymatic degradation via glycation (3). The complement system is a >40 member protein network that consists of circulating and membrane bound protein and can be activated in a stepwise manner, similar to what is seen in the coagulation system. The complement is part of the innate immune system and has been implicated in the development of type 2 diabetes mellitus (T2DM) (4, 5) and cardiovascular disease ((6), as reviewed in (7)). Interestingly, it has been reported that the glycation of complement regulators may affect complement activation (8-11).

The complement system has three main activation pathways (classical, lectin, and alternative pathway, see **Figure 1**). Activation of any of these pathways will generate a C3 convertase that can cleave C3, the central component of the complement cascade, into C3b and the anaphylatoxin C3a (12). C3b induces subsequent activation of the common terminal complement pathway and the formation of C5b-9, also known as the membrane attack complex (MAC). The alternative pathway of complement activation is continuously activated at a low level in plasma via the so-called tick-over mechanism (13). Complement activation is under strict control by complement regulators since uncontrolled complement activation can damage host cells or hyperactivate inflammatory pathways (14). To prevent excessive complement activation, the complement system is under strict control by circulating and cell-surface inhibitors ((12), as reviewed in (15)).

Decay accelerating factor (DAF) is a membrane-bound inhibitor of complement activation (see **Figure 1**). It can prevent formation of the complement convertases and accelerate their decay, thereby inhibiting the generation of C3a and C3b. Hence, DAF suppresses the amplification loop of the alternative pathway and the subsequent activation of the terminal pathway (16). Recently, it was shown that glycated DAF is present on erythrocytes of diabetes patients and was accompanied with a less efficient control of

complement activation (8). The function of another regulator of complement activation, CD59, was also hampered by glycation (10, 11, 17). CD59, also known as protectin, is a membrane-bound inhibitor that controls the final step of terminal pathway activation ((12) and shown in **Figure 1**). Inhibition or loss of function of CD59 may result in excessive formation of C5b-9/MAC on the cell surface (18, 19). Indeed, and similar to what was reported for DAF, *in vitro* studies demonstrated that glycation of CD59 impaired its inhibitory effect on C5b-9/MAC-related lysis of human erythrocytes (17). Also, erythrocytes of diabetes patients were more susceptible to complement-related lysis, likely due to glycation-induced inactivation of CD59 (11). Notably, in the kidney and nerves of diabetes patients, glycated CD59 co-localized with C5b-9, and presence of glycated, less functional CD59 was considered to be the cause of increased C5b-9 deposition (10). Glycated CD59 was also higher in the circulation of diabetes patients than in healthy individuals (9), and was detected in urine of diabetes patients (17). Glycation of other complement components, such as C3 (3, 20-23) and factor B (24) has also been reported, but biological effects of glycation on their function, if any, have not yet been reported.

Taken together, existing data suggest that dicarbonyl stress and subsequent glycation of complement regulators affect complement activation *in vivo*. In this study we investigated to what extent dicarbonyl stress, as reflected by the presence of dicarbonyls and AGEs in the circulation, is related to complement activation, as represented by the plasma concentrations of two complement activation products, i.e. C3a and soluble (s)C5b-9. In addition, we investigated whether *rs2736654* (25, 26) and *rs1049346* (27), two functional polymorphisms in the gene for glyoxalase 1 (GLO1), which is the rate-limiting detoxifying enzyme for the most important dicarbonyl compound methylglyoxal (MGO) in the formation of AGEs, were associated with plasma concentrations of C3a and/or sC5b-9.

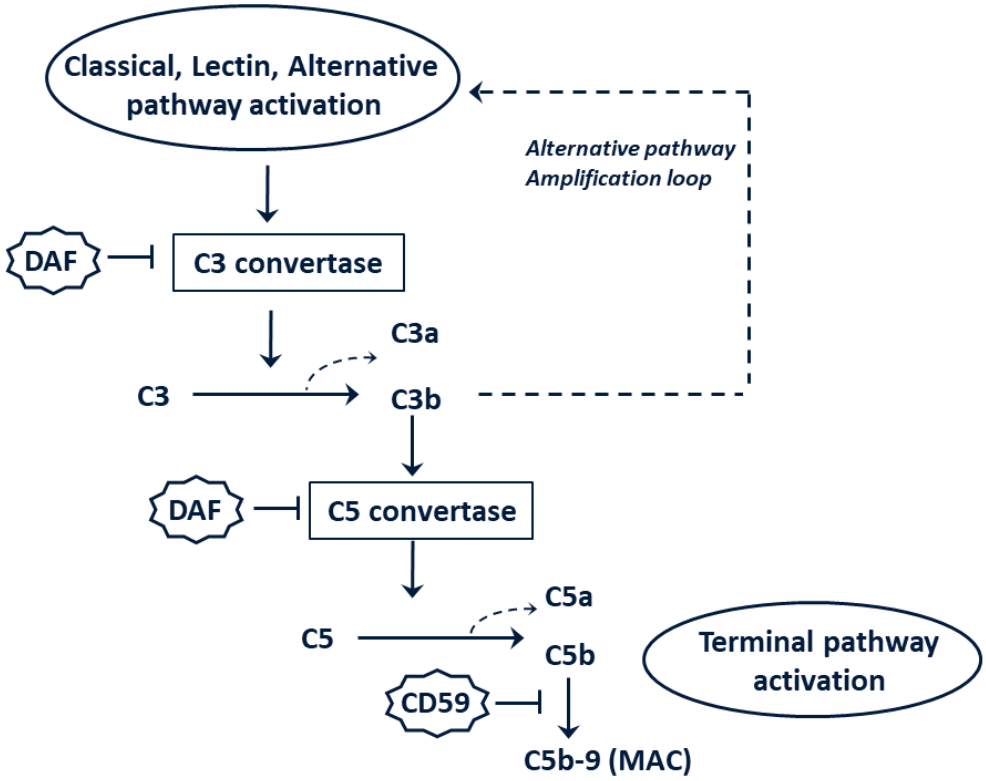

Figure 1. Activation and regulation of the complement system with focus on components that are most relevant for the current study. Complement activation starts with the activation of the classical, lectin, and/or the alternative pathway. Activation of these initial pathways leads to the generation of C3 convertases that cleave C3, the central complement product, into C3a and C3b. C3b produced by different complement pathways can contribute to the formation of alternative pathway C3 convertase. In this way, the alternative pathway functions as an amplification loop for all activation pathways. C3b can also lead to the generation of C5 convertase, which cleaves C5 into C5a and C5b and triggers the activation of the terminal pathway, leading to the formation of the membrane attack complex, C5b-9. Decay accelerating factor (DAF) and CD59 are two membrane inhibitors for complement activation. DAF controls complement activation by preventing the formation and accelerate the decay of C3 and C5 convertases. CD59 mainly functions as a specific inhibitor for of C5b-9 formation.

2 Material and methods

2.1 Study population

Participants of the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM, n=574) were selected from a large population-based observational study as described previously (28). They were of Caucasian descent and > 40 years of age with one or more of the following characteristics: body mass index (BMI) > 25 kg/m^2; use of antihypertensive medication; positive family history of T2DM; postprandial blood glucose level > 6.0 mmol/L; history of gestational diabetes and/or glycosuria. This study was approved by the medical ethics committee of Maastricht University. All participants gave written informed consent.

In the present study, individuals with missing values on the main independent variables, main dependent variables, and/or important covariates were excluded (n=44), leaving 530 individuals with complete data for the main analyses. Participants were asked to stop their lipid-modifying medication 14 days, and any other medication one day prior to the measurements.

2.2 Measurements of plasma α-dicarbonyls and AGEs

Peripheral blood samples were obtained after overnight fasting and stored at –80 °C until use. Plasma concentrations of α-dicarbonyls (MGO, glyoxal [GO], 3-deoxyglucosone [3-DG]), free AGEs (N^{ε}-(carboxymethyl)lysine [CML], N^{ε}-(carboxyethyl)lysine [CEL], N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine [MG-H1], and protein-bound AGEs (CML, CEL, pentosidine) were measured by ultra-performance liquid chromatography–tandem mass spectrometry (Waters, Milford, MA, USA) or by high performance liquid chromatography (Alltech/Grace, Breda, The Netherlands) as previously described (29, 30).

2.3 Genotyping of *GLO1 polymorphisms*

Single-nucleotide polymorphisms *rs2736654* and *rs1049346* of *GLO1* were genotyped in blood samples by the ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, California, USA), as previously described (31).

2.4 Measurements of complement factors

Fasting plasma concentrations of complement C3a and sC5b-9 were measured by ELISA (MicroVue C3a plus EIA kit, MicroVue sC5b-9 Plus EIA kit, Quidel, San Diego, USA), and concentrations of C3 was determined by auto-analyzer (Hitachi 912) using a Roche kit assays (Roche Diagnostics Nederland BV, Almere, The Netherlands), as previously described (32, 33).

2.5 Measurements of other covariates

Other covariates were obtained as described before. Briefly, BMI (kg/m^2) and waist circumference (cm) were measured at the research facility (28). Information on medication use (glucose-lowering, lipid-modifying, and/or anti-hypertensive, each yes/no), smoking status (current or previous tobacco smoking, yes/no), physical activity (METs/week), total energy intake (kJ/d), and alcohol consumption (g/d) was obtained via questionnaires (28). Normal glucose metabolism (NGM) (yes/no), impaired glucose metabolism (IGM) (yes/no) and T2DM (yes/no) were defined according to the 1999 WHO criteria based on oral glucose tolerance test data (34). Concentrations of triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C), and creatinine (to estimate glomerular filtration rate [eGFR] (35)) were measured in fasting blood samples (30).

2.6 Statistical analyses

Variables are presented as mean ± SD or percentages. Those with skewed distribution (i.e. free and protein-bound CML, free and protein-bound CEL, free MG-H1, protein-bound

pentosidine, C3a, fasting plasma glucose, alcohol intake, and triglycerides) are presented as median (interquartile range) and were log_2-transformed (except for alcohol intake) prior further analyses. General characteristics of the study population were compared between individuals with lower (< median) or higher (> median) C3a and sC5b-9 concentrations by using independent sample t-test or Mann-Whitney U test for continuous variables and Pearson chi-square test for categorical variables. All analyses were performed using IBM SPSS statistics version 25, a 2-tailed P-value < 0.10 was considered as significant for the interaction term, while a P-value < 0.05 was considered as significant in other analyses.

Main analyses: Multiple linear regression analyses were used to investigate the associations of markers of dicarbonyl stress, as represented by the plasma levels of the α-dicarbonyls and the AGEs (**main independent variables**), with markers of complement activation, i.e. plasma levels of C3a and sC5b-9 (**main outcomes**). Standardized values were calculated ([individual observed values - population mean]/standard deviation of the population) for the main independent and the main dependent variables to allow direct comparison of the effect sizes. All analyses were initially adjusted for age and sex (**model 1**), then additionally for waist circumference, lifestyle factors (i.e. smoking status, alcohol consumption, physical activity, energy intake), and use of medication (i.e. glucose-lowering, lipid-modifying, and/or anti-hypertensive), to control for potential confounding **(model 2**).

Plasma lipid levels (triglycerides, total cholesterol, HDL-C), renal function (eGFR), and C3 concentration were added separately to model 2 (the fully adjusted model). Because of the intricate biological relationships of these covariates with both dicarbonyl stress and complement, these additional models may to some extent be overadjusted. In addition, since the level of protein glycation will differ between individuals with and without (pre)diabetes, glucose metabolism status (NGM/IGM/T2DM, yes/no, as dummy variables) was added to the fully adjusted models and the effect of prevalent diabetes (T2DM, yes/no) on the associations of interest was evaluated using interaction analyses.

Sensitivity analyses: Some disease conditions may affect plasma concentrations of complement proteins. Therefore, the main analyses were repeated after excluding (1) participants with acute or chronic infections (CRP>10 mg/L, n=36), (2) participants with a (suspected) history of autoimmune disease, defined as self-reported current chronic joint

inflammation/ rheumatoid arthritis or a severe intestinal disorder that lasted for the past 3 months or longer (n=61), (3) participants with a self-reported current malignant condition or cancer (n=18), or (4) participants with self-reported liver disease (n=6). Information on autoimmune disease and malignant condition or cancer were missing in a few participants (n=10 and n=4, respectively). Therefore, the sensitivity analyses were performed in 494, 459, 508, and 524 individuals for the 4 conditions, respectively.

Additional analyses: We investigated the associations of two common functional polymorphisms in *GLO1*, *rs2736654* and *rs1049346*, with plasma concentrations of C3a and sC5b-9. Adjustment for confounders was done as mentioned for the main analyses. These additional analyses were performed in 504 individuals because information on *GLO1* polymorphisms was missing in 26 participants.

3 Results

3.1 General characteristics of the study population

General characteristics of the 530 participants included in the main analyses in total and according to the median of C3a and sC5b-9 concentrations are shown in **Table 1.** Overall, participants were middle-aged to elderly (59.5 ± 7.0 years) and 61% were men. They were, on average, overweight (BMI, 28.6 ± 4.4 kg/m^2; waist circumference, 99.3 ± 12.0 cm), with relatively normal glucose (fasting plasma glucose was 5.6 [5.2-6.5] mmol/l), lipid levels (total cholesterol, HDL-C, and total TG concentrations were 5.2 ± 1.0, 1.2 ± 0.3 and 1.4 [1.0-2.0] mmol/l, respectively), and renal function (eGFR was 91.3 ± 18.3 ml/min/1.73m^2). Overall, 22% had impaired glucose metabolism, 26% had T2DM, and 14%, 19%, 39%, respectively, were treated with glucose-lowering, lipid-modifying, and anti-hypertensive medication.

Table 1. Characteristics of the total population and according to lower and higher concentrations of C3a and sC5b-9

	Total study population*	Median of C3a concentration			Median of sC5b-9 concentration		
		< median	> median	*p*-value	< median	> median	*p*-value
Age (years)	59.5 ± 7.0	59.6 ± 6.6	59.3 ± 7.4	0.668	59.5 ± 6.9	59.4 ± 7.1	0.949
Sex (% men)	61	67	55	**0.004**	65	57	0.075
BMI (kg/m²)	28.6 ± 4.4	27.8 ± 3.9	29.3 ± 4.7	**<0.001**	28.3 ± 4.1	28.8 ± 4.6	0.166
Waist circumference (cm)	99.3 ± 12.0	97.8 ± 10.6	100.8 ± 13.2	**0.004**	98.9 ± 11.1	99.7 ± 12.9	0.435
Fasting plasma glucose (mmol/l)	5.6 (5.2-6.5)	5.7 (5.3-6.4)	5.5 (5.1-6.5)	0.324	5.7 (5.2-6.5)	5.6 (5.2-6.4)	0.288
HbA1C (%)	6.0 ± 0.8	6.0 ± 0.9	6.0 ± 0.8	0.972	6.0 ± 0.9	6.0 ± 0.7	0.997
Glucose metabolism status							
NGM / IGM / T2DM (%)	52/22/26	53/20/26	51/24/26	0.638	52/22/26	52/22/26	0.980
Current or previous smokers (%)	23	20	25	0.179	25	21	0.352
Alcohol intake (g/d)	8.6 (1.3-22.6)	10.1 (2.3-25.7)	6.6 (0.7-18.1)	**0.005**	8.6 (1.3-21.2)	8.6 (1.3-24.6)	0.686
Energy intake (10^3·kj/day)	9.3 ± 2.8	9.5 ± 2.7	9.1 ± 2.9	0.088	9.3 ± 2.8	9.2 ± 2.8	0.545
Physical activity (10^3·METs/week)	6.6 ± 4.1	7.0 ± 4.4	6.3 ± 3.7	**0.038**	6.8 ± 4.1	6.5 ± 4.1	0.461
Glucose-lowering medication (%)	14	15	13	0.447	15	13	0.447
Lipid-modifying medication (%)	19	17	22	0.227	19	20	0.742
Antihypertensive medication (%)	39	36	42	0.109	36	42	0.109
Total cholesterol (mmol/l)	5.2 ± 1.0	5.2 ± 0.9	5.2 ± 1.0	0.904	5.2 ± 1.0	5.2 ± 1.0	0.821
Total triglycerides (mmol/l)	1.4 (1.0-2.0)	1.4 (1.0-2.0)	1.4 (1.0-1.9)	0.434	1.4 (1.0-2.0)	1.4 (0.9-1.9)	0.099
HDL-cholesterol (mmol/l)	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	0.318	1.2 ± 0.3	1.2 ± 0.4	0.691
eGFR (ml/min/1.73m²)	91.3 ± 18.3	91.9 ± 17.9	90.7 ± 18.8	0.452	91.9 ± 17.7	90.7 ±18.9	0.457
C3a (µg/l)	59.2 (50.1-72.7)	50.1 (44.1-54.3)	72.7 (65.2-85.7)	---	56.6 (48.0-70.0)	62.8 (51.8-75.9)	**<0.001**
sC5b-9 (µg/l)	113.0 ± 32.9	107.6 ± 30.6	118.4 ± 34.3	**<0.001**	87.1 ± 15.5	138.8 ± 24.2	---
C3 (g/l)	1.0 ± 0.2	1.0 ± 0.1	1.1 ± 0.2	**<0.001**	0.99 ± 0.15	1.04 ± 0.16	**<0.001**
α-dicarbonyls							
MGO (nmol/l)	367.4 ± 77.7	368.8 ± 77.8	366.0 ± 77.8	0.680	369.2 ± 72.4	365.5 ± 82.9	0.579
GO (nmol/l)	1140.5 ± 359.3	1164.8 ± 365.1	1116.3 ± 352.4	0.120	1182.4 ± 354.3	1098.6 ± 360.0	**0.007**
3-DG (nmol/l)	1283.3 ± 388.7	1297.3 ± 417.5	1269.3 ± 357.9	0.408	1284.3 ± 379.3	1282.3 ± 398.6	0.952
Free AGEs							
CML (nmol/l)	78.3 (60.5-98.1)	78.4 (60.4-93.3)	78.2 (61.4-101.3)	0.204	81.8 (63.5-98.4)	76.1 (58.2-97.9)	0.505
CEL (nmol/l)	45.2 (36.5-58.2)	43.7 (36.7-58.7)	45.6 (35.7-57.0)	0.788	45.9 (38.1-59.9)	43.4 (35.5-56.0)	0.083
MG-H1 (nmol/l)	122.3 (86.3-174.7)	118.1 (85.6-170.0)	125.9 (86.6-178.1)	0.319	125.1 (88.6-176.6)	119.6 (82.8-172.6)	0.474
Protein-bound AGEs							
CML (nmol/mmol lysine)	34.4 (29.5-40.8)	34.6 (30.5-41.4)	34.1 (28.9-40.6)	0.073	34.4 (29.7-41.5)	34.5 (28.9-40.3)	0.450
CEL (nmol/mmol lysine)	23.1 (18.9-29.0)	24.4 (20.0-30.3)	21.9 (17.8-27.0)	**<0.001**	23.7 (19.1-29.5)	23.0 (18.4-28.2)	0.076
Pentosidine (nmol/mmol lysine)	0.43 (0.36-0.53)	0.45 (0.38-0.54)	0.42 (0.36-0.51)	**0.026**	0.43 (0.37-0.50)	0.43 (0.36-0.55)	0.116

***** Data from 530 participants were included in the main analyses, data for BMI were available for n=529, for HbA1C (%) were available for n=505, for fasting plasma glucose were available for n=529. **Abbreviations**: sC5b-9, soluble C5b-9; NGM, normal glucose metabolism; IGM, impaired glucose metabolism; T2DM, type 2 diabetes mellitus; HDL, high-density lipoproteins; eGFR, estimated glomerular filtration rate; MGO, methylglyoxal; GO, glyoxal; 3-DG, 3-deoxyglucosone; AGEs, advanced glycation end products; CML, N^{ε}-(carboxymethyl)lysine; CEL, N^{ε}-(carboxyethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

Individuals with higher C3a concentrations were more often women, had higher measures of adiposity, and lower daily alcohol consumption and physical activity. They also had lower plasma concentrations of protein-bound CEL and pentosidine. Individuals with higher and lower sC5b-9 concentrations did not show obvious differences in general characteristics, and those with higher sC5b-9 concentrations had lower GO concentrations. Individuals with higher plasma C3a generally had higher sC5b-9, and vice versa.

3.2 Associations of plasma α-dicarbonyls, free AGEs and protein-bound AGEs with C3a

C3a is generated during activation of C3 and higher plasma concentrations of C3a result primarily from direct activation of the alternative pathway and from activation of the amplification loop via any of the three complement activation pathways. Plasma α-dicarbonyls and free AGEs were not associated with C3a, neither in the age- and sex-adjusted models, nor in the fully adjusted models (**Table 2**). In the age- and sex-adjusted regression models, plasma concentrations of protein-bound CML, CEL, and pentosidine were inversely and significantly associated with C3a concentration (**Table 2, model 1**). In the fully adjusted models, these associations were attenuated and became non-significant for protein-bound CML and pentosidine, mainly resulting from the adjustment for waist, but remained significant for protein-bound CEL (**Table 2, model 2,** protein-bound CML, β= –0.08 [–0.17; 0.01]; protein-bound CEL, β= –0.17 [–0.25; –0.08]; protein-bound pentosidine, β= –0.05 [–0.14; 0.04]). These associations of α-dicarbonyls, free AGEs, and protein-bound AGEs with C3a were virtually unchanged after additional adjustment for plasma lipids, glucose metabolism status, renal function, or C3 levels (**Table S1**). When we evaluated if the association between dicarbonyl stress and C3a differed between individuals with and without T2DM (**Table S2**), interaction was observed for MGO ($p_{interaction}$=0.083) and protein-bound CEL ($p_{interaction}$=0.039). When the analyses were stratified on presence of T2DM, there was a trend towards a positive association of MGO with C3a in T2DM (β= 0.13 [–0.01; 0.28], n=138), but not in those without diabetes (β= –0.04 [–0.15; 0.07], n=392). The inverse

association of CEL with C3a was only observed in individuals without T2DM (β= –0.23 [–0.33; –0.12]), and not in diabetes patients (β= –0.05 [–0.19; 0.08]).

Table 2. Associations of plasma α-dicarbonyls, free and protein-bound AGEs with C3a and sC5b-9 (n=530)

		C3a			sC5b-9	
	Model	**β**	**95% CI**	***p***	**β**	**95% CI**	***p***
α-dicarbonyls							
MGO	1	0.01	[-0.07; 0.10]	*0.773*	0.01	[-0.08; 0.10]	*0.825*
	2	0.01	[-0.08; 0.10]	*0.812*	0.02	[-0.07; 0.10]	*0.745*
GO	1	-0.08	[-0.16; 0.01]	*0.074*	**-0.13**	**[-0.21; -0.04]**	***0.004***
	2	-0.06	[-0.14; 0.03]	*0.218*	**-0.12**	**[-0.21; -0.02]**	***0.013***
3-DG	1	0.02	[-0.07; 0.11]	*0.659*	0.04	[-0.05; 0.13]	*0.372*
	2	0.00	[-0.10; 0.11]	*0.955*	0.08	[-0.03; 0.18]	*0.174*
Free AGEs							
CML	1	0.03	[-0.06; 0.12]	*0.458*	-0.02	[-0.11; 0.07]	*0.637*
	2	0.01	[-0.08; 0.10]	*0.856*	-0.02	[-0.11; 0.07]	*0.638*
CEL	1	-0.02	[-0.11; 0.06]	*0.610*	-0.07	[-0.15; 0.02]	*0.139*
	2	-0.05	[-0.14; 0.04]	*0.262*	-0.07	[-0.16; 0.02]	*0.133*
MG-H1	1	-0.03	[-0.12; 0.06]	*0.508*	-0.04	[-0.13; 0.05]	*0.356*
	2	-0.05	[-0.13; 0.04]	*0.311*	-0.03	[-0.12; 0.06]	*0.488*
Protein-bound AGEs							
CML	1	**-0.14**	**[-0.23; -0.06]**	***0.001***	-0.07	[-0.15; 0.02]	*0.136*
	2	-0.08	[-0.17; -0.01]	*0.088*	-0.05	[-0.15; 0.05]	*0.334*
CEL	1	**-0.16**	**[-0.24; -0.08]**	***<0.001***	-0.07	[-0.16; 0.01]	*0.087*
	2	**-0.17**	**[-0.25; -0.08]**	***<0.001***	-0.08	[-0.17; 0.00]	*0.058*
pentosidine	1	**-0.11**	**[-0.20; -0.03]**	***0.010***	**0.10**	**[0.02; 0.19]**	***0.020***
	2	-0.05	[-0.14; -0.04]	*0.246*	**0.15**	**[0.05; 0.24]**	***0.002***

Concentrations of α-dicarbonyls, AGEs, C3a, and sC5b-9 were standardized. Concentrations of AGEs and C3a were $\log_2$ –transformed prior to standardization. **Model 1**: adjusted for age and sex. **Model 2**: **Model 1** + waist circumstance, lifestyle (smoking status, alcohol consumption, physical activity, and energy intake), and medication use (glucose-lowering, lipid-modifying, and/or anti-hypertensive). **Abbreviations**: sC5b-9, soluble C5b-9; MGO, methylglyoxal; GO, glyoxal; 3-DG, 3-deoxyglucosone; AGEs, advanced glycation end products; CML, N^{ϵ}-(carboxymethyl)lysine; CEL, N^{ϵ}-(carboxyethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

3.3 Associations of plasma α-dicarbonyls, free AGEs and protein-bound AGEs with sC5b-9

sC5b-9 is generated upon full activation of the common terminal pathway of complement activation. Plasma MGO or 3-DG were not associated with sC5b-9, while GO was inversely and significantly associated with sC5b-9, both in the age- and sex-adjusted models (**Table 2,**

model 1) and in the fully adjusted models (**Table 2, model 2,** GO, β= –0.12 [–0.21; –0.02]). Free AGEs were not associated with sC5b-9, neither in the age- and sex-adjusted nor in the fully adjusted models (**Table 2**). In contrast, a positive association was observed for the plasma concentration of protein-bound pentosidine with sC5b-9 (**Table 2, model 2,** β=0.15 [0.05; 024]), while other protein-bound AGEs were not significantly associated with sC5b-9 (**Table 2, model 2,** protein-bound CML, β= –0.05 [–0.15; 0.05]; protein-bound CEL, β= –0.08 [–0.17; 0.00]). The associations of α-dicarbonyls, free AGEs and protein-bound AGEs with sC5b-9 were virtually unchanged after additional adjustment for plasma lipids, glucose metabolism status, or renal function (**Table S1**). Only the inverse association of GO with sC5b-9 was attenuated and became non-significant after the adjustment for plasma C3 (**Table S1, model 3,** β = –0.08 [–0.17; 0.00]). The inverse association of protein-bound CEL with sC5b-9 became slightly stronger and was borderline significant after additional adjustment for eGFR (β = –0.09 [–0.17; –0.00], **Table S1**) and for plasma C3 concentration (β = –0.09 [–0.17; –0.00], **Table S1**). When we evaluated if the association between dicarbonyl stress and sC5b-9 differed between individuals with and without T2DM (**Table S2**), interaction was observed for MGO, for free and protein-bound CML, and for free and protein-bound CEL (all $p_{interaction}$<0.10). In the analyses that were stratified by presence of T2DM, a modest positive trend of MGO with sC5b-9 was observed in diabetes patients (β= 0.13 [–0.05; 0.31], n=138), and not in those without diabetes (β= –0.05 [–0.15; 0.06], n=392), similar to what was observed for C3a. In addition, and in contrast to C3a, in T2DM, both free and protein-bound CML and CEL, were inversely and significantly associated with sC5b-9 (free CML, β= –0.21 [–0.41; –0.01]; free CEL, β= –0.29 [–0.47; –0.10]; protein-bound CML, β= –0.24 [–0.42; –0.06], protein-bound CEL, β= –0.21 [–038; –0.05]). These latter associations were not observed in people without diabetes (free CML, β=0.04 [–0.06; 0.14]; free CEL, β=0.01 [–0.09; 0.11]; protein-bound CML, β=0.05 [–0.07; 0.16], protein-bound CEL, β=–0.02 [–0.12; 0.08]).

3.4 Sensitivity analyses

The main analyses were repeated after excluding participants with acute or chronic infections, with a (suspected) history of autoimmune disease, with a self-reported current malignant condition / cancer, or with a self-reported liver disease. Most of the associations were not materially changed (**Table S3**). The inverse association of GO with sC5b-9 was attenuated and became non-significant in the analyses excluding participants with acute or chronic infections.

3.5 Associations of functional *GLO1* polymorphism with C3a and sC5b-9

The frequency of *TT* (31%), *GT* (51%) and *GG* (18%) genotypes of *GLO1 rs2736654* and the *GG* (23%), *AG* (52%) and *AA* (25%) genotypes of *rs1049346* are shown in **Table 3**. The two *GLO1* polymorphisms were not significantly associated with plasma C3a (**Table 3**). Also, no significant associations were observed for *rs2736654* with sC5b-9. In contrast, significant inverse associations were observed for *rs1049346* with sC5b-9 (**Table 3, model 2,** β for *AG* = –0.32 [–0.54; –0.12]; β for *AA* = –0.40 [–0.65; –0.15]). This implies that, compared to those with the *GG* genotype of *rs1049346*, individuals with the *AG* genotype had, on average, 0.32 SD lower plasma sC5b-9 and those with the *AA* genotype, had on average, 0.40 SD lower sC5b-9. The associations of the two *GLO1* polymorphisms with plasma concentrations of MGO, free and protein-bound AGEs were also evaluated, and almost no associations were observed, except an inverse association for the *GT* genotype of *rs2736654* with MGO (**Table S4, model 2,** β = –0.21, *p*=0.036).

Table 3. Associations of *GLO1* polymorphisms with C3a and sC5b-9 (n=504)

			C3a			sC5b-9		
	N	**Model**	**β**	**95% CI**	***p***	**β**	**95% CI**	***p***
rs2736654								
TT	155	reference	–	–	–	–	–	–
GT	259	1	0.00	[-0.20; 0.20]	*0.998*	-0.01	[-0.21; 0.19]	*0.891*
		2	-0.04	[-0.24; 0.15]	*0.676*	-0.03	[-0.23; 0.17]	*0.750*
GG	90	1	-0.08	[-0.34; 0.18]	*0.559*	-0.20	[-0.46; 0.06]	*0.127*
		2	-0.08	[-0.34; 0.18]	*0.533*	-0.22	[-0.48; 0.05]	*0.105*
rs1049346								
GG	116	reference	–	–	–	–	–	–
AG	260	1	-0.06	[-0.28; 0.16]	*0.595*	**-0.30**	**[-0.51; -0.08]**	***0.008***
		2	-0.08	[-0.29; 0.14]	*0.493*	**-0.32**	**[-0.54; -0.10]**	***0.004***
AA	128	1	-0.13	[-0.38; 0.12]	*0.297*	**-0.37**	**[-0.62; -0.12]**	***0.004***
		2	-0.16	[-0.40; 0.09]	*0.217*	**-0.40**	**[-0.65; -0.15]**	***0.002***

The reference categories are the genotypes that were reported to be associated with the highest GLO1 activity (see references 25-27). Concentrations of C3a and sC5b-9 were standardized. Concentrations of C3a were $\log_2$ – transformed prior to standardization. **Model 1**: adjusted for age and sex. **Model 2**: **Model 1** + waist circumstance, lifestyle (smoking status, alcohol consumption, physical activity, and energy intake), and medication use (glucose-lowering, lipid-modifying, and/or anti-hypertensive). **Abbreviations**: GLO1, Glyoxalase 1; sC5b-9, soluble C5b-9.

4 Discussion

This study on the association between dicarbonyl stress and complement activation has several main findings. First, GO was inversely associated with sC5b-9 while no associations were observed for other α-dicarbonyls with C3a or sC5b-9 in the whole study population. Second, in the whole study population, no associations were observed between the free AGEs and C3a or sC5b-9, although free CML and CEL were inversely associated with sC5b-9 in T2DM. Third, protein-bound CEL, and to a lesser extent also CML and pentosidine, were inversely associated with C3a; protein-bound CML and CEL were not associated with sC5b-9 in the whole population but were inversely associated with sC5b-9 in T2DM patients; protein-bound pentosidine was positively and independently associated with sC5b-9, regardless of the diabetes status. Lastly, C3a did not differ between the variants of the two functional *GLO1* polymorphisms. sC5b-9 did not differ between the genotypes of *rs2736654*, while individuals with the *AA* and *AG* genotypes of *rs1049346* had lower sC5b-9 concentration than those with *GG* genotype.

Glycation of the complement regulators DAF and CD59 was, at least *in vitro,* shown to compromise their complement inhibitory function (8) (10, 11, 17). DAF controls, among others, the activation of complement C3, which leads to the production of C3a and C3b (16),

while CD59 controls the final step in complement activation, which results in formation of C5b-9/MAC on the cell surface (12). Thus, glycation of DAF and CD59 may hamper their complement regulatory function and result in enhanced complement activation with increased formation of activation products, such as C3a and sC5b-9. Because protein glycation is induced by dicarbonyl stress, we hypothesized that greater dicarbonyl stress, as represented by higher plasma concentrations of reactive α-dicarbonyl compounds and related AGEs, would be associated with more complement activation, represented by greater plasma concentrations of C3a and sC5b-9. Interestingly, our main results provide a diverse picture. This suggests that the markers of dicarbonyl stress that we included may each have their own biological effects and may therefore be associated with complement activation in its own, unique way.

We did not observe significant associations of any of the three α-dicarbonyl compounds with C3a. Likewise, MGO and 3-DG were not significantly associated with circulating sC5b-9. An inverse association was observed between GO and sC5b-9. This association was attenuated and no longer significant after adjustment of C3, and after exclusion of participants with acute or chronic infections. This suggests that the currently-observed inverse association was, at least partly, due to an ongoing inflammatory process that is somehow related to lower plasma GO concentration and/or that plasma GO may have anti-inflammatory properties. We previously showed that plasma sC5b-9 is positively associated with low-grade inflammation (33), and also GO is generally considered a pro- rather than anti-inflammatory compound, although information on the association between GO and inflammation in humans is scarce. At least one experimental study showed that exogenous GO can induce inflammatory injury in human vascular endothelial cells (36). Our current observation might thus imply a dissociation between the concentration of GO in plasma and its local concentrations and effects in tissue, similar to what was previously observed for CML (37). The lack of association for MGO and 3-DG with sC5b-9 may indicate that biological effects of these compounds differ from those of GO. In line with this, MGO and GO may trigger distinct intracellular signals involved in various cellular function in cultured endothelial cells, due to differences in their chemical structures *(38)*.

The burden of glycation and dicarbonyl stress may be higher in individuals with diabetes than those without, and the previously reported glycation of CD59 and DAF was mainly observed in diabetes patients (8, 10, 11, 17). In our cohort, there was modest but significant interaction between diabetes status and MGO on complement activation. In T2DM, positive trends, although not statistically significant, were observed for the association of MGO with C3a and sC5b-9 that were absent in individuals without diabetes. Given the small sample size (n=138) for the diabetes subgroup these data must be interpreted with caution.

Free AGEs were not significantly associated with C3a or sC5b-9, while for C5b-9, but not for C3a, there was a possible influence of diabetes status. In T2DM patients, strong inverse associations of free CML and CEL with sC5b-9 were observed. The underlying mechanism of these unexpected diabetes-specific associations is not clear yet, but similar interactions and effects were observed for protein–bound CML and CEL. This is in contrast with effects of diabetes status on the association of MGO with sC5b-9, and may again suggest that the relationships of these dicarbonyls and AGEs with complement activation occur via different routes.

The inverse association of protein-bound CML with C3a in the age- and sex-adjusted models was largely attenuated and became non-significant in the fully adjusted model, mainly due to the adjustment for obesity. We and others previously showed that plasma protein-bound CML was inversely associated with central obesity, at least partly because it was trapped by visceral adipose tissue via the receptor of AGEs (RAGE) (37, 39, 40) which, in combination with the positive association between C3a and obesity in our study cohort (41), may explain the currently-observed inverse association. Serum pentosidine was also found to be inversely correlated with BMI in one recent human study (42). Given that pentosidine can also bind RAGE (43), a similar obesity-dependent inverse association may underlie the association observed between pentosidine and C3a. In contrast to what was observed for protein-bound CML and pentosidine, the inverse association between protein-bound CEL with C3a was independent of all confounders included in our analyses. Interestingly, there was significant interaction with diabetes status, and in the stratified analyses the inverse association was only present in individuals without T2DM. Thus, it

seems that the inverse association between CEL and C3a might be diminished in hyperglycaemia/T2DM, but the underlying mechanisms need to be explored.

We observed a consistent positive association between protein-bound pentosidine and sC5b-9 that was independent of possible confounders, did not differ between individuals with and without T2DM, and remained significant in all sensitivity analyses that were performed. The absence of obesity-related inverse association for sC5b-9, which was observed for C3a, may due to the lack of association for sC5b-9 with adiposity that we showed in our previous study (41). Another reason that we observe this positive association only for sC5b-9 and not for C3a may be due to the level at which DAF and CD59 regulate complement activation. CD59 is the dedicated regulator of the formation of C5b-9/MAC, the final integrator of the common terminal pathway of complement activation. In contrast, there are, besides DAF, various other regulators that control the C3 convertase and hence affect the subsequent activation and cleavage of C3. Therefore, any glycation-induced impairment of DAF may be compensated by increased levels of other inhibitors of C3 convertases, such as factor H or CD46. This also adds to the notion discussed above that instead of a being general reflection of dicarbonyl stress, plasma protein-bound CML, CEL, and pentosidine may each represent distinct pathophysiological processes. Indeed, the possibility of different contributions for these compounds in the development of vascular disease were reported (44). For instance, via binding to RAGE, AGEs like CML can activate of inflammatory pathways (as reviewed in (45)), while, on the other hand, cross-linking AGEs, such as pentosidine may act via the direct formation of cross-links between extracellular matrix proteins (46, 47). The association between protein-bound pentosidine and sC5b-9 was the only association in this study that was in line with our pre-specified hypothesis. One reason for this positive association is that protein-bound pentosidine may, better than the other AGEs, reflect the tissue-AGE content. Previous studies from our group showed that skin autofluorescence (SAF), a non-invasive measurement of skin AGE accumulation), and plasma protein-bound pentosidine, but not protein-bound CML and CEL, were positively associated with aortic stiffening (44). In line with this, plasma pentosidine levels were shown to be correlated with SAF in Japanese hemodialysis patients (48). In another small case-control study a positive trend, although not statistically significant, was observed between

serum protein-bound pentosidine and tissue levels of pentosidine obtained by skin biopsy (49). The positive association between the plasma concentration of protein-bound pentosidine and sC5b-9 that we observed may thus reflect the effect of glycation in tissues on complement activation.

Plasma dicarbonyls produce a snapshot of the current level dicarbonyl stress while AGEs rather represent dicarbonyl stress over the last days of weeks. In our analyses, we also included a measure that may reflect the life-long effects of differences in dicarbonyl stress, i.e. potentially functional polymorphisms in the MGO-detoxifying enzyme GLO1. Most of the human studies on *GLO1 rs2736654* reported that the individuals with *AA* (i.e. *TT*) genotype have the best GLO1 function/activity (25, 26). This implies that individuals with the *AC* (i.e. *GT*) or *CC* (i.e. *GG*) genotype may have been exposed to life-long greater dicarbonyl stress, due to less GLO1 activity. Given our hypothesis that greater dicarbonyl stress is associated with more complement activation, we hypothesized that presence of the *AC* (*TG*) of *AA* (*TT*) genotype would lead to enhanced complement activation. Contrary to our hypothesis, *rs2736654* was not associated with C3a concentrations. In addition, individuals carrying the genotype with the lowest predicted GLO1 activity (i.e. the *GG* genotype) had lower sC5b-9 concentrations, although non-significant. The other functional *GLO1* polymorphism, *rs1049346*, was also not associated with C3a concentration, while individuals with *AG* and *AA* genotypes of *rs1049346*, which were demonstrated to have lower GLO1 enzyme activity (27), had significantly lower sC5b-9 concentrations. These results do not support our pre-specified hypothesis, but do agree with some of the inverse associations we observed for the dicarbonyls and protein-bouns AGEs. Critical re-evaluation of the available literature shows that the claims that are made on functionality of these polymorphisms are partly based on *in vitro* data and partly inconsistent (25-27, 50, 51). Our current observations may thus imply that the biological effects of these polymorphisms on functional GLO1 enzyme activity in humans is limited. Moreover, also in case of genuine functional polymorphisms, genotype-related alterations in protein concentration, which may compensate its influence in enzyme activity, may have occurred. In this respect, it is noteworthy that also the associations between the *GLO1* polymorphisms and plasma concentrations of MGO or AGEs were virtually absent (**Table S4**).

The main strength of our study is the availability of plasma concentrations of α-dicarbonyls, free AGEs and protein-bound AGEs, as well as complement activation products, within one well-phenotyped cohort. The availability of DNA allowed us to evaluate functional *GLO1* polymorphisms and add an additional level of information to our data. The detailed phenotyping of the study population additionally provided us with the opportunity to thoroughly evaluate the effects of potential confounders and perform relevant sensitivity analyses. Our study also has several limitations. Most importantly, despite the detailed information we have on the overall levels of dicarbonyl compounds and AGE concentration for each participant, we do not have information on the extent to which their individual complement regulators were actually carbonylated and/or glycated. Other limitations include the cross-sectional design, which prohibits conclusions on causality. We aimed to mitigate this limitation by including the functional *GLO1* polymorphisms which, theoretically, represent a lifelong exposure to greater dicarbonyl stress, at least to MGO. Further exploration on the effects of *GLO1* polymorphisms on GLO1 function and dicarbonyl stress is needed since the two *GLO1* polymorphisms evaluated in the present study were poorly associated with plasma MGO and other AGEs. Moreover, in the analyses stratified for presence of T2DM, as well as in the analyses for the *GLO1* polymorphism, the smaller sample size in subgroups may have decreased the statistical power to identify relevant relationships. Lastly, our participants are middle-aged to older individuals characterized by increased risks of cardiovascular disease. This selection of the study population would limit the generalizability of present findings. For this reason, it is important that these evaluations will, in due time, be confirmed in a population-based cohort.

In conclusion, this study shows that plasma concentrations of dicarbonyl stress markers display various associations with complement activation. This suggests different biological effects of the individuals plasma α-dicarbonyls and AGEs in circulation, potentially with different clinical relevance. The most striking findings herein are that the genotype that represented less GLO1 activity (hence greater dicarbonyl stress) was associated with less activation of the terminal pathway, that protein-bound and free CML and CEL were all inversely associated with sC5b-9, but only in the T2DM patients, while protein-bound CEL showed a consisted inverse association with C3a. Pentosidine, on the other hand, was

positively and significantly associated with sC5b-9. This latter finding was in line with our pre-specified hypothesis. Taken together, these data illustrate that the complex underlying physiological processes of these circulating markers should be taken into account in future work. The solid associations observed for plasma pentosidine may indicate its higher priority as a marker for tissue AGEs and future human studies investigating the association of tissue AGEs, obtained from biopsy or reflected by SAF, with complement activation would be of great interest. Moreover, the consistent inverse associations between AGEs and complement activation in this moderately-sized cohort need to be confirmed and extended in larger study population.

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Supplementary data

Table S1. Associations of plasma α-dicarbonyls, free and protein-bound AGEs with C3a and sC5b-9 — Additional adjustment for plasma lipids, renal function, C3 levels, and glucose metabolism status (n=530)

		C3a			sC5b-9		
	Full model plus:	**β**	**95% CI**	***p***	**β**	**95% CI**	***p***
α-dicarbonyls							
MGO	Lipids	0.02	[-0.07; 0.10]	*0.735*	0.02	[-0.07; 0.11]	*0.607*
	GMS	0.01	[-0.08; 0.10]	*0.803*	0.01	[-0.09; 0.10]	*0.902*
	eGFR	0.02	[-0.07; 0.10]	*0.719*	0.01	[-0.08; 0.10]	*0.851*
	C3	0.00	[-0.08; 0.09]	*0.940*	0.01	[-0.08; 0.10]	*0.826*
GO	Lipids	-0.05	[-0.15; 0.04]	*0.232*	**-0.11**	**[-0.21; -0.02]**	***0.019***
	GMS	-0.06	[-0.15; 0.03]	*0.213*	**-0.12**	**[-0.21; -0.03]**	***0.011***
	eGFR	-0.06	[-0.14; 0.03]	*0.227*	**-0.12**	**[-0.21; -0.03]**	***0.012***
	C3	0.00	[-0.09; 0.09]	*0.967*	-0.08	[-0.17; 0.01]	*0.086*
3-DG	Lipids	0.01	[-0.10; 0.11]	*0.885*	0.09	[-0.02; 0.20]	*0.100*
	GMS	-0.00	[-0.12; 0.12]	*0.972*	0.06	[-0.06; 0.18]	*0.326*
	eGFR	0.00	[-0.10; 0.11]	*0.981*	0.08	[-0.03; 0.19]	*0.162*
	C3	-0.02	[-0.12; 0.08]	*0.708*	0.06	[-0.05; 0.17]	*0.269*
Free AGEs							
CML	Lipids	0.01	[-0.08; 0.10]	*0.860*	-0.03	[-0.12; 0.07]	*0.576*
	GMS	0.01	[-0.08; 0.10]	*0.808*	-0.02	[-0.11; 0.07]	*0.658*
	eGFR	0.02	[-0.07; 0.11]	*0.678*	-0.04	[-0.13; 0.06]	*0.450*
	C3	0.01	[-0.07; 0.10]	*0.803*	-0.02	[-0.11; 0.07]	*0.662*
CEL	Lipids	-0.05	[-0.14; 0.04]	*0.264*	-0.07	[-0.16; 0.02]	*0.140*
	GMS	-0.05	[-0.13; 0.04]	*0.278*	-0.07	[-0.16; 0.02]	*0.125*
	eGFR	-0.04	[-0.13; 0.05]	*0.362*	-0.09	[-0.18; 0.00]	*0.062*
	C3	-0.05	[-0.14; 0.03]	*0.229*	-0.07	[-0.16; 0.02]	*0.121*
MG-H1	Lipids	-0.05	[-0.14; 0.04]	*0.272*	-0.04	[-0.13; 0.06]	*0.431*
	GMS	-0.04	[-0.13; 0.05]	*0.341*	-0.03	[-0.12; 0.06]	*0.496*
	eGFR	-0.04	[-0.13; 0.05]	*0.405*	-0.05	[-0.14; 0.05]	*0.339*
	C3	-0.04	[-0.12; 0.05]	*0.377*	-0.03	[-0.12; 0.06]	*0.549*
Protein-bound AGEs							
CML	Lipids	-0.09	[-0.18; 0.01]	*0.070*	-0.06	[-015; 0.04]	*0.245*
	GMS	-0.08	[-0.17; 0.01]	*0.097*	-0.04	[-0.14; 0.06]	*0.410*
	eGFR	-0.07	[-0.17; 0.02]	*0.114*	-0.06	[-0.15; 0.04]	*0.246*
	C3	-0.04	[-0.13; 0.05]	*0.364*	-0.02	[-0.12; 0.07]	*0.662*
CEL	Lipids	**-0.16**	**[-0.25; -0.08]**	***<0.001***	-0.07	[-0.16; 0.01]	*0.100*
	GMS	**-0.17**	**[-0.25; -0.08]**	***<0.001***	-0.08	[-0.17; 0.01]	*0.069*
	eGFR	**-0.16**	**[-0.25; -0.08]**	***<0.001***	**-0.09**	**[-0.17; -0.00]**	***0.048***
	C3	**-0.17**	**[-0.25; -0.09]**	***<0.001***	**-0.09**	**[-0.17; -0.00]**	***0.043***
pentosidine	Lipids	-0.06	[-0.15; 0.03]	*0.217*	**0.14**	**[0.05; 0.23]**	***0.003***
	GMS	-0.05	[-0.14; 0.04]	*0.264*	**0.15**	**[0.05; 0.24]**	***0.002***
	eGFR	-0.05	[-0.14; 0.05]	*0.314*	**0.14**	**[0.05; 0.23]**	***0.003***
	C3	-0.03	[-0.12; 0.06]	*0.534*	**0.16**	**[0.07; 0.25]**	***<0.001***

Concentrations of α-dicarbonyls, AGEs, C3a, and sC5b-9 were standardized. Concentrations of AGEs, C3a, and triglycerides were $\log_2$–transformed prior to standardization. **Lipids**: adjusted for age, sex, waist circumstance, lifestyle (smoking status, alcohol consumption, physical activity, and energy intake), and medication use (glucose-lowering, lipid-modifying, and/or anti-hypertensive) (**Full Model**) + plasma lipids (concentrations of triglycerides, total cholesterol, HDL- cholesterol). **GMS**: **Full Model** + glucose metabolism status (GMS). **eGFR**: **Full Model** + renal function (estimated glomerular filtration rate, eGFR). **C3**: **Full Model** + C3 concentration. **Abbreviations**: sC5b-9, soluble C5b-9; MGO, methylglyoxal; GO, glyoxal; 3-DG, 3-deoxyglucosone; AGEs, advanced glycation end products; CML, N^{ε}-(carboxymethyl)lysine; CEL, N^{ε}-(carboxyethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

Table S2. Stratified analyses for the associations of stressplasma α-dicarbonyls, free and protein-bound AGEs with C3a and sC5b-9 according to the presence of diabetes (n=530)

		C3a			sC5b-9		
	Model	**β**	**95% CI**	***p***	**β**	**95% CI**	***p***
α-dicarbonyls							
MGO	NDM	-0.04	[-0.15; 0.07]	*0.487*	-0.05	[-0.15; 0.06]	*0.393*
	T2DM	0.13	[-0.01; 0.28]	*0.073*	0.13	[-0.05; 0.31]	*0.155*
			p for interaction	***0.083***		*p for interaction*	***0.098***
GO	NDM	-0.10	[-0.23; 0.04]	*0.154*	**-0.15**	**[-028; -0.02]**	***0.024***
	T2DM	-0.04	[-0.15; 0.08]	*0.537*	-0.10	[-0.24; 0.04]	*0.171*
			p for interaction	*0.598*		*p for interaction*	*0.602*
3-DG	NDM	-0.06	[-0.24; 0.12]	*0.485*	-0.03	[-0.20; 0.15]	*0.774*
	T2DM	0.07	[-0.08; 0.21]	*0.371*	0.12	[-0.06; 0.30]	*0.193*
			p for interaction	*0.168*		*p for interaction*	*0.248*
Free AGEs							
CML	NDM	0.03	[-0.08; 0.24]	*0.637*	0.04	[-0.06; 0.14]	*0.413*
	T2DM	-0.05	[-0.21; 0.12]	*0.567*	**-0.21**	**[-0.41; -0.01]**	***0.040***
			p for interaction	*0.535*		*p for interaction*	***0.010***
CEL	NDM	-0.04	[-0.15; 0.07]	*0.455*	0.01	[-0.09; 0.11]	*0.872*
	T2DM	-0.10	[-0.25; 0.05]	*0.188*	**-0.29**	**[-0.47; -0.10]**	***0.003***
			p for interaction	*0.719*		*p for interaction*	***0.004***
MG-H1	NDM	-0.03	[-0.14; 0.08]	*0.581*	-0.02	[-0.13; 0.09]	*0.687*
	T2DM	-0.09	[-023; 0.06]	*0.252*	-0.05	[-0.23; 0.14]	*0.616*
			p for interaction	*0.588*		*p for interaction*	*0.578*
Protein-bound AGEs							
CML	NDM	-0.09	[-0.21; 0.03]	*0.143*	0.05	[-0.07; 0.016]	*0.410*
	T2DM	-0.06	[-0.21; 0.08]	*0.393*	**-0.24**	**[-0.42; -0.06]**	***0.010***
			p for interaction	*0.743*		*p for interaction*	***0.027***
CEL	NDM	***-0.23***	***[-0.33; -0.12]***	***<0.001***	-0.02	[-0.12; 0.08]	*0.703*
	T2DM	-0.05	[-0.19; 0.08]	*0.429*	**-0.21**	**[-0.38; -0.05]**	***0.013***
			p for interaction	***0.039***		*p for interaction*	***0.066***
pentosidine	NDM	-0.02	[-0.14; 0.09]	*0.717*	**0.17**	**[0.06; 0.28]**	***0.003***
	T2DM	-0.13	[-0.27; 0.01]	*0.070*	0.11	[-0.07; 0.29]	*0.214*
			p for interaction	*0.146*		*p for interaction*	*0.555*

Concentrations of α-dicarbonyls, AGEs, C3a, and sC5b-9 were standardized. Concentrations of AGEs and C3a were log_2–transformed prior to standardization. **NDM**, analyses were performed in individuals without diabetes (n=392). **T2DM**, analyses were performed in individuals with T2DM (n=138). All adjusted for age, sex, waist circumstance, lifestyle (smoking status, alcohol consumption, physical activity, and energy intake), and medication use (glucose-lowering, lipid-modifying, and/or anti-hypertensive). **Abbreviations:** sC5b-9, soluble C5b-9; MGO, methylglyoxal; GO, glyoxa; 3-DG, 3-deoxyglucosone; AGEs, advanced glycation end products; CML, N^{ε}-(carboxymethyl)lysine; CEL, N^{ε}-(carboxyethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

Table S3. Sensitivity analyses for the associations of plasma α-dicarbonyls, free and protein-bound AGEs with C3a and sC5b-9

	Outcome					
	C3a			**sC5b-9**		
	β	**95 % CI**	***p***	**β**	**95 % CI**	***p***
	No chronic or acute infections; N=494 [a]					
α-dicarbonyls						
MGO	0.01	[-0.08; 0.10]	0.883	-0.02	[-0.11; 0.08]	0.740
GO	-0.05	[-0.14; 0.05]	0.327	-0.08	[-0.18; 0.01]	0.087
3-DG	-0.01	[-0.13; 0.10]	0.831	0.03	[-0.09; 0.14]	0.670
Free AGEs						
CML	0.03	[-0.06; 0.12]	0.518	-0.02	[-0.11; 0.08]	0.727
CEL	-0.04	[-0.13; 0.05]	0.383	-0.06	[-0.15; 0.04]	0.245
MG-H1	-0.04	[-0.13; 0.05]	0.399	-0.02	[-0.11; 0.08]	0.737
Protein-bound AGEs						
CML	-0.08	[-0.18; 0.01]	0.088	-0.05	[-0.15; 0.05]	0.316
CEL	**-0.18**	**[-0.26; -0.09]**	**<0.001**	**-0.09**	**[-0.18; 0.00]**	**0.042**
pentosidine	-0.05	[-0.14; 0.04]	0.300	**0.14**	**[0.04; 0.23]**	**0.006**
	No autoimmune disease; N=459 [b]					
α-dicarbonyls						
MGO	-0.01	[-0.1; 0.08]	0.805	0.01	[-0.09; 0.11]	0.832
GO	-0.06	[-0.15; 0.04]	0.253	**-0.11**	**[-0.20; -0.01]**	**0.038**
3-DG	-0.01	[-0.12; 0.10]	0.836	0.06	[-0.05; 0.18]	0.280
Free AGEs						
CML	0.00	[-0.09; 0.10]	0.933	-0.03	[-0.12; 0.07]	0.572
CEL	-0.07	[-0.16; 0.03]	0.158	-0.08	[-0.18; 0.01]	0.096
MG-H1	-0.05	[-0.14; 0.05]	0.329	-0.05	[-0.15; 0.04]	0.285
Protein-bound AGEs						
CML	**-0.11**	**[-0.21; -0.01]**	**0.029**	-0.06	[-0.16; 0.04]	0.263
CEL	**-0.20**	**[-0.28; -0.11]**	**0.000**	-0.09	[-0.18; 0.00]	0.054
pentosidine	-0.05	[-0.15; 0.04]	0.284	**0.14**	**[0.04; 0.24]**	**0.006**
	No malignant disease and/or cancer; N=508 [c]					
α-dicarbonyls						
MGO	0.01	[-0.07; 0.10]	0.741	0.03	[-0.06; 0.12]	0.568
GO	-0.06	[-0.15; 0.03]	0.168	**-0.11**	**[-0.20; -0.02]**	**0.018**
3-DG	0.01	[-0.10; 0.12]	0.846	0.09	[-0.02; 0.20]	0.111
Free AGEs						
CML	0.00	[-0.09; 0.09]	0.969	-0.02	[-0.11; 0.07]	0.701
CEL	-0.06	[-0.15; 0.03]	0.200	-0.07	[-0.16; 0.02]	0.114
MG-H1	-0.05	[-0.14; 0.04]	0.273	-0.03	[-0.12; 0.06]	0.524
Protein-bound AGEs						
CML	-0.08	[-0.17; 0.01]	0.096	-0.06	[-0.16; 0.03]	0.213
CEL	**-0.17**	**[-0.25; -0.08]**	**<0.001**	-0.09	[-0.17; 0.00]	0.051
pentosidine	-0.06	[-0.15; 0.03]	0.201	**0.14**	**[0.05; 0.24]**	**0.002**
	No liver disease; N=524 [d]					
α-dicarbonyls						
MGO	0.01	[-0.07; 0.10]	0.749	0.02	[-0.07; 0.11]	0.602
GO	-0.05	[-0.14; 0.04]	0.273	**-0.11**	**[-0.20; -0.02]**	**0.018**
3-DG	0.01	[-0.10; 0.11]	0.925	0.07	[-0.04; 0.18]	0.194
Free AGEs						
CML	0.01	[-0.08; 0.10]	0.834	-0.02	[-0.11; 0.07]	0.712
CEL	-0.05	[-0.13; 0.04]	0.286	-0.06	[-0.15; 0.03]	0.163
MG-H1	-0.05	[-0.14; 0.04]	0.298	-0.03	[-0.12; 0.06]	0.573
Protein-bound AGEs						
CML	-0.08	[-0.17; 0.01]	0.074	-0.05	[-0.14; 0.05]	0.327
CEL	**-0.17**	**[-0.25; -0.08]**	**0.000**	-0.08	[-0.16; 0.01]	0.073
pentosidine	-0.05	[-0.14; 0.04]	0.278	**0.15**	**[0.05; 0.24]**	**0.002**

Concentrations of α-dicarbonyls, AGEs, C3a, and sC5b-9 were standardized. Concentrations of AGEs and C3a were $\log_2$–transformed prior to standardization. All adjusted for age, sex, waist circumstance, lifestyle (smoking status, alcohol consumption, physical activity, and energy intake), and medication use (glucose-lowering, lipid-modifying, and/or anti-hypertensive). **a**, 36 participants with acute or chronic infections were excluded. **b**, 71 participants with a (suspected) history of autoimmune disease were excluded. **c**, 22 participants with a self-reported current malignant condition/cancer were excluded. **d**, 6 participants with a self-reported current liver disease were excluded. **Abbreviations:** sC5b-9, soluble C5b-9; MGO, methylglyoxal; GO, glyoxal; 3-DG, 3-deoxyglucosone; AGEs,

advanced glycation end products; CML, N^{ε}-(carboxymethyl)lysine; CEL, N^{ε}-(carboxyethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

Table S4. Associations of *GLO1* polymorphism with plasma α-dicarbonyls, free and protein-bound AGEs (n=504)

		rs2736654					*rs1049346*				
		TT (reference)	*GT*		*GG*		*GG* (reference)	*AG*		*AA*	
	Model		β	*p*	β	*p*		β	*p*	β	*p*
Outcomes											
MGO	1	–	-0.18	0.074	-0.19	0.156	–	0.09	0.446	0.02	0.861
	2	–	**-0.21**	**0.036**	-0.15	0.258	–	0.09	0.410	0.08	0.508
Free AGEs											
CML	1	–	-0.04	0.717	-0.21	0.099	–	-0.10	0.340	-0.15	0.232
	2	–	-0.04	0.708	-0.18	0.160	–	-0.14	0.197	-0.19	0.120
CEL	1	–	-0.10	0.326	-0.12	0.375	–	-0.11	0.317	0.09	0.478
	2	–	-0.11	0.271	-0.08	0.529	–	-0.14	0.212	0.09	0.478
MG-H1	1	–	-0.03	0.776	-0.01	0.961	–	-0.08	0.443	0.02	0.845
	2	–	-0.01	0.885	0.04	0.739	–	-0.14	0.198	-0.04	0.751
Protein-bound AGEs											
CML	1	–	-0.06	0.576	-0.03	0.796	–	-0.05	0.625	0.08	0.513
	2	–	0.01	0.908	-0.11	0.355	–	-0.07	0.511	0.03	0.824
CEL	1	–	-0.16	0.121	-0.00	0.990	–	0.02	0.831	0.05	0.697
	2	–	-0.15	0.140	-0.01	0.964	–	-0.01	0.962	0.01	0.930
pentosidine	1	–	-0.03	0.757	-0.11	0.401	–	-0.08	0.491	-0.12	0.360
	2	–	-0.01	0.924	-0.18	0.141	–	-0.07	0.528	-0.10	0.417

The reference categories are the genotypes that were reported to be associated with the highest GLO1 activity (see reference 25-27 in the main text). Concentrations of MGO and AGEs were standardized. Concentrations of AGEs were $\log_2$ –transformed prior to standardization. **Model 1**: adjusted for age and sex. **Model 2**: **Model 1** + waist circumstance, lifestyle (smoking status, alcohol consumption, physical activity, and energy intake), and medication use (glucose-lowering, lipid-modifying, and/or anti-hypertensive). **Abbreviations:** MGO, methylglyoxal; AGEs, advanced glycation end products; CML, N^{ε}-(carboxymethyl)lysine; CEL, N^{ε}-(carboxyethyl)lysine; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine.

Chapter 7

Summary and general discussion

Obesity is a worldwide epidemic, affecting not only adults but nowadays also children and adolescents. Globally, about 39% of adults are overweight, 13% of adults are obese, and over 18% of children and adolescents are overweight or obese in 2016 (World Health Organization (1)). People with obesity are at high risk of chronic metabolic disorders such as dyslipidaemia, hypertension, metabolic syndrome, type 2 diabetes mellitus (T2DM), and cardiovascular disease (CVD) (2). Obesity is also associated with increased mortality from various causes, in particular CVD (3). According to the literature, about 4 million of deaths worldwide among adults were attributable to overweight and obesity in 2015, of which the most common cause was CVD (4). The higher presence of obesity and associated comorbid conditions leads to increased morbidity, mortality, which will cause higher medical and economic burdens. Therefore, exploration of the aetiology for obesity and related comorbid diseases is urgently needed.

The complement system is part of the innate immune system and, as such, a system for host defence. It is now becoming increasingly clear that complement is also involved in whole body homeostasis and metabolism. Many complement factors can be produced by adipose tissue as well as other metabolic organs such as the liver and the pancreas and can contribute to metabolic (dys)regulation (5, 6). Until recently, human studies on the associations between the complement system and metabolic disorders were mostly focused on the central complement component, C3 (7-9). Activation of C3 results from activation of the classical, the alternative, or the lectin pathway, and can lead to activation of the terminal pathway (10). Previous work from our group and others has indicated distinct roles of these complement pathways in metabolic disorders in humans. Most of these studies pointed towards an adverse association of the alternative pathway (11-13), while a few showed also adverse associations of classical and terminal pathway activation, with metabolic disorders (8). The lectin pathway was reported to play a dual role especially in insulin resistance and CVD (8, 14). Therefore, the major objective of this thesis was to investigate the associations of the components involved in activation of the alternative pathway, and to some extent also the classical and terminal pathway, with obesity and related metabolic disorders, such as dyslipidaemia, metabolic syndrome, and T2DM. Our studies were performed in a human observational cohort study, the CODAM study. In this

final chapter, we summarize the main findings and place them in the context of the available literature. We additionally discuss possible methodological considerations in our studies, and address several future research directions.

1 Main findings

In **chapter 2**, we investigated longitudinal associations of complement components of the alternative (C3, C3a, Bb, factor D, factor H, and properdin) and terminal (C5a, sC5b-9) pathways with adiposity (BMI and waist circumstance). We found that, during a 7-year period, higher baseline plasma concentrations of most alternative pathway components (i.e. C3, C3a, factor D, factor H, and properdin), but not Bb, an alternative pathway activation product, were associated with more adiposity, i.e. higher BMI and waist circumstance. The terminal pathway activation products (i.e. C5a, or sC5b-9) were not associated with the development of adiposity. These observations point towards a role for the alternative pathway of complement activation in the development of human obesity. This may be attributed to the effect of C3-C3a-C3adesarg/ASP axis on lipid metabolism and adipocyte differentiation that was demonstrated in previous experimental studies (5, 15). We further evaluated the associations between changes in complement concentrations and changes in adiposity over time. These latter analyses were performed for C3, factor D, factor H, C5a, and sC5b-9. We only observed significant positive associations for C3 and factor H. This observation suggests that the longitudinal association of C3 and factor H with adiposity may reflect both within-individual changes and interindividual differences during the 7-year period (16). For factor D, the longitudinal association with adiposity was mainly attributed to interindividual differences that remained consistent over time. These findings indicate that the longitudinal associations of various aspects of the alternative complement pathway with adiposity may be attributed to distinct underlying mechanisms.

Abnormality in plasma lipids and lipoproteins is an important risk factor for various metabolic disorders that are often seen in obesity (17). In **chapter 3**, the associations between the complement system and plasma lipoproteins were investigated. For this, we investigated the cross-sectional associations of the central complement component, C3,

and other complement regulators and activation products of the alternative pathway (C3a, Bb, factor D, factor H, properdin, and MASP-3) with the distribution and lipid composition of 14 lipoprotein subclasses. We showed that a higher concentration of C3 was associated with an adverse lipoprotein distribution and composition, i.e. more TG-enriched lipoproteins and fewer larger-sized HDL lipoproteins. Consistent but weaker associations were observed for properdin, factor H, factor D, and MASP-3 (effect size in descending order). In addition, we found that the associations for most components (i.e. properdin, factor D, and MASP-3 but not factor H,) were largely independent of C3. If we take into account the lack of associations for the alternative pathway activation products (i.e. C3a and Bb), it is possible that instead of representing the activation of the alternative pathway, these complement components may themselves play a distinct role in lipid and lipoprotein metabolism. Although the underlying mechanisms are not fully understood, the causal role of some complement proteins in lipid metabolism has been demonstrated in several genetically modified animal models (18-20). We found that the observed associations with lipoproteins were (largely) independent of obesity, insulin resistance, low-grade-inflammation, and liver enzymes, the major biological pathways that are involved in lipid and lipoprotein metabolism. Previous human studies demonstrated the presence of complement proteins on plasma VLDL, LDL, and HDL lipoproteins, of which C3 was the most dominant complement component (21-23). This raises the possibility that complement components, especially C3, could have a direct physical effect on the metabolism of these lipoproteins, which might lead to an altered distribution and composition of the lipoprotein subclasses. In addition, it has been shown that under pathological conditions, plasma HDL can be modified into a dysfunctional, pro-inflammatory particle that contains more inflammatory proteins (24). In line with this, our results suggest that some (dysfunctional) lipoprotein subfractions may have substantial affinity for binding complement components (22, 24). Taken together, our observations suggest that the alternative complement pathway, especially C3, was associated with a pro-atherogenic lipoprotein profile. This may be a possible mechanism through which these complement components can contribute to dyslipidaemia and cardiometabolic disorders.

In **chapter 4**, we investigated the cross-sectional and prospective associations of complement proteins, regulators and activation products from the alternative (i.e. C3, C3a, Bb, FD, FH, and properdin) and classical (i.e. C1q, C1-INH, and C4) pathway, with prevalence and incidence of the metabolic syndrome. We showed that not only C3 and C4 but also several regulators and activation products of the alternative pathway (i.e. C3a, factor H, and properdin) are indeed positively associated with the prevalence of the metabolic syndrome. However, in the prospective analyses, only complement C3 and C4, but none of their regulators or activation products, were associated with the development of the metabolic syndrome. This suggests that the relation of C3 and C4 with the development of the metabolic syndrome might be independent of traditionally recognized complement activation. For instance, recent findings showed that C3 could be activated intracellularly by cathepsin L, without the presence of C3 convertase, and this intracellular activation of C3 was involved in T-cell homeostasis (25). This suggests a specific biological effect of C3, that is independent of complement proteins and regulators of the activation pathways. We also explored potential underlying mechanisms for the associations we observed for C3 and C4 with incident metabolic syndrome. We found that the prospective association for C3 can be explained by the baseline profile of components of the metabolic syndrome, as well as plasma concentrations of liver enzymes. Thus, higher baseline C3 concentration was related to a worse metabolic profile and probably also related to impaired liver function, via which it promotes the development of metabolic syndrome. However, the prospective association for C4 was independent of the baseline metabolic syndrome profile and also independent of the other potential pathways that were explored. This may suggest that C3 and C4 have distinct effects on (metabolic) pathways that lead to development of the metabolic syndrome.

In **chapter 5**, we evaluated longitudinal associations of components of the alternative complement pathway (C3, C3a, Bb, factor D, factor H, properdin, and MASP-3) with insulin sensitivity/resistance (Matsuda index, Stumvoll's index, and HOMA2-IR), β-cell function (basal and overall insulin secretion rate, C-peptidogenic index, β-cell glucose sensitivity, β-cell potentiation, and β-cell rate sensitivity), and T2DM. The most prominent observation was that, over the 7-year period, a higher baseline C3 concentration was associated with

less insulin sensitivity, indicating worse insulin resistance. This association was independent of several potential confounders and β-cell function. We also found that higher C3 was associated with more insulin secretion, which was largely attributed to insulin resistance. Although at first sight C3 also appeared to be associated with worse β-cell function, these associations were mostly confounded by other factors such as obesity, or related to insulin resistance. Similar but weaker associations were observed for several alternative pathway complement regulators, i.e. properdin, factor H, and MASP-3 (effect size in descending order). One possible explanation for these observations is that the complement components may contribute to obesity-induced whole body insulin resistance via their pro-inflammatory properties, and at the same time may induce insulin secretion by islets to compensate for the inflammation-induced insulin resistance. In this way, complement may serve as a metabolic feedback system in response to insulin resistance (26). In addition, based on the available experimental data, mostly obtained in rodents, alternative pathway activation was anticipated to be positively associated with some measures of better β-cell function. In contrast, we found almost no associations for the activation product C3a and a positive association for another activation product Bb with insulin sensitivity. We hypothesized that this might be due to intrinsic differences between human and rodent islets, and that in humans it may be the activation of C5 and the terminal pathway, rather than C3 and the alternative pathway, that is associated with enhanced β-cell function. Lastly, C3 was the only complement factor that was found to be associated with the 7-year incidence of T2DM. This association was mediated by insulin resistance, but not by β-cell function. Taken together, these findings indicate multiple and dynamic roles of (activation of) alternative pathway in the development of T2DM in humans, which at the whole body level mainly points to an association with worse insulin resistance rather than better β-cell function.

The most prominent metabolic disturbance in T2DM is hyperglycaemia, which may underlie the development of various complications that are observed in T2DM patients. Hyperglycaemia can cause, among others, dicarbonyl stress (27, 28). In the circulation, dicarbonyl stress may be reflected by a higher presence of glycating agents, i.e. α-dicarbonyls (e.g. MGO, GO, 3-DG), as well as related AGEs (e.g. free and protein-bound CML,

CEL, free MG-H1, and protein-bound pentosidine) (27). Data in the literature suggested that the functionality of complement inhibitors is hampered by glycation (29-31) and glycated complement membrane inhibitors were observed in diabetes patients (29, 31, 32). We therefore hypothesized that dicarbonyl stress may induce the glycation of complement inhibitors and impaired their regulatory function, leading to enhanced complement activation. In **chapter 6**, we explored the possible relationship of dicarbonyl stress with complement activation (as reflected by plasma levels of the activation products, C3a and sC5b-9). We found distinct associations of these markers of dicarbonyl stress with complement activation: in the whole population, GO was not associated with C3a and was inversely associated with sC5b-9, while MGO, 3-DG, and free AGEs were not associated with either C3a or sC5b-9; protein-bound CEL and to some extent also protein-bound CML and pentosidine were inversely associated with C3a; and protein-bound pentosidine was positively associated with sC5b-9 while no associations were observed for protein-bound CML and CEL. We also found that the association of GO with sC5b-9 may be related to an ongoing inflammation process, and that the associations of protein-bound CML and pentosidine with C3a were explained by obesity, while the associations for protein-bound CEL with C3a and for pentosidine with sC5b-9 were independent of the potential confounders evaluated in our study. This suggests that these associations may be attributed to different underlying mechanisms. In line with this, we found that the presence of T2DM, which may lead to a higher burden of glycation and dicarbonyl stress, influenced some, but not all of, the association between dicarbonyl stress and complement activation. Notably, the positive association of protein-bound pentosidine with sC5b-9, which is the only association in this study that was in line with our hypothesis, was not influenced by diabetes status. Previous human studies indicated that plasma protein-bound pentosidine may better represent tissue AGE accumulation (33-35). Therefore, this positive association may reflect the effect of dicarbonyl stress on complement activation in tissues. In addition, when we evaluated associations of potential markers for life-long MGO stress, i.e. two functional polymorphisms in the MGO-detoxifying enzyme GLO1, the direction of association with complement activation was unexpected. *Rs2736654* was not associated with either C3a or sc5b-9, while the genotypes that were reported to have less GLO1 activity (hence greater

dicarbonyl stress) of *rs1049346*, were associated with lower sC5b-9 concentration (hence less activation of the terminal pathway). *Rs1049346* was also not associated with the C3a concentration. Overall, the associations of plasma dicarbonyl stress markers with complement activation, mostly pointed towards an inverse direction, except for protein-bound pentosidine.

2 Methodological considerations

2.1 Potential sources of bias in observational studies

In observational studies, the association of interest can be improperly estimated and interpreted as a result of bias (systematic error) and/or confounding (distortion of the association) (36).

2.1.1 Selection bias

In a (population-based) cohort study, selection bias occurs when the selection or retention of the participants is related to both the exposure(s) and the outcome(s) that are evaluated in the association of interest (36). At the enrolment stage, selection bias is usually a result of nonresponse (37). This is a common issue in observational studies. In CODAM, the nonresponse rate was at least 58%. Due to self-selection, participants in the CODAM study may have a higher education level and health consciousness, thus usually have a healthier lifestyle than the general population. The result of this may be that CODAM participants represent a relatively healthy subset of the target population. Such a selection bias may harm the generalizability of our results, but is less likely to affect internal validity.

In prospective studies, selection bias is mainly caused by loss to follow-up (38). The proportion of participants that was lost to follow-up was 14% in CODAM and those who were lost to follow-up had at baseline a somewhat worse health status (e.g. were more likely to use medication and had a higher prevalence of T2DM). In **chapter 4 and 5,** the outcome was the incidence of disease (the metabolic syndrome and T2DM, respectively). In these analyses, we excluded the participants without follow-up data. The relatively

healthy individuals that remained may have had lower risks to develop metabolic syndrome and/or have been progress to T2DM. Therefore, in these chapters, loss to follow-up may have caused some differential misclassification with unknown effect on the estimation of the effect size. However, in the sensitivity analyses in our evaluations, we did not find influences of medication use or diseases status on our observations. This may mean that our findings are not likely to be affected by health status. In **chapter 2**, on the other hand, the levels of most exposures (i.e. the concentrations of the complement components) and outcomes (BMI and waist circumference) at baseline did not differ between those who were lost to follow-up and those who attended the follow-up. Therefore, in this chapter our results might have been affected by non-differential misclassification, leading to underestimation of the real association due to the diminution in the number of participants. But taken together, given the relatively high retention rate in CODAM, we expect the effects of loss-to-follow up to be quite modest.

2.1.2 Information bias

Information bias occurs when the exposures and/or outcomes are not well measured and defined (36). In the evaluations presented in this thesis, the exposures and outcomes were often biomarkers that were measured in blood samples (e.g. levels of complement components, lipoproteins, dicarbonyls, and AGEs), or were defined based on laboratory or physical measurements (e.g. adiposity, metabolic syndrome, insulin resistance, β-cell function, and T2DM). These measurements were performed in duplicate by trained researchers who were not aware of the other characteristics of the participants. Therefore, errors in these measurements are likely to be randomly distributed across the study population, which may lead to non-differential misclassification, and thus greater uncertainty in the estimation of the strength of an association. The result of this may be that some of the (weaker) true associations may have been missed in our analyses. On the other hand, the various associations that we did observe and discuss are likely to be true findings. In addition, some covariates in our studies, such as lifestyle factors (smoking status, calorie intake), were obtained by a self-reported questionnaire. This kind of health-related

information could be recalled differently by participants with a worse health status, such as those with obesity or T2DM, than by healthy participants. Therefore, the use of questionnaires to collect information may have resulted in some differential misclassification of these data. In our evaluations, these variables were mainly used as potential confounders, so such differential misclassification may have resulted in some residual confounding (39).

2.1.3 Confounding

Confounding describes the situation when there is a third factor that is associated with the exposure and at the same time is a risk factor for the outcome, but is not included in the causal path between the exposure and the outcome (36). In case of an existing observational cohort, as was used in this thesis, it can be reduced by statistical strategies such as stratification or multivariate analysis after the completion of the study (40). In the evaluations presented in this thesis, we therefore adjusted for a substantial number of potential confounders, including age, sex, lifestyle factors, and use of medication, when investigating the association of interest.

In the field of biology, the relationship between two factors is not always straightforward. Variables considered as potential confounders in the evaluations presented in this thesis sometimes have multiple associations with the exposure and/or the outcome. Under such conditions, adjusting for these variables simply as confounders may bias the results and harm the estimation of the real associations (41). For instance, in **chapter 3**, in the association of complement system with lipoproteins, obesity may be considered a potential confounder because many complement components are partly derived from adipose tissue and higher plasma levels of complement were demonstrated in people with obesity (42). At the same time, data in the literature showed that complement may be involved in the development of obesity (43), which would place obesity in the causal path between complement and lipoprotein (metabolism), and in that case it should be regarded as a potential mediator, and adjusting for obesity may cause overadjustment and lead to underestimation of the association (41). Another example is in

chapter 6, in the association of dicarbonyl stress with complement activation, renal function may be regarded a potential confounder since the kidney is involved in clearance and metabolism of plasma AGEs (44), and complement activation was also involved in diverse renal diseases (45). On the other hand, accumulation of AGEs in the kidney was reported to contribute to worsening of renal function (46), and complement activation may contribute to the progressive loss of renal function (45). In the latter situation, renal function may be regarded a consequence of dicarbonyl stress and complement activation, in which cases it can be regarded as a collider (i.e. a variable that can be affected by both exposure [in this case dicarbonyl stress] and outcome [in this case complement activation]). Adjusting for renal function can thus cause collider bias, which may introduce associations where there are none (47). Therefore, in our evaluations, we used separate models to adjust for a potential confounder that could also be a mediator or collider in the association of interest, and interpreted the results with care. In addition, even if the variable is not in the causal path, adjustment for a variable that is measured more precisely than the exposure can lead to the underestimation of real association (48). In our studies, some covariates, such as BMI and lifestyle factors (e.g. use of medication), may have been measured more precisely than the exposure, which are mostly blood markers and may suffer from both technical (measurement) error and biological variation. Adjusting for them may have affected our results and attenuated the causal relationship. Again, result of this could be that some of the (weaker) true associations may have been missed in our analyses, but the associations that we did observe are likely to be true.

Overall, failure to adjust for potential confounding may lead to incorrect estimation of the association. On the other hand, adjustment for mediator, collider, or variable with better precision may also bias the results. Therefore, when investigating the relationship between two variables by using a multivariate strategy, we should always take into account the intricate biological associations between variables and interpret the results from different points of view and with caution.

2.2 External validity

External validity describes to what extent the results of a study can be extrapolated to other populations, which is also called generalization (49). The CODAM study is an observational cohort study that consists of middle-aged to elderly Caucasian individuals with an increased risk of cardiometabolic diseases. The criteria that were used to selection our study population may have limited the generalization of our findings to other population groups with different geographic distribution, ethnicities, health status, and/or age-range. However, the primary goal of the evaluations in this thesis was to explore the aetiological role of complement in obesity and related metabolic disorders. Therefore, to provide valid results that were less biased had higher priority than generalizability. In addition, the participants of CODAM study do represent a large proportion of Western populations, particularly those who are relatively older and have higher risks to develop T2DM and CVD over time. Thus, our findings can be extrapolated to this subpopulation and may add novel knowledge on the mechanisms involved in the high presence of metabolic disorders in this population.

2.3 Causality

The main purpose of the research presented in this thesis was to investigate the possible aetological role of the complement system in obesity and related metabolic disorders. All evaluations were conducted in a human observational study that has known limitations with respect to assessment of causal relationships (50), with which we have dealt to the best of our capabilities. In the cross-sectional analyses, the exposures and outcomes were simultaneously assessed and the direction of the relationship may therefore be unclear. In the GEE analyses in which both baseline and follow-up data are included, the results reflect a combination of within and between subject effects and therefore suffer from the same limitation, although the power is likely better. The causal inference of our observations is supported by the fact that our hypotheses were based on numerous experimental studies, e.g. genetically modified mouse models or cell work, where the potential confounders are

better controlled than non-experimental studies and thus the causal relationship is more pronounced (51). In addition, the comprehensive adjustment for potential confounders in our analyses could strengthen the possibility of a causal relationship (51).

3 Implications and future perspectives

People with obesity are vulnerable to various metabolic disorders that may progress to advanced disease outcomes, such as T2DM and CVD. Increased prevalence of obesity and obesity-related metabolic disorders can thus lead to higher health and economic burden (17). The exploration of aetological factors involved in these pathophysiological processes can provide novel knowledge on prevention and/or treatment strategies. In this thesis, by using a human observational cohort study that consisted of participants with a moderately increased risk of cardiometabolic disease, we showed that the complement system, especially the alternative pathway, was associated with obesity and related metabolic disorders. Although the association of C3 with several metabolic disorders had already been quite widely studied in humans (7-9), the evidence for other alternative pathway components had so far been limited. The novel data presented in this thesis provide several opportunities for future research. These future evaluations should perhaps not all be done in similar cohorts given the different lines of information that were yielded.

Clearly, the findings in our cross-sectional studies need further evaluation in prospective studies to assess the temporality of the relations. For instance, the association between baseline levels of complement components and the development of dyslipidaemia will need to be addressed. Likewise, the associations between (trajectories of) changes in dicarbonyl stress and changes in complement activation, over several time-points, will provide information on the temporality of their relationship. Also, the results will have to be confirmed in, and expanded to, larger population-based Caucasian studies with detailed phenotypes and high-quality biobank materials such as for instance the Maastricht study (52). Moreover, taking into account the requirement of generalization, future studies are needed that replicate our findings in populations with non-Caucasian ethnicities, particularly those that comprise large numbers of individuals and face a steeply increasing

prevalence of obesity such as e.g. the Asian (e.g. Chinese) and South-Asian (e.g. Indian) populations (53).

The stratified analyses that were based on diabetes status and the analyses of incident diseases that are presented in this thesis (**chapter 4, 5, and 6**), are potentially very interesting but are currently limited by the relatively small sample sizes that underlie these results. These observations therefore need to be confirmed in larger cohorts. Also for this purpose, the above-mentioned Maastricht Study is of great interest since it is enriched with individuals with type 2 diabetes (52) .

In this thesis, we also present some unexpected observations. For instance, in **chapter 5** we showed that, in CODAM, the pathways that underlie the associations of C3 and C4 with development of the metabolic syndrome may differ. C4 is a main component of the classical and lectin pathways, while C3 is a main component of the alternative pathway and the amplification loop. Since these three complement activation pathways all converge on C3 and the amplification loop, these data suggest that the proximal part of the classical and lectin pathway may be associated with development of the Metabolic Syndrome via currently unknown mechanisms. Identification of such mechanisms may open new routes towards intervention in the development of the metabolic syndrome. However, given the small numbers of incident cases of the metabolic syndrome in CODAM, these observations first need to be confirmed in a larger prospective cohort. Likewise, in **chapter 6**, we observed partly unexpected associations of plasma markers of dicarbonyl stress with complement activation products. Therefore, we need to further investigate how well these plasma dicarbonyl stress markers can represent the intracellular or tissue glycation burden. To identify a convincing and accessible measurement for tissue dicarbonyl stress, as well as its association with complement activation, may be another direction of future research. Investigation on the proportion of glycated complement components compared to the total amount may also help to better understand the observed results.

Although strong conclusions on causality cannot be drawn based on our results obtained in an observational human study, the fact that higher baseline levels of some complement components were associated with the incidence of the metabolic syndrome (C3 and C4) and T2DM (C3) suggests that they may have a potential causal or at least

prognostic role in these disorders. Therefore, early interventions, such as promoting a healthy lifestyle and improved control of already exist abnormalities (obesity, impaired glucose metabolism, dyslipidaemia) in people with higher plasma complement levels may have beneficial effects. Several experimental studies showed that deficiency in complement components (54, 55), or blocking their effector pathways (56, 57), can protect study animals from diet-induced metabolic disorders. Therefore, modulation of the complement system may be a promising strategy in the prevention and treatment of these metabolic disorders. However, the multiple functions of the complement system, e.g. function in innate immunity, inflammation, cell and tissue homeostasis, and in cancer, makes it challenging to develop complement-targeted drugs (6, 58). To date, clinically available complement inhibitors are very few and expensive, mainly limited to two types of complement therapeutic: an anti-C5 antibody (eculizumab), and a C1 esterase inhibitor (Sanquin, Pharming) (59). Their clinical application is also limited to the treatment of rare diseases, such as paroxysmal nocturnal haemoglobinuria and atypical haemolytic uraemic syndrome. Therefore, the development of a new generation of complement-modulating therapeutic with potential implications for general metabolic diseases and accessible cost is eagerly awaited. Fortunately, there are already some “alternative” solutions (8). For instance, lifestyle intervention (physical exercises (60, 61) and healthy diet (62-64)) and some commonly prescribed medications (65-68) appear to beneficially affect (i.e. lower) some systemic complement concentrations.

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Valorisation addendum

The ultimate aim of scientific research is to transfer the research findings into practice, via which they can not only contribute to academic but also to society and economy. This process is called valorisation. In this chapter, the social and economic value of our findings and how they can be translated into practical use are discussed.

1 Social and economic relevance

Obesity is a rising epidemic worldwide. According to a survey of 195 countries in 2017, the prevalence of obesity has continuously increased and has doubled in most of the countries in the past 3 decades (1). The higher prevalence of obesity can have a negative impact on society and the economy (2). First, obese individuals have a lower quality of life due to impaired psychological and physical health (3). For instance, individuals with obesity may experience emotional and psychological problems (e.g. lower self-esteem, anxiety, and depression) that result from perceived lower social acceptance. They may also experience physical problems, such as back pain and shortness of breath. Such psychological and physical impairments in obese individuals can lead to a lower employment rate, which may further reduce their quality of life. Second, obese individuals are at a higher risk for obesity-related diseases, such as hypertension, T2DM, CVD, and cancer, which are related to higher morbidity and mortality. In 2015, global deaths among adults due to high BMI (BMI equal to or greater than 22.5 kg/m^2, which is the midpoint of the optimal range of 20-25 kg/m^2) were estimated to be 4 million, and about 60% of them occurred in obese individuals (1). In addition, the above-mentioned obesity-related issues can all contribute to increased economic burden on the individuals, their family, and the society due to higher costs of health care, as well as productivity losses (4). In 2014, the estimated worldwide economic impact of obesity is 2.0 trillion dollars, which equals 2.8 percent of global gross domestic product (5). Therefore, efforts on controlling the (global) prevalence and incidence, as well as the (health) consequences, of obesity is in urgent need. For this purpose, steps could be taken to explore the related intervention programs and to provide better predictions of obese individuals with higher risk to develop comorbid conditions. In addition, we must

obtain better knowledge of the aetiology of obesity and its health consequences prior to these attempts.

In this thesis, we showed that the complement system, especially the alternative complement pathway, was associated with the prevalence and the development of adiposity. We also found that higher plasma levels of (some) alternative pathway components were associated with several common metabolic disorders related to obesity, including adverse lipoprotein profile, higher prevalence and incidence of metabolic syndrome, worse insulin resistance and higher prevalence and incidence of T2DM. Although our present findings cannot be directly applied to real practice and policy development, it proposed the possibility for plasma complement components to serve as candidate biomarkers in risk prediction of these obesity-associated metabolic disorders, and as a novel target for disease treatment.

2 Target group

Our main findings on the possible aetiological role of the complement system in obesity and related metabolic disorders can benefit other academics by adding novel knowledge on the pathophysiological mechanisms on obesity and its complications. For instance, the current results can provide basis for relevant future intervention programs by inducing the possibility of plasma complement markers as intervention targets or as treatment response markers. It can also promote the pharmaceutical companies to develop novel therapeutic drugs targeting complement reduction for the treatment of chronic metabolic disease. In addition, our findings on the potential predictive and therapeutic value of these blood complement markers can help healthcare professionals with decision making in medicine and health care, after its clinical application.

3 Innovation and implementation

Our studies investigated in humans the associations of complement, not only the most discussed central complement factor C3 but also its regulators and activation products, with

a series of obesity-related metabolic disorders. Our current findings confirmed and expanded the previously reported role of C3 in cardiometabolic diseases in our study population. For the associations of other complement components with these disorders, our data fill the knowledge gap in humans, since such information was, previously, mainly derived from animal studies. We observed in **chapter 4** and **chapter 5**, positive associations of C3, but not other complement regulators and activation products, with the incidence of metabolic syndrome and T2DM. Our results thus indicate potential biological effects for individual complement components, which may independent of complement activation, the commonly accepted underlying route in these pathophysiological processes. We also found in **chapter 3** that the associations of most alternative pathway components with lipoproteins were independent of C3. In **chapter 6**, we showed the intricate associations of plasma markers of dicarbonyl stress with complement activation. It can facilitate future research on the underlying mechanisms of the association between them, which may contribute to a better understanding of cardiovascular complications in diabetes.

In this thesis, we observe elevated plasma levels of alternative pathway components in metabolic disorders in middle-aged to elderly Caucasian individuals with moderately increased risk of cardiometabolic disease. In our prospective studies in **chapter 2**, **3**, **4**, we also show that higher plasma level of C3, and to a lesser extent C4 and factor H, are associated with the development of obesity and related metabolic disorders, such as metabolic syndrome and T2DM. Our findings thus suggest a potential role for plasma complement as predictive biomarkers in clinical practice. They can also contribute to personalized health care by their potential function as a supplementary marker for classical risk factors in selecting high-risk individuals. Other potential future applications of our findings include the preventive and therapeutic value of plasma complement markers in the treatment of chronic metabolic disorders. For instance, they could help to guide the therapeutic interventions of these diseases. The effect of lifestyle interventions (6-10) and some commonly prescribed medications (11-14) on limiting systemic levels of some complement components have been reported previously. This potential beneficial effect on complement and its application in diseases prevention and treatment should be further investigated in large-scale randomized clinical trials. In addition, plasma levels of these

complement markers can be used to evaluate the effect of medical treatment on these metabolic diseases. Lastly, our findings raise the possibility of a novel therapeutic strategy for these metabolic diseases that targeting the reduction of blood complement levels. As discussed in **chapter 7**, future studies are needed to develop sensitive, accurate, effective, and lower cost complement modifying drugs, which take into account the multiple functions of the complement system in humans. Possible directions for drug development may include anti-complement antibodies, small molecular inhibitors for complement, as well as blockers for the relevant effector pathways. Nevertheless, further technical and clinical validation and standardization of these complement markers are necessary prior to their clinical application. Plasma level of C3, the most abundant complement components, is widely used in the clinic and is generally regarded as a marker of inflammation or immune response that are most likely linked to diseases like acute infection or autoimmune disease. Similar to what is seen in other human studies investigating the cardiometabolic risk of C3 in the general population or in specific populations (e.g. individuals with obesity or T2DM), the distribution of C3 in our study population is mostly within the normal reference range (15). Although a moderately higher C3 concentration has been consistently shown to be present in and/or to predict chronic metabolic diseases, a reliable cut-off reference for abnormal C3 concentration under these disease conditions is still lacking. Future exploration of this aspect, which should also take into account the characteristics of the target population (e.g. age, sex, ethnicity), is needed for its clinical use.

Taken together, with the investigations in this thesis we show a potential role of complement in obesity and related metabolic disorders. Our results may open new paths for the prediction, prevention, and/or treatment of these diseases. Efforts on validation, standardization, as well as intervention programs will be critical to warrant future clinical practice.

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A

Curriculum vitae

Ying Xin was born on September 8th, 1988 in Henan, China. In 2007, she was admitted to Henan University of Science and Technology and started her study in clinical medicine. In 2012, she obtained her bachelor's degree, after which she started to do her master of Medicine at Zhengzhou University. Ying obtained her medical practitioner's Qualification Certificate in 2013. She obtained her master's degree in 2015, and in the same year, she was awarded a national grant from the Chinese Scholarship Council, which sponsored her 4-year PhD study at the department of Internal Medicine of the Maastricht University. During her Ph.D. study, Ying worked on the role of the complement system in obesity and related metabolic disorders under the supervision of Dr. van Greevenbroek, Prof. Schalkwijk, and Prof. Stehouwer. The results of her research are presented in this thesis. In February 2019, she received an offer from the First Affiliated Hospital of Zhengzhou University. With this offer, she will start her resident physician training at the First Affiliated Hospital of Zhengzhou University, after which she will be specialized in Endocrinology at the same hospital.

Scientific output

List of publications

- **Xin Y**, Hertle E, van der Kallen CJH, Schalkwijk CG, Stehouwer CDA, van Greevenbroek MMJ. Longitudinal associations of the alternative and terminal pathways of complement activation with adiposity: The CODAM study. Obesity Research & Clinical Practice, 2018, 12(3): 286-292.

- **Xin Y**, Hertle E, van der Kallen CJH, Schalkwijk CG, Stehouwer CDA, van Greevenbroek MMJ. Complement C3 and C4, but not their regulators or activated products, are associated with incident metabolic syndrome: The CODAM study. Endocrine, 2018: 1-11.

- **Xin Y**, Hertle E, van der Kallen CJH, Vogelzangs N, Arts ICW, Schalkwijk CG, Stehouwer CDA, van Greevenbroek MMJ. Complement C3 and components of the alternative pathway are associated with an adverse lipid profile: The CODAM study. (Submitted)

- **Xin Y**, Hertle E, van der Kallen CJH, Schalkwijk CG, Stehouwer CDA, van Greevenbroek MMJ. The role of carbonyl stress in complement activation: The CODAM study. (Submitted)

- van Greevenbroek MMJ, **Xin Y**, van der Kallen CJH, Hertle E, Schuitemaker T, Sep S, den Biggelaar L, Eussen S, Ferrannini E, Mari A, Schalkwijk CG, Stehouwer CDA. The alternative pathway of complement is a determinant of insulin resistance, rather than β-cell function in humans: The CODAM study. (Submitted)

Oral presentations

- Annual Dutch Diabetes Research Meeting 2016 (Oosterbeek, The Netherlands): Longitudinal associations of the alternative and terminal pathways of complement activation with obesity The CODAM study.

- Annual Dutch Diabetes Research Meeting 2017 (Oosterbeek, The Netherlands): Complement proteins, regulators and activated products are cross-sectionally and prospectively associated with the Metabolic Syndrome: The CODAM study.

- Dutch Complement Symposium 2018 (Nunspeet, The Netherlands): The alternative complement pathway is associated with an adverse lipid profile: The CODAM study.

- Annual Dutch Diabetes Research Meeting 2018 (Oosterbeek, The Netherlands): Associations of the alternative complement pathway with ^{1}H-NMR lipoprotein profiles: The CODAM study.

Poster presentations

- Annual Meeting of the European Association for the Study of Diabetes 2016 (München, Germany): The alternative pathway of complement activation is longitudinal association with obesity: The CODAM study.

- European Meeting on Complement in Human Diseases 2017 (Copenhagen, Denmark): Associations of the alternative pathway and classical pathway of complement activation with incident metabolic syndrome: The CODAM study.

- Annual Meeting of the European Association for the Study of Diabetes 2018 (Berlin, Germany): Complement C3 and C4, but not their regulators or activated products, are associated with incident Metabolic Syndrome: The CODAM study.

- European Atherosclerosis Society Congress 2019 (Maastricht, The Netherlands): The alternative complement pathway is associated with an adverse lipid profile: The CODAM study.