The alternative pathway of the complement system in vascular comorbidities of obesity and type 2 diabetes

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Dissertation

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Chapter 1

General introduction

1. Complement system

As early as in the 1890s, complement was discovered. It was observed to aid antibodies to kill pathogenic microorganisms, and was therefore named 'complement' [1] Complement system is one crucial arm of innate immune system and is the bridge between innate and adaptive immunity [2, 3]. It also plays a crucial role in tissue homeostasis [4]. Complement system is a group of proteins, which consists of over 50 proteins [5]. These proteins are mainly produced in the liver and are present in plasma as well as on the cell membrane as inactive precursors [6]. Concentrations of these proteins in plasma are over 3 g/l, which accounts for a large fraction of plasma proteins [2]. Recent studies showed that complement activation occurs not only in plasma and at tissue, but also within cells [7].

1.1 The complement activation pathways

Complement can be activated by three pathways, i.e. classical pathway, lectin pathway and the alternative pathway (see figure 1.1). Once complement activation occurs, it will lead to a hierarchical proteolytic cascade [3]. The three activation pathways converge at the level of complement C3, followed by activation of the shared terminal pathway of complement activation [8]. In additional to the three canonical pathways of complement activation, a 'fourth' pathway of complement activation has been proposed in which C3 and C5 are activated by proteases of the coagulation, fibrinolysis and kinin system, which may be particularly relevant under pathophysiological conditions [9, 10]. Uncontrolled complement activation will lead to self-attack and damage of cells. For this reason, many circulating and cell-bound complement inhibitors exist that control the different steps of the complement activation cascade [6].

The classical pathway: The classical pathway was the first complement pathway discovered [6]. The classical complement pathway is initiated via its recognition

molecule C1q (the first protein involved in the classical pathway cascade). Binding of C1q to its target, e.g. antibody-antigen complexes that contain IgM or IgG, leads to activation of the C1q-associated proteases C1r and C1s. These activated serine proteases then cleave complement C4 and C2, which then form C4b2a: the classical pathway C3 convertase. C4b2a cleaves C3 into C3a and C3b [11]. C3b binds to C4b2a to form C4b2a3b: the classical pathway C5 convertase that can activate C5 by cleaving it into C5a and C5b. The classical complement pathway activation can also be activated independent of antibodies [12-15].

The lectin pathway: Mannose-binding lectin (MBL) and ficolins, which are multimeric lectin complexes, are the initiating molecules of the lectin pathway. MBL and ficolins bind to specific carbohydrate patterns, i.e. PAMPS (pathogen-associated molecular patterns) and DAMPS (damage-associated molecular patterns) on foreign microbes or altered self-surfaces. Analogous to C1q, the initiation factors of the lectin pathway circulate in complex with their serine proteases. Upon target binding, the lectin pathway is activated via enzymatic activity of MBL-associated serine proteases (MASPs). The lectin pathway shares a pattern with the classical pathway since MBL and the C1 complex are similar in structure [16]. The serine protease MASP-2 can cleave C4 and C2 to form C4b2a, a C3 convertase that is identical to the classical pathway C3 convertase.

The alternative complement pathway: Approximate half a century after the discovery of the classical pathway, the alternative pathway was proposed [17, 18]. The alternative complement pathway is initiated by spontaneous hydrolysis of C3 forming C3(H_2O) [19]. In the fluid phase, C3(H_20) can bind to factor B and interact with factor D to form C3(H_20)Bb, which is an alternative pathway C3 convertase. This convertase cleaves C3 into C3b and C3a. Similar to C3(H_2O) in the fluid phase, C3b on the surface also binds with factor B and interacts with factor D to form C3bBb, the alternative pathway C3 convertase [11]. C3b generated by alternative C3 convertases, but also C3b generated by C3 convertases of the classical and the lectin pathway, can generate more

C3 convertases by interacting with factors B and D, thus acting as an amplification loop [14]. C3bBb cleaves C3 into C3a and C3b, which binds to C3bBb to form C3bBbC3b: the alternative pathways C5 convertase.

The terminal complement pathway: C5b, which is generated upon activation of all complement pathways, binds to C6. This C5b6 complex is inserted in membranes and then binds C7 and C8 to form the C5b-7 and C5b-8 complexes, which are more firmly embedded in the membrane. Finally, multiple C9 molecules associate with C5b-8 to form the C5b-9 complex, also known as Membrane Attack complex (MAC) or terminal complement complex (TCC). MAC leads bacterial lysis via inserting into its membrane and creating functional pores [20, 21]. In addition, and in contrast to this lytic effect on pathogens, insertion of MAC in the membrane of nucleated cells will generally induce a proinflammatory response [22-24].

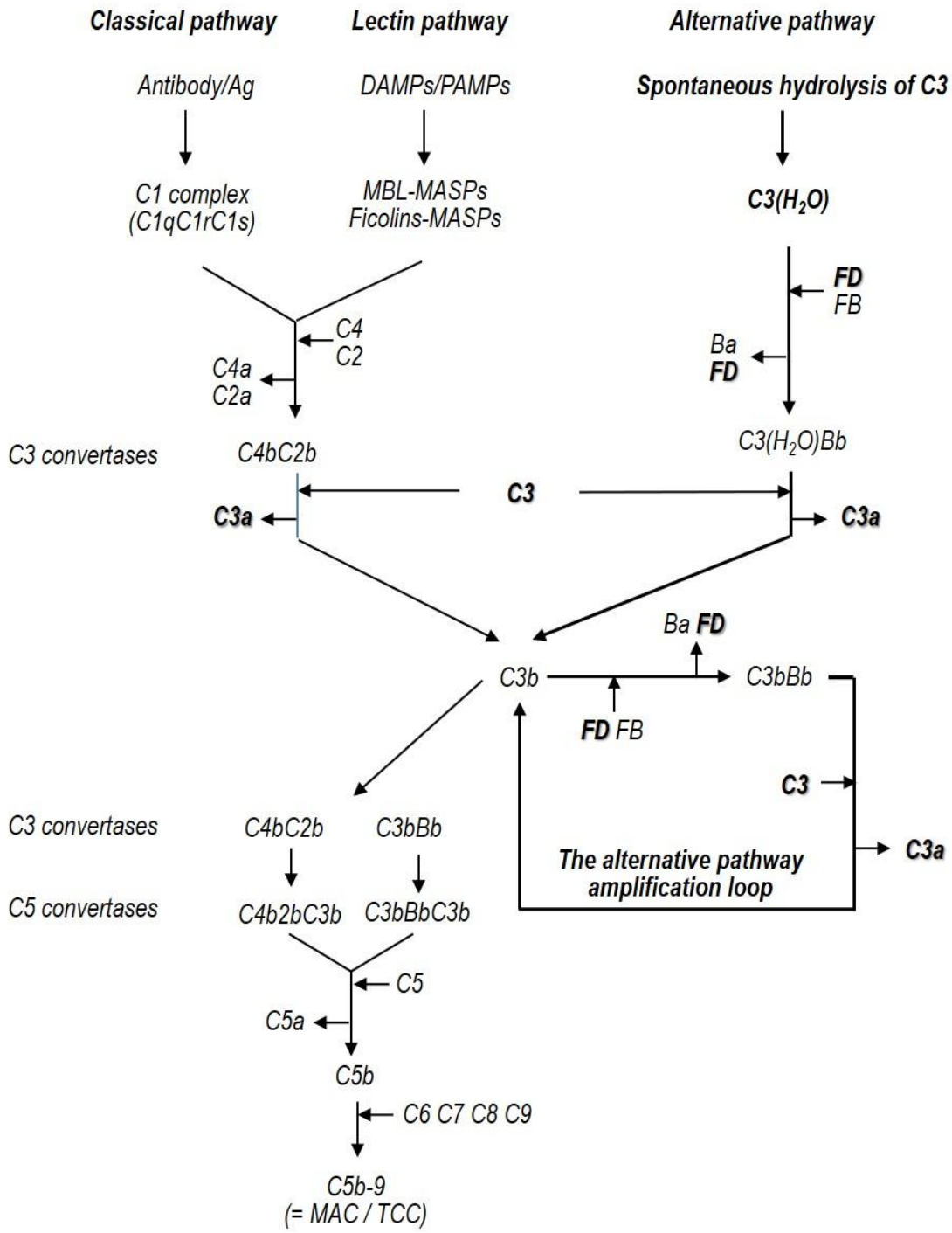

Figure 1.1 Schematic overview of the activation of the complement system via the 3 main pathways. The alternative pathway of complement activation, the amplification loop and the complement components that are the focus in this thesis are bold.

1.2 Regulation of complement activation

The activation of the complement system is strictly regulated by both circulating and membrane-bound regulatory proteins that prevent excessive complement activation and injury from activated complement. The first regulation way is accelerating the rate of decay of primarily the C3 convertases which is called decay-acceleration activity. Another main regulatory mechanism is preventing the reformation of C3 convertase [2, 25]. Complement regulators that are involved in these processes include plasma regulatory proteins such as C1-inhibitor, C4 binding protein, factor I, and factor H as well as membrane-bound proteins such as CD35, CD46, and CD55 ((also known as decay accelerating factor [DAF]) [6, 26-31]. Complement inhibition also occurs at the level of the terminal pathway, for instance by CD59 (also known as protectin) which inhibits the formation of C5b-9/MAC by preventing the interaction of C5b-8 and 9 [32].

2. Focus of this thesis: Factors C3, D and C3a of the alternative complement pathway and cardiometabolic diseases

The studies in this thesis focus on the alternative pathway of complement activation and, therein, particularly on C3, C3a and factor D.

Complement C3 is the central component of alternative complement pathway. Circulating concentration of complement C3 is around 1 to 1.5 g/l , which makes it the most abundant complement factor in the circulation [33]. Its structure is highly conserved in mammalian species [34, 35]. C3 is mainly produced by hepatocytes [33] but it is also synthesized by adipocytes, capillary and vascular endothelium, uterine epithelium, kidney tubular epithelium, mononuclear phagocytes, polymorphonuclear neutrophils, fibroblasts, type 2 alveolar cells pneumocytes, activated T-cells, osteoblastic and marrow-derived stromal cells, and astroglia [33].

C3a is a product of cleavage of C3. C3a is an anaphylatoxin and stimulates recruitment and activation of inflammatory cells, thus contributing to the inflammatory response [6]. Once C3a is released, circulating carboxypeptidases rapidly cleave its C-terminal arginine to generate a more stable and less potent form: C3a desArg [36].

Complement factor D is the rate-limiting enzyme in the activation of alternative pathway [37]. Circulating concentration of complement factor D is around 1 to 2 mg/l [38], which makes it a low-abundance complement factor [37]. The larger part of factor D that circulated in plasma is in the activated form [38]. Hence, the amount of factor D in plasma determines the activity of not only the initiation of the alternative pathway, but also of the amplification loop. One way to control the overall activity of factor D is by maintaining a low concentration via an extremely rapid catabolic rate [39]. Factor D is not only synthesized by adipose tissue, may also by e.g. monocytes[40]/ macrophages [41] and brain astrocytes [40-42], which makes factor D of importance for complement-dependent roles in tissue sites.

Each of the three main pathways of complement activation have been implicated in cardiometabolic diseases, but in the context of this thesis the focus is on the alternative pathway and the amplification loop on which all three activations pathways converge.

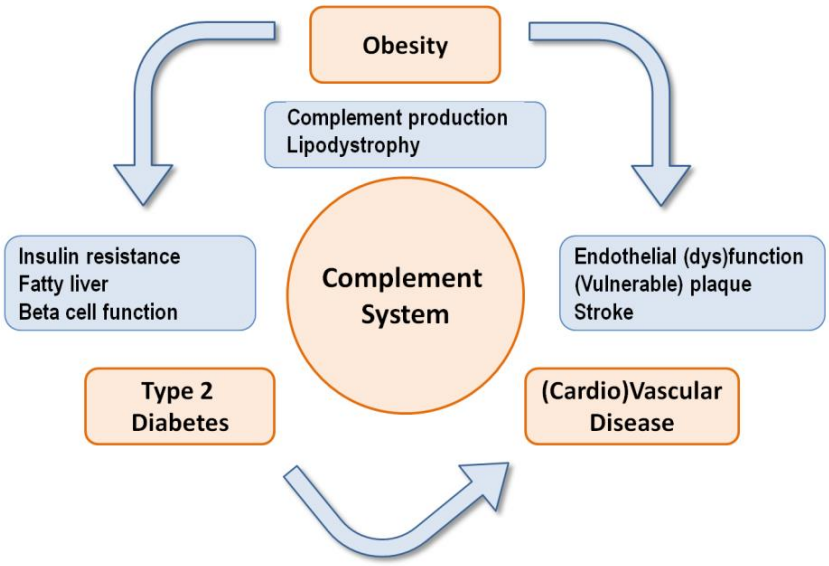

Figure 1.2 The complement system in cardiometabolic diseases

2.1 The alternative complement pathway and obesity

Obesity, which is characterized by abnormal or excessive accumulation of adipose tissue, is a chronic disease spread all over the world [43, 44]. According to the World Health Organization, nowadays over 1.9 billion adults are overweight of whom more than 650 million are obese. Moreover, around 400 million children and adolescents are overweight or obese, worldwide. Obesity can contribute to life-threatening medical complications, for instance dyslipidaemia, type 2 diabetes (T2D), cardiovascular disease (CVD), and metabolic syndrome [45]. These serious consequences make research into the etiology of obesity and its related complications urgent.

In individuals with obesity, the adipose tissue becomes hypertrophic. This induces a local inflammatory response that contributes to the development of cardiometabolic diseases e.g. by promoting adipose tissue insulin resistance and attracting more inflammatory cells. Adipose tissue consists of adipocytes, endothelial cells, immune cells and fibroblast-like cells [46]. Therefore, adipose tissue cannot only store fat and energy, but can also produce and secrete numerous endocrine substances, known as adipokines [47]. Many adipokines have been identified to play a role in

metabolic disorders [48, 49]. Notably, a substantial number of components of the complement system have been identified as adipokines [50], among which are the complement components that are the focus of this thesis.

The most clear-cut adipose-tissue derived complement component is factor D, which is also known as adipsin. Factor D was reported to be produced mainly by adipose tissue, particularly by adipocytes [51, 52]. While the liver is the main source of plasma C3 [53, 54], substantial C3 production also occurs in adipose tissue [50]. C3a on the other hand is primarily generated extracellularly, upon generation of C3 convertases that cleave C3 into C3a and C3b [4]. Consequently, plasma concentrations of complement C3, factor D and C3a are strongly and positively associated with adiposity in humans [55].

2.2. The alternative complement pathway and type 2 diabetes

T2D is one of the major causes of morbidity and mortality, world-wide [56]. Obesity is considered the primary risk factor for T2D. The diagnosis T2D is defined by elevated plasma glucose higher than 7.0 mmol/L when fasting and/or higher than 11.1 mmol/L when non-fasting [57]. This increased blood glucose results from insulin resistance combined with insufficient insulin secretion from (relative) beta cell dysfunction [58]. Insulin resistance is defined as decreased physiological responsiveness, in the liver, adipose tissue and muscle, to the effect of insulin [59]. In the presence of insulin resistance the beta cell must synthesize and secrete more insulin in order to maintain the circulating glucose at a normal level. Once an insufficient amount of insulin is secreted by the beta cell, the circulating glucose level will increase and prediabetes and then T2D occur [60].

The complement factors that are studied in this thesis have been reported to be involved in the process of insulin resistance and T2D. Firstly, factor D [61] and C3 [62, 63] have both been implicated in obesity-associated insulin resistance, and we [64]and

others [65] have identified C3 as an independent risk factor for incident T2D. On the other hand, however, factor D [66, 67], C3 [68] , and C3a [66] also been implicated in beta-cell preservation. This combination of potentially opposing effects of complement C3, factor D, and C3a on insulin resistance and beta cell function would fit the concept that adipose tissue releases beta cell-preserving factors in a situation of enhanced insulin resistance. This combination of effects could prevent the development of hyperglycemia and thereby post-pone development of T2D. It might, however, at the same time prolong period of the prediabetic state that is characterized by insulin resistance and hyperinsulinemia. This prediabetic state is known to be related to dyslipidemia and the metabolic syndrome which, in turn, are associated with an increased risk of CVD.

2.3 The alternative complement pathway and cardiovascular diseases

Cardiovascular disease is a major challenge for global health. According to WHO, cardiovascular disease is a one of the primary causes of death all over the world. Cardiovascular disease is regarded as a low-grade inflammatory disease of the vascular wall [69-71]. The pathophysiological processes involved in the development of CVD include endothelial dysfunction, atherosclerosis and atherothrombosis.

Complement activation, including the alternative pathway of complement activation, and complement-mediated inflammation has been implicated in CVD in humans [49, 72]. Complement activation is shown in atherosclerotic plaques [73]. Moreover, a large array of complement factors, primarily of classical and alternative pathway, is strongly upregulated in human early atherosclerotic tissue [74], suggesting that complement activation may be an early event in the atherosclerotic process.

3. Outline of the thesis and study populations

3.1 Aims

The main aim of this thesis was to investigate the potential roles of components of the alternative complement pathway in (i) processes that underlie CVD, including arterial stiffness and vascular dysfunction and (ii) processes that underlie T2D, including fasting glucose and insulin resistance. In addition, we investigate the effect of weight loss on circulating levels of alternative complement factors.

The added value of the analyses presented in this thesis to information that is already available in the literature is (i) the simultaneous measurements of three components of the alternative pathway in the observational cohort and randomized controlled trial, which allow more direct comparisons, in contrast to measurements in separate cohorts, (ii) the availability of several measures of sub-clinical disease in the deeply-phenotyped Maastricht Study, and (iii) the large number of participants in The Maastricht study, which provides sufficient power to evaluate potential differences between the sexes and between persons with and without diabetes.

3.2 Study populations

3.2.1 The Maastricht Study

The Maastricht Study is an observational prospective population-based cohort study. The rationale and methodology have been described previously [75]. In brief, the study focuses on the aetiology, pathophysiology, complications and comorbidities of T2D and is characterized by an extensive phenotyping approach. Eligible for participation were all individuals aged between 40 and 75 years and living in the southern part of the Netherlands. Participants were recruited through mass media campaigns and from the municipal registries and the regional Diabetes Patient Registry via mailings.

Recruitment was stratified according to known T2D status, with an oversampling of individuals with T2D, for reasons of efficiency. The analyses in this thesis include cross-sectional data from the first 7689 participants, who completed the baseline survey between November 2010 and December 2017. The examinations of each participant were performed within a time window of three months. The study has been approved by the institutional medical ethical committee (NL31329.068.10) and the Minister of Health, Welfare and Sports of the Netherlands (Permit 131088-105234-PG). All participants gave written informed consent.

3.2.2 Weight loss study

Caucasian men were recruited via advertisements in local newspapers or among participants involved in earlier studies [76, 77]. They were included if they met the following inclusion criteria: age between 18 and 65 years, weight change <3 kg within the previous 3 months, non-smokers, without diabetes, without cardiovascular disease, no drug or alcohol abuse, no use of medication known to affect lipid or glucose metabolism or hypertension, and no participation in another biomedical trial during the past 30 days. Twenty-five normal-weight (waist circumference: <94 cm) and 53 men with abdominal obesity (waist circumference: 102-110 cm) completed the baseline measurements. The men with obesity were allocated into 2 age groups (18-49 years or 50-65 years). Men in the same age group were randomly divided into the weight stable control group or to the weight loss group. All participants gave written informed consent before entering the study. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center, performed in accordance with the Declaration of Helsinki, and registered at clinicaltrials.gov as NCT01675401.

Study design: At the start of the study, all men with normal weight and with abdominally obesity underwent baseline measurements at the research facilities [77].

Briefly, participants in the weight loss group visited our research dietitian every week and had a very-low-calorie diet (VLCD, Modifast; Nutrition et Sante Benelux) for at least 4 weeks, under strict guidance. After the VLCD period, a period of 1-2 weeks followed in which they were provided a calorie-restricted diet in line with the Dutch dietary guideline. In week 7 and week 8 the participants were kept in energy balance (weight-maintenance period). Participants in the weight stable control group maintained their normal diet, physical activities, and alcohol consumption, and were also monitored by the dietician through whole period.

3.3 Outline of the thesis

As indicated paragraph 3.1 and figure 1.3, the potential roles of factor C3, C3a and factor D in obesity, T2D and CVD were investigated.

In **chapter 2**, we investigated the associations of complement factor D with vascular dysfunction and cardiovascular disease in the Maastricht Study. In **chapter 3**, we investigated the associations of factor D and C3 with arterial stiffness represented by carotid-femoral pulse wave velocity (cfPWV), carotid distensibility coefficient (DC) and carotid Young's elastic modulus (YEM) in the Maastricht Study. In **chapter 4**, we investigated whether complement C3, factor D and C3a could explain (part of) the association of obesity with disturbed metabolism homeostasis represented by fasting glucose, insulin resistance, prevalence of T2D in the Maastricht Study. Finally, in **chapter 5** the effects of weight loss intervention in obese men on the plasma concentration of C3, factor D and C3a were investigated. The data are summarized and reflected upon in **chapter 6**

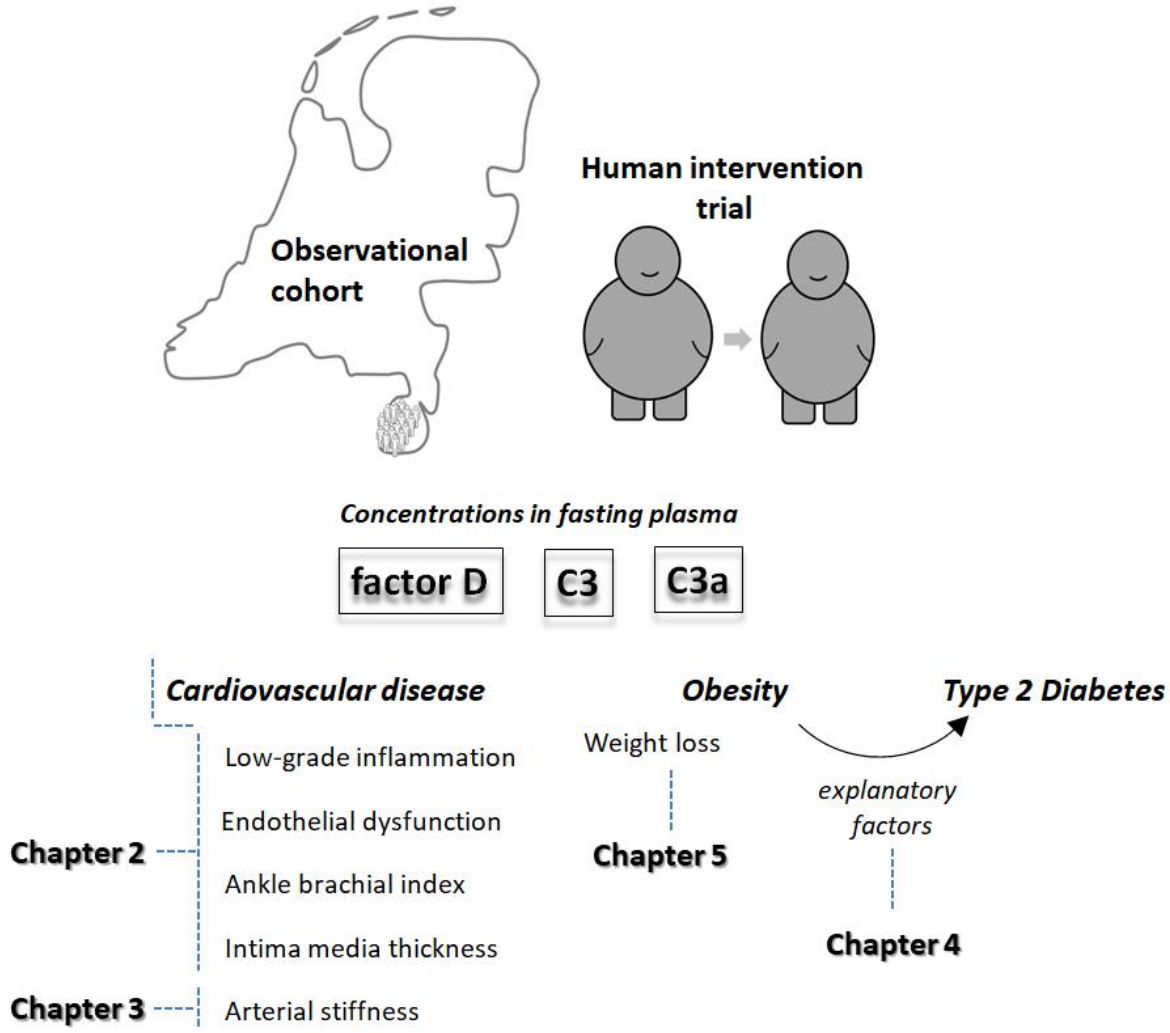

Figure 1.3 Thesis outline

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Chapter 2

Plasma factor D is cross-sectionally associated with low-grade inflammation, endothelial dysfunction and cardiovascular disease: The Maastricht Study.

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In Revision

Chapter 3

Complement factor D and C3 cross-sectionally associate with arterial stiffness, but not independently of metabolic risk factors: The Maastricht Study

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Abstract

Background: Arterial stiffness predicts cardiovascular outcomes. The complement system, particularly the alternative complement pathway, has been implicated in cardiovascular diseases. We herein investigated the associations of factor D, the rate-limiting protease of the alternative pathway, and C3, the central complement component, with arterial stiffness.

Methods: In 3019 population-based participants (51.9% men, 60.1±8.2 years, 27.7% type 2 diabetes [T2D], oversampled]) we measured carotid-femoral pulse wave velocity (cfPWV), carotid distensibility coefficient (DC) and carotid Young's elastic modulus (YEM), and plasma concentrations of factor D and C3. We conducted multiple linear regression to investigate the association of factor D and C3 (main independent variables, standardized) with cfPWV (primary outcome) and DC and YEM (secondary outcomes), adjusted for potential confounders.

Results: Per SD higher factor D and C3, cfPWV was 0.41 m/s [95%CI: 0.34;0.49] and 0.33 m/s [0.25;0.41] greater respectively. These associations were substantially attenuated when adjusted for age, sex, education, mean arterial pressure, and heart rate (0.08 m/s [0.02;0.15] and 0.11 m/s [0.05;0.18], respectively), and were not significant when additionally adjusted for T2D, waist circumference and additional cardiovascular risk factors (0.06 m/s [-0.01;0.13] and 0.01 m/s [-0.06;0.09], respectively). Results were comparable for carotid YEM and DC. In persons with T2D, but not in those without, the association between factor D and cfPWV was significant in the fully adjusted model (0.14 m/s, [0.01;0.27], p=0.038， $P_{interaction}$<0.05).

Conclusion: The strong association of plasma factor D and C3 with arterial stiffness in this population-based cohort was not independent of T2D and other metabolic risk factors. Our data suggest that a possible causal pathway starting from alternative complement activation may via hypertension and T2D contribute to greater arterial stiffness.

Introduction

Arterial stiffening is one of the key processes in the development of cardiovascular diseases (CVD). Greater arterial stiffness may contribute to a higher risk CVD via an increase in systolic blood pressure but the association of arterial stiffness with CVD is, at least in part, independent of blood pressure [1]. Arterial stiffness is higher in obese individuals of all ages than in their non-obese peers [2]. Arterial stiffness is, among others, determined by properties of elastin and collagen in the arterial wall [3]. It is also affected by changes in endothelial cell signaling and vascular smooth muscle cell tone [4].

The complement system, an intricate protein network that is part of the innate immune system, has been suggested as a potential modulator of arterial stiffness. Complement factor D and C3 comprise major components of the alternative pathway of complement activation which has consistently been implicated in CVD in humans [5]. Complement factor D is the rate-limiting serine-protease in activation of the alternative complement pathway. Complement activation results in the activation of C3, the central complement component.

Factor D, also known as adipsin, is produced in high amounts in adipocytes as well as monocytes/macrophages of adipose tissue [6]. It is also expressed in the aortic endothelium and its expression is higher in endothelial cells derived from diabetic mice [7]. Notably, in a mouse model of vascular calcification, factor D was identified as the major elastase involved in elastin fragmentation and subsequent elastocalcinosis [8]. In line with the above, we previously showed that higher factor D concentration was associated with worse endothelial dysfunction [9]. We also showed that a greater plasma factor D concentration was significantly associated with incident cardiovascular events after adjustment for age, sex and glucose metabolism status, although the association was strongly attenuated upon full adjustment for potential confounders [9]. Factor D was also identified as a biomarker for poor prognosis in patients with coronary artery disease [10]. The Women's Health Initiative observational study cohort

confirmed factor D as a risk marker for coronary heart disease (CHD), but not for stroke [11] while in another large cohort consisting only of men, factor D was positively associated with the risk of developing stroke [12] but not with risk of CHD [13].

C3 is mainly synthesized in the liver, but is also produced by e.g. the vascular endothelium [14] and adipocytes, including perivascular adipocytes [15, 16]. In humans, circulating C3 is strongly associated with adiposity [17, 18], is longitudinally associated with cardiovascular disease [5] and was identified as a risk factor for myocardial infarction [19]. C3 was also identified as a risk factor for hypertension, at least in men [20], and recently an association between complement C3 and carotid-femoral pulse wave velocity (cfPWV) was proposed in a large population-based cohort [21], which awaits confirmation in a large independent study. C3 binds to collagen and elastin fibers within the adventitia and may, thereby, contribute to vascular stiffening [22]. C3 is also produced by aortic smooth muscle cells of spontaneously hypertensive rats and contributes to vascular smooth muscle cell proliferation and extracellular matrix synthesis [23].

Taken together, experimental data suggest that factor D and C3 may be associated with changes in the vascular wall that are functionally involved in processes related to large artery function [8, 22] but human data to support this are limited for complement C3 and, to the best of our knowledge, absent for factor D. In our current study we addressed, in a large human cohort enriched for individuals with type 2 diabetes (T2D), the cross-sectional associations of plasma factor D and C3 with aortic stiffness defined as cfPWV, and carotid stiffness defined as carotid distensibility coefficient (DC) and carotid Young's elastic modulus (YEM).

Research design and methods

Study population and design

We used data from The Maastricht Study, an observational prospective population-based cohort study. The rationale and methodology have been described previously.[24]. In brief, the study focuses on the etiology, pathophysiology, complications and comorbidities of T2D and is characterized by an extensive phenotyping approach. Eligible for participation were all individuals aged between 40 and 75 years and living in the southern part of the Netherlands. Participants were recruited through mass media campaigns and from the municipal registries and the regional Diabetes Patient Registry via mailings. Recruitment was stratified according to known T2D status, with an oversampling of individuals with T2D, for reasons of efficiency. A flow-chart of the inclusion of study participants in the current analyses is presented in **Figure 3-1**. The present report includes cross-sectional data from the first 7689 participants, who completed the baseline survey between November 2010 and December 2017. The examinations of each participant were performed within a time window of three months. The study has been approved by the institutional medical ethical committee (NL31329.068.10) and the the Minister of Health, Welfare and Sports of the Netherlands (Permit 131088-105234-PG). All participants gave written informed consent.

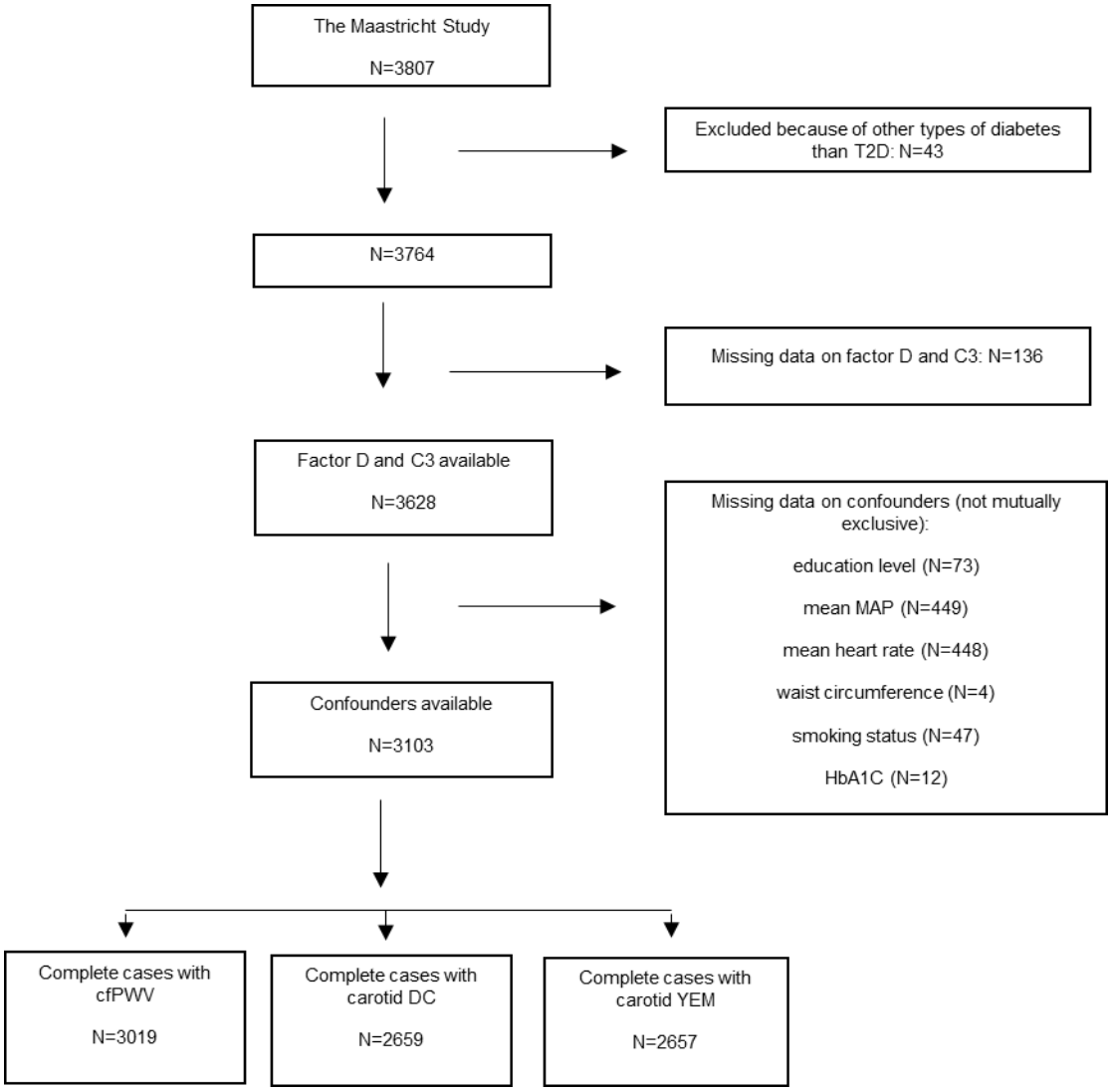

Figure 3-1. Derivation of final study population. MAP indicates mean artery pressure; HbA1C, hemoglobin A1c (glycated hemoglobin); cfPWV, carotid-femoral pulse wave velocity; DC, distensibility coefficient; YEM, Young's elastic modulus.

Factor D and C3 measurement

After overnight fasting, venous blood samples were collected in EDTA on ice. Blood was immediately centrifuged and plasma samples were stored at -80°C until use. Complement factor D was measured in EDTA plasma using an R&D duoset kit assay, as described before [9]. The inter-assay variation was 4.6%. Complement C3 was measured in EDTA plasma using an MSD R-plex Human Complement C3 Antibody Set.

The assay was performed according to the manufacturer's instruction except for the use of a 1:20.000 instead of 1:300.000 dilution, which resulted in better stability of the measurements. The inter-assay variation was 8.9%.

Arterial Stiffness Measurements

All measurements were done by trained vascular technicians unaware of the participants' clinical or diabetes mellitus status, in a dark, quiet temperature-controlled room (21°C–23°C), as described previously [25]. Participants were asked to refrain from smoking and drinking coffee, tea or alcoholic beverages 3 hours before the measurements. Participants were allowed to have a light meal (breakfast or lunch). All measurements were performed in supine position after 10 minutes of rest. Talking or sleeping was not allowed during the examination. During the vascular measurements (≈45 minutes), brachial systolic, diastolic, and mean arterial pressure (MAP) were determined every 5 minutes with an oscillometric device (Accutorr Plus, Datascope Inc, Montvale, NJ). The mean MAP and heart rate (HR) during these measurements were used in the statistical analysis. A 3-lead ECG was recorded continuously during the measurements to facilitate automatic signal processing.

*Carotid-Femoral Pulse Wave Velocity***:** Carotid-femoral pulse wave velocity (cfPWV) was determined according to guidelines with the use of applanation tonometry (SphygmoCor, Atcor Medical, Sydney, Australia). Pressure waveforms were determined at the right common arteries and right common femoral arteries. Difference in the time of pulse arrival from the R-wave of the ECG between the 2 sites (transit time) was determined with the intersecting tangents algorithm. The pulse wave travel distance was calculated as 80% of the direct straight distance (measured with an infantometer) between the 2 arterial sites. The median of 3 consecutive cfPWV (defined as traveled distance/ transit time) recordings was used in the analyses.

Local Carotid Arterial Properties: Diameter and distension of the left common carotid artery were measured with an ultrasound scanner equipped with a 7.5-MHz

linear probe (MyLab 70, Esaote Europe B.V., Maastricht, The Netherlands). Exactly as reported before [25], during the ultrasound measurements a B-mode image on the basis of 19 M-lines was captured on screen and an online echo-tracking algorithm showed real-time anterior and posterior arterial wall displacements. The M-mode recordings were comprised of 19 simultaneous recordings at a frame rate of 498 Hz. The distance between the M-line recording positions was 0.96 mm; therefore, a total segment of 18.24 mm of each artery was covered by the scan plane. For offline processing, the radiofrequency signal was fed into a dedicated PC-based acquisition system with a sampling frequency of 50 MHz. Data processing was conducted in MatLab. The distension waveforms were acquired from the radio frequency data with the use of a wall track algorithm. The median diameter and distension of 2 measurements were used in the analyses. Local arterial elastic properties were quantified by calculating the following indices:

Distensibility coefficient (DC)

$DC = (2\Delta D * D + \Delta D^2) / (PP * IAD^2)$ [in 10^3 kPa^{-1}]

Young's elastic modulus (YEM)

$YEM = D / (IMT * DC)$ [in 10^3 kPa]

where ΔD is distension; D, arterial diameter; and PP, brachial pulse pressure (calculated as systolic BP minus diastolic BP); and IMT, intima-media thickness. DC represents arterial distensibility and YEM represents the stiffness of the arterial wall material at operating pressure.

Other characteristics of participants

Covariates were measured as reported before [24]. In short, smoking (never, former or current smoker), alcohol consumption (none, low (women ≤7 and men ≤14 glasses/week,) and high (women >7 and men >14 glasses/week)), physical activity (hours/week) and education status (low, medium, high) were obtained through web-based questionnaires. Use of lipid-modifying and antihypertensive medication was

collected by means of an interview. Weight and height were measured to calculate body mass index (BMI, kg/m^2) and waist circumference was measured midway between the lower rib margin and the iliac crest. T2D was diagnosed according to World Health Organization criteria, using a 75 g oral glucose tolerance test [26]. Serum total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured using an automatic analyzer (Beckman Synchron LX20, Beckman Coulter Inc.). MAP was calculated as 1/3 Systolic Blood Pressure + 2/3 Diastolic Blood Pressure. Twenty-four-hour blood pressure was measured using an ambulatory device (WatchBP O3, Microlife, Switzerland). Readings were taken each 15 minutes between 8.00-23.00h and each 30 minutes from 23.00-8.00h. A validated food frequency questionnaire [27] was administered and used to derive the Dutch Healthy Diet (DHD)15-index that contains 15 components representing the 15 food-based Dutch dietary guidelines of 2015 [28]. Since the FFQ did not distinguish between filtered and unfiltered coffee, the DHD score in the present study ranged from 0-140. Kidney function can be affected by arterial stiffness [29], and therefore, the glomerular filtration rate (eGFR) was included as descriptive variable, estimated by means of CKD-EPI equation based on the combination of serum creatinine and serum cystatin C [24].

Statistical analyses

All analyses were performed using IBM SPSS Statistics 27 for windows (version 27; IBM Corp, Somers, NY). A two-tailed p value <0.05 was considered significant. Normality of variable distribution was tested. Normal distributed variables are presented as mean ± SD. Categorical variables are presented as proportions (%). Characteristics were compared across quartiles of factor D and C3, using an ANOVA for continuous variables, or X^2-test in case of categorical variables.

Multiple linear regression was used to investigate the associations of plasma concentrations of factor D and C3 (main independent variables) with cfPWV (primary outcome), and carotid DC and YEM (secondary outcomes). Regression models were as

follows: (I) Model 1 was the crude association; (II) Model 2 was additionally adjusted for age (years) and sex (male or female), education status (medium high, each yes/no); (III) Model 3 was additionally adjusted for MAP (mmHg) and HR (bpm); (IV) model 4 was additionally adjusted for T2D (as yes/no, because of oversampling of T2D) (V) Model 5 was additionally adjusted for waist circumference (in cm) Model 6 was additionally adjusted for smoking habits (current or former smoker, each yes/no), lipid-modifying and/or anti-hypertensive medication (each yes/no), total/HDL cholesterol ratio triglycerides (mmol/l) and HbA1c (mmol/mol).

Note model 4 and model 5 may be overadjusted for the association of factor D and arterial stiffness, because factor D [30, 31] has been implicated in β-cell preservation, strongly expressed in adipose tissue, and additionally implicated in adipocyte differentiation and progression of obesity [32]. Model 3 and 4 may be overadjusted for the association of C3 and arterial stiffness, because complement C3 has been implicated in development of hypertension [20], and T2D [33].

We tested for interaction with T2D because of the oversampling of T2D in The Maastricht Study and for interaction with sex to explore potential differences in these relationships between men and women, by including interaction terms of interest, i.e. factor D*T2D, factor D*sex, C3*T2D, C3*sex, respectively, plus T2D or sex*all confounders included in the model [34]

To assess the robustness of the findings in the main analyses, we performed several sensitivity analyses. First, associations between factor D or C3 and arterial stiffness were re-evaluated using income level and occupation status substituted for education status. Second, associations between factor D or C3 and arterial stiffness were re-evaluated using MAP calculated from 24 hours blood pressure measurements substituted for MAP at the time of ultrasound measurement. Third, associations between complement factor D or C3 and arterial stiffness were re-evaluated using BMI instead of waist circumference. Fourth, associations between complement factor D or C3 and arterial stiffness were re-evaluated by additionally adjusting for physical activity

(hours/week), and for DHD15 (score). This reduced the number of participants in the analyses because of a relatively large number of missing in these variables. We also substituted energy intake (kJ/day) and alcohol consumption (low alcohol consumption and high alcohol consumption, each yes/no) for DHD15 (score).

Results

General characteristics of the study population

Factor D concentration was 0.92 ± 0.23 mg/l (range 0.33 to 2.32), C3 concentration was 1.15 ± 0.23 g/l (range 0.45 to 2.50). The correlation between factor D and C3 was r=0.123, p<0.001 (n=3019). General characteristics of the study population across quartiles of factor D and C3 are presented in **Table 1**. Participants in the higher quartiles of both factor D and C3 were older, which was more pronounced for factor D. Those with higher factor D were more often men, while this was less so for C3. Both were strongly and positively associated with measures of obesity (BMI and waist). The higher prevalence of prediabetes and T2D in those with higher factor D and C3 was more pronounced for C3. The prevalence of CVD was comparable for factor D and C3, and was higher in the higher quartiles. Higher C3 was more prominently than higher factor D characterized by higher fasting triglycerides and glucose, which was also reflected in the use of medication. With respect to the primary and secondary outcomes of this study, those with higher factor D and C3 had higher cfPWV, lower carotid DC and higher carotid YEM, all consistent with worse arterial stiffness.

***Table 1**: General characteristics of the study population based on quartiles of factor D and C3 concentration*

	Quartiles of plasma factor D concentration					Quartiles of plasma factor C3 concentration				
	1st quartile N=755 0.33-0.78 mg/l	**2rd quartile** N=755 0.78-0.90 mg/l	**3rd quartile** N=755 0.90-1.04 mg/l	**4th quartile** N=754 1.04-2.32 mg/l	**P-value** [a]	**1st quartile** N=757 0.45- 0.99 g/l	**2rd quartile** N=767 0.99-1.13 g/l	**3rd quartile** N=751 1.13-1.28 g/l	**4th quartile** N=744 1.29- 2.50 g/l	**P-value** [a]
Demographics										
Age (years)	56.8±7.9	58.9±8.1	60.8±8.0	63.8±7.1	<0.001	58.7±8.3	60.5±8.0	60.7±7.9	60.4±8.5	<0.001
Sex (%men)	40.5	50.2	56.3	60.7	<0.001	46.8	54.0	55.8	51.2	0.003
Medium/high Education status (%)	31.4/39.5	28.2/41.9	27.8/38.4	25.3/35.1	<0.001	25.0/52.0	30.9/39.4	27.6/33.8	29.3/29.4	<0.001
lifestyle										
BMI (kg/m^2) [b]	25.4±3.8	26.5±4.1	27.2±4.1	28.7±4.8	<0.001	24.6±3.2	26.2±3.7	27.5±3.9	29.7±4.8	<0.001
Waist circumference (cm)	90.3±12.5	94.0±12.7	96.7±12.6	101.5±13.1	<0.001	88.2±11.1	93.5±11.9	97.7±12.0	103.3±13.6	<0.001
Physical activity (Hours/Week) [b]	15.4±8.3	14.6±8.0	13.8±8.0	12.9±7.8	<0.001	15.2±7.9	14.7±8.0	13.9±8.4	12.7±7.8	<0.001
Former smokers/current smokers (%)	49.9/14.2	51.9/13.2	49.9/13.8	59.3/12.2	0.005	52.6/12.4	49.4/15.6	53.8/13.4	55.4/11.8	0.195
Energy (kcal) [b]	2181±611	2137±601	2212±602	2160±602	0.113	2185±591	2199±591	2179±607	2126±628	0.118
Low/high alcohol consumption (%)	53.4/29.1	54.7/28.2	55.9/27.3	58.9/20.1	0.001	53.6/34.4	56.8/30.2	60.1/20.9	52.4/19.0	<0.001
DHD15-sum [b]	83.7±15.4	84.2±14.4	82.9±14.6	83.0±14.3	0.266	85.4±14.5	84.0±14.5	83.0±14.8	81.3±14.7	<0.001
Biological/clinical										
Fasting plasma glucose (mmol/l) [b]	5.8±1.6	5.9±1.6	6.1±1.7	6.3±1.6	<0.001	5.5±1.2	5.8±1.2	6.2±1.5	6.7±2.1	<0.001
HbA1c (mmol/mol)	39.5±9.3	39.9±9.2	41.2±10	42.5±9.8	<0.001	37.7±7.1	39.0±7.7	41.7±9.2	44.8±12.4	<0.001
Systolic blood pressure (mmHg) [b]	131.9±17.3	133.9±17.8	136.6±18.0	137.2±18.3	<0.001	129.9±17.7	133.9±17.4	137.3±17.6	138.2±17.8	<0.001
Diastolic blood pressure (mmHg) [b]	74.9±10.0	75.8±9.5	77.2±10.0	76.2±9.9	<0.001	73.8±9.8	75.6±9.8	77.0±9.6	77.7±9.9	<0.001
Mean of MAP (mmHg)	95.8±10.6	95.9±9.6	97.7±10.3	97.5±10.5	<0.001	95.3±10.6	96.3±10.2	97.6±10.4	97.8±9.8	<0.001
Heart rate-mean (bpm)	63.0±9.5	62.4±9.2	62.8±9.4	62.9±9.6	0.676	60.6±8.5	61.6±8.9	63.1±9.1	65.7±10.3	<0.001
Total/HDL cholesterol	3.5±1.1	3.6±1.2	3.7±1.1	3.9±1.2	<0.001	3.4±1.1	3.7±1.1	3.7±1.1	3.9±1.3	<0.001

Triglycerides (mmol/l)	1.3±0.9	1.3±0.8	1.4±0.8	1.6±0.9	<0.001	1.1±0.6	1.3±0.7	1.5±0.9	1.8±1.0	<0.001
eGFR(ml/min/1.73m2) [b]	99.0±10.8	92.5±10.1	86.6±11.0	74.8±14.2	<0.001	90.3±13.6	87.8±14.2	88.2±14.5	85.9±16.1	<0.001
Cardiovascular disease (%)	11.6	15.1	18.8	23.0	<0.001	13.1	13.0	19.3	23.2	<0.001
prediabetes/T2D (%)	12.8/22.3	13.2/23.6	15.5/27.9	17.4/36.9	<0.001	10.8/11.1	15.9/19.8	14.8/32.8	17.5/47.4	<0.001
Medication use										
Use of glucose-lowering medication (%)	16.7	18.1	21.3	29.7	<0.001	8.9	15.5	24.6	37.2	<0.001
Ues of antihypertensive medication (%)	27.2	30.9	40.0	58.4	<0.001	24.4	35.3	42.2	54.7	<0.001
Use of lipid-modifying medication (%)	29.7	30.5	38.1	47.5	<0.001	19.4	30.9	44.5	51.3	<0.001
Outcome variables										
cfPWV (m/s)	8.6±1.9	8.9±2.1	9.2±2.1	9.6±2.4	<0.001	8.5±1.9	9.0±2.2	9.2±2.2	9.5±2.4	<0.001
Carotid DC (10^{-3}KPa) [b]	15.4±5.4	14.7±5.3	14.0±4.9	13.1±4.6	<0.001	15.2±5.3	14.3±5.0	13.7±4.9	13.9±5.1	<0.001
Carotid YEM (10^{3}KPa) [b]	0.69±0.41	0.72±0.32	0.76±0.35	0.83±0.40	<0.001	0.70±0.36	0.74±0.33	0.79±0.39	0.78±0.41	<0.001

Legend to Table 1: Data are presented as mean ± SD (continuous variables) or proportion (%, categorical variables). [a] P-values were obtained by ANOVA or Pearson Chi-square. [b] BMI, n=3018; Physical activity, n= 2669; energy intake, n=2850; DHD15-sum, n= 2850; fasting plasma glucose, n= 3017; diastolic blood pressure, n=3017; systolic blood pressure, n=3017, eGFR, n=2701; Carotid DC, n = 2659; Carotid YEM, n = 2657. Abbreviations: BMI, body mass index; DHD-15, Dutch health diet index; HbA1c, hemoglobin A1c (glycated hemoglobin); MAP, mean artery pressure; HDL, high-density lipoprotein; eGFR, estimated glomerular filtration rate; cfPWV, carotid-femoral pulse wave velocity; DC, distensibility coefficient; YEM, Young's elastic modulus.

Associations of factor D and C3 with carotid-femoral Pulse Wave Velocity

The associations of factor D and C3 with cfPWV are presented in **Table 2**. Factor D was positively associated with cfPWV (per SD higher factor D, cfPWV was 0.413 m/s greater, 95% confidence interval (CI) [0.337; 0.490], model 1). After adjustment for age, sex, education status, MAP and HR, the association was attenuated to 0.083 m/s ([0.017; 0.150], model 3) and after additional adjustment for T2D it was no longer significant (0.062 m/s, [-0.003; 0.127], model 4). C3 was also positively associated with cfPWV (0.331 m/s, [0.254; 0.407], model 1). After adjustment for age, sex, education status, MAP and HR, the association was attenuated to 0.113 [0.048; 0.177] (model 3). After additional adjustment for T2D, the association was no longer significant (0.028 m/s, [-0.037; 0.094], model 4). Note that the associations in model 4 may to some extent be overadjusted because of reasons indicated in the methods.

***Table 2**: associations of factor D and factor C3 with aortic stiffness measurements*

	cfPWV (m/s) [a]					
	factor D [b]			C3 [b]		
	β	[95%CI]	P-value	β	[95%CI]	P-value
Model 1	**0.413**	**[0.337; 0.490]**	**<0.001**	**0.331**	**[0.254; 0.407]**	**<0.001**
Model 2	0.074	[0.000; 0.147]	0.051	**0.247**	**[0.177; 0.317]**	**<0.001**
Model 3	**0.083**	**[0.017; 0.150]**	**0.014**	**0.113**	**[0.048; 0.177]**	**0.001**
Model 4	0.062	[-0.003; 0.127]	0.062	0.028	[-0.037; 0.094]	0.395
Model 5	0.064	[-0.003; 0.132]	0.062	0.029	[-0.041; 0.099]	0.419
Model 6	0.058	[-0.010; 0.126]	0.097	0.014	[-0.058; 0.085]	0.707

Legend to table 2: [a] n=3019, [b] factor D and C3 are standardized. Bold typefont represents statistically significant data.
Model 1: Crude association
Model 2: adjusted for age, sex, education status
Model 3: additionally adjusted for mean arterial pressure, mean heart rate
Model 4: additionally adjusted for yes/no T2D
Model 5: additionally adjusted for waist circumference
Model 6: additionally adjusted for smoking habits, lipid-modifying and/or anti-hypertensive medication, Total/high-density lipoprotein cholesterol ratio, triglycerides and HbA1c
Abbreviation: cfPWV, carotid-femoral pulse wave velocity

Next, we tested whether the associations of factor D and C3 with cfPWV differed between participants with or without T2D or between men and women. T2D, but not sex, modified the association of factor D, but not C3, with cfPWV. In the fully adjusted models, $P_{interaction}$ with T2D was 0.04 and 0.83 and $P_{interaction}$ with sex was 0.22 and 0.87, respectively, for factor D and C3. Subsequent stratified analyses on T2D (**Table 3**) showed that the crude associations between factor D and cfPWV did not differ between participants with (0.310 m/s [0.168; 0.452]) and without T2D (0.322 m/s [0.263; 0.408]). This association was fully attenuated in the non-diabetic participants (-0.004 m/s [-0.083; 0.075]), but remained positive and significant in those with T2D (0.139 m/s [0.008; 0.271]).

***Table 3**: Associations of factor D with aortic stiffness measurements stratified on T2D*

	cfPWV (m/s)					
	Participants with T2D [a]			Participants without T2D [a]		
	β factor D [b]	[95%CI]	P-value	β factor D [b]	[95%CI]	P-value
Model 1	**0.310**	**[0.168; 0.452]**	**<0.001**	**0.322**	**[0.236; 0.408]**	**<0.001**
Model 2	0.058	[-0.081; 0.196]	0.414	0.027	[-0.058; 0.111]	0.536
Model 3	0.120	[-0.007; 0.246]	0.064	0.019	[-0.056; 0.095]	0.615
Model 4	**0.136**	**[0.006; 0.267]**	**0.041**	0.007	[-0.072; 0.085]	0.869
Model 5	**0.139**	**[0.008; 0.271]**	**0.038**	-0.004	[-0.083; 0.075]	0.916

Legend to table 3: [a] Persons with T2D; n=835, persons without T2D; n=2184, [b] factor D is standardized. Bold typefont represents statistically significant data.
Model 1: Crude association
Model 2: adjusted for age, sex, education status
Model 3: additionally adjusted for mean arterial pressure, mean heart rate
Model 4: additionally adjusted for waist circumference
Model 5: additionally adjusted for smoking habits, lipid-modifying and/or anti-hypertensive medication, Total/high-density lipoprotein cholesterol ratio, triglycerides and HbA1c
Abbreviation: cfPWV, carotid-femoral pulse wave velocity

In subsequent sensitivity analyses, exchanging income level and occupation status for education status, 24-hour MAP for MAP at the time of ultrasound measurement, BMI for waist circumference, or additionally adjusting for physical

activity and DHD15, with subsequently substituting energy intake and alcohol consumption for DHD15 did not materially alter the results (data not shown).

Associations of factor D and C3 with Carotid DC and carotid YEM

Factor D was inversely associated with carotid DC (per SD higher factor D, the β for carotid DC was $-0.832 \cdot 10^{-3}$/kPa [-1.027; -0.638], **Table 4**, model 1), indicating that a higher concentration of factor D was associated with stiffer arteries. After adjustment for age, sex and education status, this association was no longer significant $-0.090 \cdot 10^{-3}$/kPa, [-0.277; 0.096], model 2). C3 was also inversely associated with carotid DC ($-0.547 \cdot 10^{-3}$/kPa [-0.742; -0.353], **Table 4**, model 1). After adjustment for age, sex, education status, MAP, HR, and T2D, the association attenuated to $-0.176 \cdot 10^{-3}$/kPa [-0.344; -0.008], model 4) and in the fully adjusted model it was no longer significant ($-0.023 \cdot 10^{-3}$/kPa [-0.207; 0.161], model 6).

Factor D was positively associated with carotid YEM (per SD higher factor D, carotid YEM was higher $0.049 \cdot 10^3$ kPa [0.035 0.063], **Table 4**, model 1), but after adjustment for age this association was not significant ($0.011 \cdot 10^3$ kPa [-0.003 0.026], model 2). C3 was positively associated with carotid YEM (per SD higher C3, carotid YEM was higher $0.034 \cdot 10^3$ kPa [0.020; 0.049], model 1), which was attenuated after adjustment for age, sex, education status, MAP ($0.017 \cdot 10^3$ kPa, 95%CI [0.004; 0.030], model 3) and no longer significant after additional adjustment for T2D ($0.007 \cdot 10^3$ kPa [-0.006; 0.021], model 4). Neither T2D nor sex modified the association of factor D or C3 with carotid DC or carotid YEM ($P_{interaction}$ ranging from 0.29 to 1.0).

In subsequent sensitivity analyses, exchanging income level and occupation status for education status respectively, 24-hour MAP for MAP at the time of ultrasound measurement, BMI for waist circumference, or additionally adjusting for physical activity and DHD15, with subsequently substituting energy intake and alcohol consumption for DHD15 did not materially alter the results (data not shown).

***Table 4**: associations of factor D and factor C3 with carotid stiffness measurements*

	Carotid DC (10^{-3}/kPa) [a]						**Carotid YEM** ($10^{\wedge 3}$ kPa) [a]					
	factor D [b]			**C3** [b]			**factor D** [b]			**C3** [b]		
	β	**[95%CI]**	**P-value**	**β**	**[95%CI]**	**P-value**	**β**	**[95%CI]**	**P-value**	**β**	**[95%CI]**	**P-value**
Model 1	**-0.832**	**-1.027; -0.638**	**<0.001**	**-0.547**	**[-0.742; -0.353]**	**<0.001**	**0.049**	**0.035; 0.063**	**<0.001**	**0.034**	**[0.020; 0.049]**	**<0.001**
Model 2	-0.090	-0.277; 0.096	0.342	**-0.432**	**[-0.607; -0.256]**	**<0.001**	0.011	-0.003;0.026	0.123	**0.028**	**[0.014; 0.041]**	**<0.001**
Model 3	-0.118	-0.288; 0.052	0.173	**-0.268**	**[-0.429; -0.107]**	**0.001**	0.013	0.000;0.027	0.058	**0.017**	**[0.004; 0.030]**	**0.010**
Model 4	-0.093	-0.263; 0.076	0.281	**-0.176**	**[-0.344; -0.008]**	**0.040**	0.011	-0.003;0.025	0.120	0.007	[-0.006; 0.021]	0.279
Model 5	-0.030	-0.206; 0.145	0.736	-0.097	[-0.278; 0.084]	0.293	0.005	-0.009;0.020	0.452	-0.001	[-0.015; 0.014]	0.909
Model 6	-0.004	-0.180; 0.173	0.968	-0.023	[-0.207; 0.161]	0.804	0.003	-0.011;0.018	0.657	-0.002	[-0.017; 0.013]	0.776

Legend to table 4: [a] Carotid DC, n=2659, Carotid YEM, n=2657; [b] Factor D and C3 are standardized. Bold typefont represents statistically significant data.

Model 1: Crude association

Model 2: adjusted for age, sex, education status

Model 3: additionally adjusted for mean arterial pressure

Model 4: additionally adjusted for yes/no T2D

Model 5: additionally adjusted for waist circumference

Model 6: additionally adjusted for smoking habits, lipid-modifying and/or anti-hypertensive medication, Total/high-density lipoprotein cholesterol ratio, triglycerides and HbA1c

Abbreviations: carotid DC, distensibility coefficient; carotid YEM, Young's elastic modulus

Discussion

In a large population-based cohort, we examined cross-sectional associations of the alternative complement factors D and C3 with arterial stiffness. The main finding of this study was that higher concentrations of factor D and C3 were associated with greater aortic and carotid stiffness, as represented by cfPWV, carotid DC and carotid YEM, but not independently of age, sex, education status, HR, MAP, and presence of T2D.

The positive associations of factor D and C3 with adiposity were as expected, given their strong expression in adipose tissue, and consistent with previous reports [6, 17]. The higher concentration of factor D in older individuals was shown before [9], although in a small group of healthy Caucasians an inverse association was reported [35]. Notably, when we restricted our analysis to individuals without T2D and CVD, the associations remained positive and significant (data not shown). The slightly higher concentration of C3 with increasing age is not consistently confirmed by existing literature [36] and may be related to higher prevalence of T2D in the older individuals. The higher concentration of factor D in men is consistent with our previous observation in an independent cohort [9] but not always confirmed [35]. Again, when we restricted our analyses to the participants without T2D and CVD, this sex-difference remained (data not shown). Factor D and C3 were both associated with higher MAP. This is corroborated by our own observations on systolic and diastolic blood pressure in an independent cohort [9, 37], as well as by reports that C3 is associated with development of hypertension [20] and that a polymorphism in *CFD* is associated with pulmonary hypertension in patients with systemic sclerosis [38].The association of C3, but not factor D, with HR has, to the best of our knowledge, not been reported before.

Arterial stiffness is, among others, determined by properties of the extracellular matrix such as collagen and elastin, and by vascular endothelial and smooth muscle cell function [3, 4, 39]. Factor D may contribute to arterial stiffness via cleavage of elastin fibers [8], while C3 may bind to collagen and elastin fibers within adventitia [22].

Factor D and C3 are implicated in worse endothelial dysfunction and low-grade inflammation via alternative pathway activation [9]. Complement activation results in generation of anaphylatoxins which are potent soluble mediators of inflammation [40], and induces membrane disruption in target cells [41], hence activating endothelial and smooth muscle cells [40, 41]. Low-grade inflammation results in functional stiffening of large arteries through impairment of endothelial function [42, 43], proliferation of smooth muscle cells [3], and increased synthesis of extracellular matrix proteins [3].

Approximately 80% of the crude association between factor D and cfPWV was explained by age, sex and education status and this was largely attributable to age. Little to no additional effect of MAP and HR was observed. Age-induced arterial wall remodeling contributes to arterial stiffness [44]. This is generally attributed to 'wear and tear' but the proposed function of factor D as a locally produced elastase [18] combined with strong relationship between age and factor D (Pearson's correlation 0.328, $P<0.001$) is striking. In contrast, age, sex and education status explained only 25% of the association between C3 and cfPWV, while strong additional attenuation was attributable to MAP and HR. A role for complement activation in hypertension-related vascular dysfunction has been proposed [45], and complement C3 has been implicated in development of hypertension [20]. Our current observation corroborates a potential contribution of C3 to cfPWV via the induction of hypertension.

Upon adjustment for T2D, the associations of factor D and C3 with cfPWV were further attenuated and non-significant. A previous study reported a positive association between C3 and cfPWV [21], but did not adjust for presence of diabetes at the time of cfPWW measurements, and the findings reported are hence in line with our current results. Factor D [30, 31] and C3 [46] have both been implicated in β-cell preservation. At the same time, C3 has been implicated in obesity-associated insulin resistance, and we [47] and others [33] identified C3 as an independent risk factor for T2D. As such, adjustment for T2D may to some extent represent overadjustment, as

the relationships between these complement factors and arterial stiffness may partly result from their effects on glucose metabolism and insulin resistance.

Upon full adjustment neither factor D nor C3 remained statistically significantly associated with greater carotid stiffness, as represented by carotid DC and carotid YEM. This does not corroborate the idea that carotid stiffness may act as mediator of the previously-reported association between factor D and stroke [12]. The association of factor D with cfPWV differed somewhat according to T2D status, as indicated by a significant interaction term, and in the fully adjusted models in stratified analyses the association was only significant in those with T2D, not in those without T2D. Interaction with T2D was only observed for aortic (cfPWV), not for carotid (YEM or DC) stiffness, and only for factor D, not for C3. This result should therefore be interpreted with caution, as it may represent a chance finding.

Strengths of our study include the large population-based cohort and the extensive phenotyping, which allowed thorough adjustment for potential confounders. Moreover, this is, for as far as we are aware, the first large human study in which both factor D and arterial stiffness are available. This study also has limitations. First, factor D and C3 have various sites of production including adipose tissue, perivascular fat, immune cells, β-cells, and for C3 also liver. Plasma measurements represent the integral of different cellular sources. Moreover, factor D and C3 exert multiple biological functions. Some of their effects place some of the potential confounders we included in our analyses, at least partly, in the causal path towards arterial stiffness. Indeed, as noted above, factor D and C3 have implicated in in β-cell preservation, while C3 has been particularly implicated in the development of hypertension, and development of insulin resistance and T2D. This may have introduced overadjustment. Also, factor D and C3 were measured systemically, while their production and effects in the vessel wall may be partly local. Moreover, our cross-sectional design hampers causal inference. Lastly, our study only focused on Caucasian individuals aged 40-75 years, which prohibits extension of the findings to other ethnicities and ages.

Conclusions

In this population-based study the association of factor D with arterial stiffness is for a large part explained by age, while the association of C3 with arterial stiffness is primarily explained by HR and MAP. A small part of the observed associations might be attributed to a causal path leading from alternative complement activation via hypertension and T2D to arterial stiffness.

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Chapter 4

Plasma concentrations of complement C3 explain part of the associations of adiposity with insulin resistance, fasting glucose and type 2 diabetes: The Maastricht Study.

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Chapter 5

A randomized diet-induced weight loss intervention reduces plasma complement C3: possible implication for endothelial dysfunction

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Abstract

Objective. Complement C3 and other components of the alternative pathway are higher in individuals with obesity. Moreover, C3 has been identified as a risk factor for cardiovascular disease (CVD). We investigated whether, and how, a weight loss intervention reduced plasma C3, activated C3 (C3a) and factor D and explored potential biological effects of such a reduction.

Methods. We measured plasma C3, C3a and factor D by ELISA, and visceral (VAT), subcutaneous adipose tissue (SAT) and intrahepatic lipid (IHL) by MRI in lean men (n=25) and men with abdominal obesity (n=52). The men with obesity were randomized to habitual diet or an 8-wk dietary weight loss intervention.

Results. The intervention significantly reduced C3 (-0.15 g/L [95%CI -0.23; -0.07]), but not C3a or factor D. The C3 reduction was mainly explained by reduction in VAT but not SAT or IHL. This reduction in C3 explained a part of the weight loss-induced improvement of markers of endothelial dysfunction, particularly the reduction in sE-selectin and sICAM.

Conclusions. Diet-induced weight loss in men with abdominal obesity could be a way to the lower plasma C3 and thereby improve endothelial dysfunction. C3 reduction may be part of the mechanism via which diet-induced weight loss could ameliorate the risk of CVD in men with abdominal obesity.

Introduction

Obesity is a global epidemic and the number of persons with excess body weight approaches 2 billion, worldwide, with one third being people with obesity [1, 2]. Particularly excess visceral adipose tissue (VAT) is associated with low-grade inflammation and may contribute to development of cardiovascular disease (CVD) [3, 4] and type 2 diabetes (T2D) [5, 6].

Obesity-induced endothelial dysfunction is one of the mechanisms via which obesity contributes to cardiometabolic diseases [7, 8]. Vascular endothelial cells are a major target of inflammatory damage [9]. Vascular endothelial dysfunction is a hallmark of the early stages of most CVD and is, among others, characterized by a higher expression of biomarkers such as soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), and soluble endothelial selectin (sE-selectin) [10].

The complement system is an intricate protein network that is part of the innate immune system. The endothelial lining of all blood vessels is, by virtue of its location, in close contact with circulating complement components. Several complement factors can directly or indirectly target the endothelium [9, 11]. Endothelial cells express anaphylatoxin receptors and complement regulators on their surface and are a direct target of complement [12, 13].

A substantial number of complement factors are produced in adipose tissue [14], and this production may be higher in individuals with obesity. In humans, plasma concentrations of factor D and complement C3, two main circulating components of the alternative complement pathway, are strongly associated with adiposity [15], and both are produced in adipose tissue [16, 17]. The anaphylatoxin C3a was also reported to be positively associated with body mass index (BMI) [18]. C3a is released by C3 upon complement activation [19] and may directly activate endothelial cells via the C3a receptor [20]. There are indications that the plasma concentrations of some of these complement components may change upon changes in body weight. Circulating

complement C3 was higher in obesity and decreased after non-randomized weight loss in patients with obesity [21, 22] while this was not the case for factor D [22]. In addition, the concentrations of C3, C3a and factor D, as well as other components of the alternative complement pathway, were lower in persons with anorexia [22]. While C3 and factor D increased upon weight gain in anorexia, the C3a level remained comparable to that at low weight [22]. Moreover, C3 was identified as key marker for change of body fat as found in a human proteomics study [23]. In addition, in an observational human cohort, we have shown that factor D, C3 as well as C3a were strongly associated with adiposity, but only changes in C3 were associated with changes in BMI over time [15].

The aim of our study was to evaluate whether diet-induced weight loss intervention reduced circulating concentrations of complement C3, factor D and/or C3a in a post-hoc evaluation of a previously-published weight loss intervention trial in apparently healthy, men with abdominal obesity [24, 25]. We also evaluated whether weight loss-induced changes in complement, if observed, were explained by a reduction of specific fat depots i.e. subcutaneous, visceral adipose tissue and/or intrahepatic lipid (VAT, SAT, IHL, respectively). We additionally explored whether changes in circulating complement components, if any, could explain the previously published observation that diet-induced weight loss improved markers of endothelial dysfunction [25, 26].

Methods

Study cohort

As described before [24, 25], Caucasian men were recruited via advertisements in local newspapers or among participants involved in earlier studies. They were included if they met the following inclusion criteria: age between 18 and 65 years, weight change

<3 kg within the previous 3 months, non-smokers, without diabetes, without cardiovascular disease, no drug or alcohol abuse, no use of medication known to affect lipid or glucose metabolism or hypertension, and no participation in another biomedical trial during the past 30 days. Twenty-five normal-weight (waist circumference: <94 cm) and 53 men with abdominal obesity (waist circumference: 102-110 cm) completed the baseline measurements. The men with obesity were allocated into 2 age groups (18-49 years or 50-65 years). Men in the same age group were randomly divided into the weight stable control group or to the weight loss group. Three men did not complete the weight loss study and one violated the protocol (see flow chart in **supplemental figure S1**). All participants gave written informed consent before entering the study. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center, performed in accordance with the Declaration of Helsinki, and registered at clinicaltrials.gov as NCT01675401.

Study design

At the start of the study, all men with normal weight and with abdominally obesity underwent baseline measurements at the research facilities. Details of the intervention have been published before [25]. Briefly, participants in the weight loss group visited our research dietitian every week and had a very-low-calorie diet (VLCD, Modifast; Nutrition et Sante Benelux) for at least 4 weeks, under strict guidance. After the VLCD period, a period of 1-2 weeks followed in which they were provided a calorie-restricted diet in line with the Dutch dietary guideline. In week 7 and week 8 the participants were kept in energy balance (weight-maintenance period). Participants in the weight stable control group maintained their normal diet, physical activities, and alcohol consumption, and were also monitored by the dietician through whole period.

Clinical measurements

As published previously [24], a 3.0T Philips Achieva MRI scanner with a dedicated 16-element torso coil (XLTorso coil; Philips Healthcare) was used to assess subcutaneous and visceral adipose tissue volumes. Two-dimensional T1-weighted turbo spin-echo images were acquired centered at the top of the L4 vertebral body. Images were analyzed offline with dedicated software (Hippo Fat; IFC CNR).

The same MRI scanner and coil were used to assess IHL content through mDixon imaging. Two 6-mm-thick transverse slices through the liver were acquired using a 2D three-point T1-fast field echo (T1-FFE) mDixon pulse sequence, to correct for T2* relaxation. The intrahepatic fat percentage was calculated in three regions of interest within the liver parenchyma, carefully avoiding blood vessels. The fat content was expressed as the weighted mean fat signal, divided by the sum of the weighted mean water and fat signal, as described before [24].

Blood analyses

After an overnight fast, blood was drawn through an intravenous catheter into NaF–containing vacutainer tubes (Becton, Dickinson and Company) and EDTA-coated vacutainer tubes (Becton, Dickinson and Company) on ice. Within 30 min after blood sampling, the tubes were centrifuged at 1300 x g for 15 min at 4°C to obtain plasma. Blood drawn in vacutainer serum tubes (Becton, Dickinson and Company) was allowed to clot for 30 min at 21°C and centrifuged at 1300 x g for 15 min at 21°C. Plasma and serum aliquots were stored at -80°C until use.

Endothelial function markers [soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), soluble endothelial selectin (sE-selectin)] were measured in EDTA plasma on a multiarray detection system based on electrochemiluminescence technology (SECTOR Imager 2400; Meso Scale Discovery), while Von Willebrand factor (vWf) was assessed by ELISA in citrate plasma, all as previously described [25]. The inter-assay coefficients of variation were 3.1%, 4.2%, 5.7% and 7.2% for sICAM-1, vWf, sE-selectin and sVCAM-1, respectively.

To reduce the influence of the biological variability of each marker and achieve statistical efficiency, we standardized sum score for endothelial dysfunction. To obtain standardized sum scores we first standardized each individual biomarker, then z-scores were averaged into overall standardized endothelial dysfunction score.

Complement factor D was measured in EDTA plasma using an R&D duoset kit assay, as described before [27]. Complement C3 was measured in EDTA plasma using an MSD R-plex Human Complement C3 Antibody Set (Mesoscale Discoveries). The assay was performed according to the manufacturer's instruction except for the use of a 1:20.000 instead of 1:300.000 dilution, which resulted in better stability of the measurements. Complement C3a was measured in EDTA plasma by ELISA (MicroVue C3a plus EIA kit, Quidel, San Diego, USA) [19]. The inter-assay coefficients of variation were 4.0%, 8.9%, and 4.2% for factor D, C3 and C3a, respectively.

Glucose concentrations were measured in NaF–plasma (Horiba ABX). Serum samples were analyzed for total cholesterol (CHOD-PAP method; Roche Diagnostics), HDL-cholesterol (precipitation method; Roche Diagnostics), triacylglycerol (GPO Trinder; Sigma-Aldrich Corp.), HbA1c (Bio-Rad). LDL cholesterol was calculated by using the Friedewald formula and triacylglycerol was corrected for free glycerol [25].

Statistical analyses

Normally distributed variables are presented as mean ± SD. Skewed variables are presented as median with interquartile range (IQR). Differences at baseline between men with normal weight and with abdominal obesity were examined by an independent Student's t test in case of normally distributed data or Mann-Whitney *U* test in case of a skewed distribution. One-factor analysis of covariance (ANCOVA), using baseline measurements as covariates, was performed to evaluate the effect of weight loss intervention. To take into account the age-stratification in the randomization process, adjustment for age was performed an additional analysis Linear regression was performed to investigate the association of changes in fat measures, with changes

in complement concentration. Linear regression with adjustment for age was used to investigate the association of (i) the cross-sectional association of BMI and measures of body composition, with complement concentration, and (ii) the assocations of the intervention and changes in measures of body components with changes in complement concentrations. Multiple mediator analysis was used to study whether (i) a specific fat depot (i.e. SAT, VAT and/or IHL) independently mediated the cross-sectional association of BMI with plasma complement concentrations or (ii) whether changes in a specific fat depot (i.e. SAT, VAT and/or IHL) independently mediated the effect the weight loss intervention on changes in complement concentrations. To further explore possible effects of weight loss-induced changes in circulating complement on biomarkers of endothelial dysfunction, their associations were evaluated using linear regression. and single mediator analyses were done to investigate whether (change in) complement concentration significantly mediated the association of BMI or intervention with (changes in) markers for endothelial dysfunction. Statistical analyses were performed using SPSS 25.0 (SPSS Inc, Chicago, IL, USA). A two-sided p-value of < 0.05 was considered statistically significant. Mediation analyses were conducted with the PROGRESS plug-in for SPSS version 3.5.2 (Andrew F. Hayes, The Ohio State University, Columbus, Ohio, USA). Bootstrapped confidence intervals (5000 samplings) were generated and effects were deemed significant when the confidence interval did not include zero.

Results

Study participants: Comparison of participants with normal weight and with abdominal obesity

Men with normal weight (n=25) and with abdominal obesity (n=52) that completed the baseline measurements were analyzed, as reported before [24]. Baseline

characteristics of the participants are shown in **Table 1.** The median age was similar for the men with normal weight and with abdominal obesity. Anthropometric measures, which include weight, waist circumference, and BMI, were higher in the participants with abdominal obesity, as were VAT, SAT, and IHL (P<0.001). The metabolic profile, i.e. blood pressure, lipid metabolism (total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides) and glucose metabolism (fasting plasma glucose and HbA1c), was generally worse in men with abdominal obesity. Plasma sE-selectin was significantly higher in men with abdominal obesity (P<0.001), sICAM-1 also tended to be higher, albeit not statistically significantly (p=0.086), whereas sVCAM and vWf were comparable between the two groups. Factor D (13%) and C3 (22%) were higher in men with abdominal obesity, compared to lean men (P=0.027 and P<0.001, respectively). While plasma C3a levels did not statistically differ between lean men and men with abdominal obesity (p=0.194).

Effect of weight loss intervention on plasma concentrations of factor D, C3 and C3a

Table 2 and **supplemental figure S2** show the effect of the weight loss intervention on the complement components. The intervention significantly reduced plasma C3 (-0.15 g/L; [95% confidence interval -0.23; -0.07 g/l], p<0.001), which is approximately 10% of the baseline concentration. No significant changes were observed in plasma factor D (-0.03 mg/L [-0.09; 0.02]) or C3a levels (3.74 µg/L [-2.50; 9.98]). Additional adjustment for age did not affect these results.

Supplemental table S1 shows that, in the men with abdominal obesity, the changes in BMI, SAT and VAT that were observed over the 8 weeks follow-up period associated with the change in C3 (β=0.042 to 0.156 g/l, P=0.030 to P<0.001). No significant associations were observed between changes in adiposity and changes in factor D or C3a.

***Table 1**: Baseline characteristics of the study population*

	Lean (n=25)	Obese (52)	P value
	Baseline	Baseline	
Age (yrs)	53.7 [25.0-61.6]	51.8 [45.7-60.7]	0.965
Body weight measures			
Weight (kg)	74.9±8.3	96.9±8.4	<0.001
Waist circumference (cm)	84.9±6.3	106.5±3.6	<0.001
BMI (kg/m^2)	23.3±1.8	30.1±2.1	<0.001
Subcutaneous fat volume (l) [1]	1.45±0.51	3.09±0.78	<0.001
Visceral fat volume (l) [1]	0.89±0.42	2.34±0.72	<0.001
Intrahepatic lipid content (%) [1]	3.43 [3.13-3.78]	4.96 [3.90-7.86]	<0.001
Blood pressure			
24-hour systolic blood pressure (mmHg)	117.5±8.8	123.4±8.7	0.007
24-hour diastolic blood pressure (mmHg)	72.5±9.4	80.4±7.3	<0.001
Lipid metabolism status			
Total cholesterol (mmol/l)	4.55±0.78	5.56±0.97	<0.001
HDL cholesterol (mmol/l)	1.26±0.26	1.11±0.21	0.008
LDL- cholesterol (mmol/l) [2]	2.82±0.70	3.68±0.89	<0.001
Triglycerides (mmol/l)	0.95 [0.67-1.11]	1.66 [1.17-2.19]	<0.001
Glucose metabolism status			
HbA1c (%)	5.18±0.37	5.30±0.37	0.193
Fasting plasma glucose (mmol/l)	5.35±0.29	5.64±0.48	0.006
Markers of Endothelial dysfunction			
sE-selectin (ng/mL)	70.4±28.6	108.0±44.6	<0.001
sICAM-1(ng/mL)	234.7±37.7	255.0±51.9	0.086
vWf (%)	125.9±38.2	125.1±44.2	0.937
sVCAM-1 (ng/mL)	398.3±82.8	413.5±79.1	0.439
Components of the alternative complement pathway			
Factor D (mg/l)	0.86±0.17	0.97±0.21	0.027
C3 (g/l)	1.29±0.26	1.57±0.24	<0.001
C3a (µg/l)	32.5 [27.5-38.5]	35.4 [30.3-47.7]	0.194

Legend to table 5-1: [1] analyzed in 24 lean and 52 obese men; [2] in 25 lean and 50 obese men. Data presented as mean ± SD (normal distribution) or median [IQR] (skewed distribution), as partially published before [24]. P values were obtained by independent Student's *t* test or Mann-Whitney *U* test, where appropriate. Abbreviations: BMI: body mass index; MAP: mean arterial pressure; HR: heart rate; HbA1c, glycated hemoglobin; sICAM-1, soluble intercellular adhesion molecule 1; sVCAM-1, soluble vascular cell adhesion molecule 1; sE-selectin, soluble endothelial selectin; vWf: von Willebrand factor

***Table 2**: Plasma complement concentration before and after the 8-wk dietary intervention.*

	Weight stable control group [1]		Weight loss group [2]		Treatment effect	
	Baseline	Follow-up	Baseline	Follow-up	Mean change (95% CI)	P value
Factor D (mg/l)	0.96±0.20	0.97±0.19	0.99±0.22	0.96±0.20	-0.03 [-0.09; 0.02]	0.237
C3 (g/l)	1.62±0.22	1.58±0.22	1.53±0.25	1.37±0.18	**-0.15 [-0.23; -0.07]**	**<0.001**
C3a (ug/l)	35.3 [32.3-46.7]	35.0 [29.5-42.3]	35.5 [26.9-49.4]	40.0 [26.4-60.7]	3.74 [-2.50; 9.98]	0.234

Legend table 2: [1] n=26; [2] n=23. P value of treatment effect was obtained by 1-factor ANCOVA with baseline value as covariate. C3a concentrations at baseline and follow-up were skewed distributed, while the change of C3a concentration showed a normally distribution. When C3a was ln-transformed in a sensitivity analysis, the effect weight loss intervention was comparable.

***Table 3**. Multivariate linear associations of the (changes in) different fat depots (independent variables) with (changes in) C3 (g/L, dependent variable).*

	Intervention study [1]			Cross-sectional study [2]		
		Δ C3 (95% CI)	P value		C3 (95% CI)	P value
Model 1	Δ SC fat (L)	**0.103 [0.005; 0.200]**	**0.040**	SC fat (L)	**0.121 [0.068; 0.174]**	**<0.001**
Model 2		-0.034 [-0.163; 0.095]	0.600		**0.055 [0.003; 0.108]**	**0.040**
Model 1	Δ V fat (L)	**0.157 [0.075; 0.239]**	**<0.001**	VI fat (L)	**0.199 [0.141; 0.256]**	**<0.001**
Model 2		**0.173 [0.054; 0.292]**	**0.005**		**0.112 [0.036; 0.189]**	**0.005**
Model 1	Δ IHL (%)	0.019 [-0.003; 0.042]	0.093	Ln IHL lipid (%)	**0.308 [0.193; 0.422]**	**<0.001**
Model 2		0.003 [-0.021; 0.026]	0.827		**0.161 [0.039; 0.282]**	**0.011**

Legend to table 3: [1] N=49; [2] N=78. Model 1: Adjusted for age, Model 2: Additionally adjusted for the other 2 fat depots. Abbreviations: SC fat; Subcutaneous fat, V fat; Visceral fat; IHL, intrahepatic lipid

Next, we evaluated whether the significant effects of weight loss intervention on plasma complement C3 were attributable to changes in one of more of the individual fat depots. In the intervention study, only the change in VAT was associated with the change in C3, independent of age and independent of the other fat depots (**Table 3**). In multiple mediator models, we subsequently observed that the association between the weight loss intervention and changes in C3 was substantially and

independently mediated by changes in VAT (C3: -0.147 g/L, [-0.285; -0.001]) but not by changes in SAT or IHL (**Figure 5-1A**).

In the cross-sectional linear regression analyses, SAT, VAT and IHL were each associated with C3 concentration (**Table 3**). In multiple mediator models, the association between BMI, as a measure of generalized adiposity, and plasma C3 was independently mediated by VAT (0.020 g/L, [0.005; 0.040]) and by IHL (0.011g/L, [0.003; 0.021]), but not by SAT (**Figure 5-1B**).

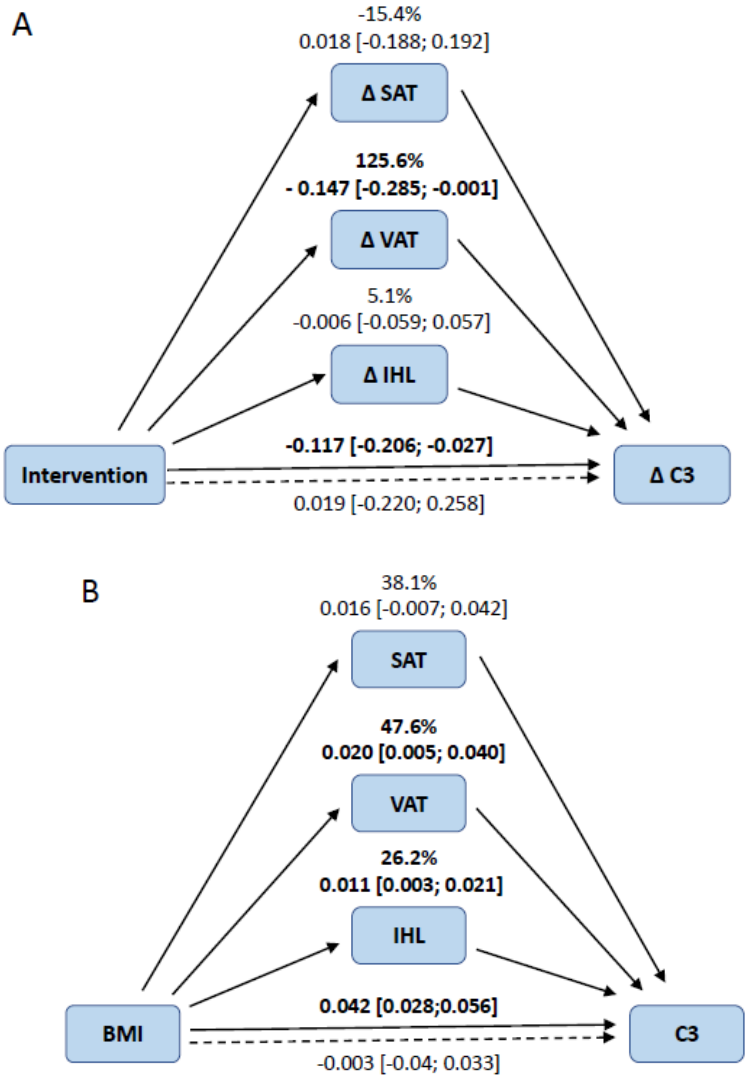

Figure 5-1: Multiple mediator models to determine the contribution of the different fat depots (subcutaneous adipose tissue [SAT], visceral adipose tissue [VAT] and intrahepatic lipid [IHL]) on the difference (or change) in C3. (A) Multiple mediator model where Δ VAT, but not ΔSAT or Δ IHL, was an independent mediator of the association between the weight loss intervention and Δ C3 (n=49). (B) Multiple mediator model adjusted for age

where VAT and ln-transformed IHL, but not SAT, were significant mediators of the cross-sectional association between BMI and plasma C3 (n=76).

Effect of abdominal obesity and the weight loss intervention on markers of endothelial dysfunction via change in plasma C3

To explore the potential biological consequences of the effects of weight loss intervention on alternative complement pathway components, we also investigated whether changes in complement associated with changes in circulating markers of endothelial dysfunction. **Table 4** shows that the association of the change in C3 associated with the change in the overall endothelial dysfunction score (β=2.500 SD [0.866; 4.125]). In line with this, the change in C3 also significantly associated with changes in sICAM (78 ng/mL [37; 119]) and sE-selectin (83 ng/mL [39; 127]), but not vWF or sVCAM.

***Table 4**: Linear association between change in plasma C3 concentrations and changes in plasma concentrations of biomarkers for endothelial dysfunction*

	Δ C3 (g/l)	
	95% CI	P value
Δ endothelial dysfunction score	**2.500 [0.866; 4.125]**	**0.003**
Δ sE-selectin (ng/mL)	**83.00 [39.17; 126.83]**	**<0.001**
Δ sICAM-1 (ng/mL)	**78.12 [36.94; 119.29]**	**<0.001**
Δ vWF (%)	17.21 [-30.77; 65.18]	0.474
Δ sVCAM-1 (ng/mL)	29.00 [-67.11; 125.11]	0.547

Legend to table 4: Crude unstandardized associations between changes in plasma C3 and changes in plasma endothelial biomarkers. Results are shown for all obese men who participated in the intervention study (n=49). sE-selectin, soluble endothelial selectin; sICAM-1, soluble intercellular adhesion molecule 1; vWf: von Willebrand factor; sVCAM-1, soluble vascular cell adhesion molecule 1

We subsequently explored whether C3 could be a mediating variable in the associations of adiposity (BMI, cross-sectional analyses) or the weight loss intervention, with endothelial dysfunction. In **Table 5**, the results are shown of single mediation analyses in which we explored the mediating effect of C3 on the associations of either BMI or the weight loss intervention with the combined endothelial dysfunction score or the individual endothelial markers. The intervention-induced change in C3 partially mediated the effect of the intervention on the overall score for endothelial dysfunction (-0.22 SD [-0.66; 0.01]), on sE-selectin (-6.10 ng/ml, [-14.2; -0.92]), and on sICAM (-6.65 ng/ml, [-17.2; -0.54]). In subsequent sensitivity analyses, we evaluated the mediating effects of C3 on the associations of waist circumference and VAT with the endothelial dysfunction markers (data shown in **Supplemental Table S2**). Additional adjustment for age did not affect any of the associations observed in the weight loss intervention.

***Table 5**: The mediating effect of (changes in) C3 on the association of weight loss intervention or BMI (kg/m^2) with (changes in) markers of endothelial dysfunction (dependent variables) in simple mediator models.*

Dependent	**Independent: Intervention (Y/N) [1]**				**Dependent**	**Independent: BMI (kg/m^2) [2]**			
	path [3]	β	95% CI	% [4]		path [3]	β	95% CI	% [4]
Δ EndDys score (SD)	c	**-0.86**	**[-1.39; -0.33]**		EndDys score (SD)	c	**0.06**	**[0.00; 0.12]**	
	c'	**-0.64**	**[-1.18; -0.10]**			c'	0.02	[-0.05; 0.08]	
	*a*b Δ C3*	*-0.22*	*[-0.66; 0.01]*	*26%*		a*b C3	***0.05***	***[0.01; 0.10]***	***83%***
Δ sE-selectin (ng/ml)	c	**-36.5**	**[-48.9; -24.1]**		sE-selectin (ng/ml)	c	**4.81**	**[2.38; 7.23]**	
	c'	**-30.4**	**[-42.8; -17.9]**			c'	**3.88**	**[1.14; 6.63]**	
	*a*b Δ C3*	***-6.10***	***[-14.2; -0.92]***	***17%***		a*b C3	*0.92*	*[-0.60; 2.60]*	*19%*
Δ sICAM (ng/ml)	c	**-26.5**	**[-39.9; -13.2]**		sICAM (ng/ml)	c	**3.13**	**[0.27; 5.98]**	
	c'	**-19.9**	**[-33.3; -6.47]**			c'	1.24	[-1.91; 4.39]	
	*a*b Δ C3*	***-6.65***	***[-17.2; -0.54]***	***25%***		a*b C3	***1.88***	***[0.11; 4.78]***	***60%***
Δ vWF (ng/ml)	c	-5.47	[-20.5; 9.60]		vWF (ng/ml)	c	-0.69	[-3.08; 1.71]	
	c'	-3.02	[-19.2; 13.2]			c'	-1.96	[-4.64; 0.72]	
	*a*b Δ C3*	*-2.44*	*[-16.6; 5.61]*			a*b C3	*1.27*	*[-0.44; 3.19]*	
Δ sVCAM (ng/ml)	c	3.47	[-28.3; 35.2]		sVCAM (ng/ml)	Cc	1.25	[-3.64; 6.14]	
	c'	7.64	[-26.6; 41.9]			c'	-1.99	[-7.38; 3.41]	
	*a*b Δ C3*	*-4.17*	*[-15.2; 7.63]*			a*b C3	*3.24*	*[-0.89; 6.29]*	

Legend to table 5: [1] N=49; [2] N=77; [3] c is the total effect i.e. the regression coefficient of the association of BMI or the intervention as independent and the respective marker of endothelial dysfunction as outcome, c' is the direct effect, a*b is the indirect affect via (change in) plasma C3.[4] The proportion mediated effect [a*b/c] was only calculated

when the total effect (c path) was significant. EndDys score, endothelial dysfunction score; sE-selectin, soluble endothelial selectin; sICAM-1, soluble intercellular adhesion molecule 1; vWf: von Willebrand factor; sVCAM-1, soluble vascular cell adhesion molecule 1

Discussion

This randomized controlled dietary weight loss intervention study in men with abdominal obesity has several main observations. First, the weight loss intervention reduced C3, but not factor D or C3a. Second, the effect of intervention on plasma C3 is partially explained by changes in VAT. Third, the effect of weight loss intervention on plasma markers of endothelial dysfunction was partly mediated by changes in C3.

The weight loss intervention reduced the plasma concentration of complement C3. Also in the cross-sectional comparison, the C3 concentration was significantly higher in men with obesity than in the normal weight group, as was reported previously [15, 22]. The observed reduction in C3 after weight loss intervention agrees with reports from non-randomized, non-controlled weight loss trials in men and women of various degrees of obesity, yet all with a BMI above 40 kg/m^2 [21, 22, 26]. A reduction in C3 generally results from either hypoproduction, i.e. less production of C3 in tissue, including adipose tissue, or hyperconsumption, i.e. reduction of C3 upon of activation of the C3 cascade, when C3 is converted into C3a and C3b. Since plasma C3a in our study did not change with weight loss intervention, we consider it most likely that the decrease in C3 with weight loss intervention resulted from decreased C3 production, rather than increased consumption. As the reduction in C3 with weight loss intervention was mainly explained by VAT, we speculate that the weight loss intervention reduced the production of C3 in this adipose tissue depot. It could be argued that there may be an indirect effect as well, e.g. via reduction of C3 production in the liver, which is the main source of plasma C3 [17]. However, the most likely mechanism via which a reduction in VAT would lead to a lower hepatic C3 production would be via a decrease in circulating inflammatory factors [26], which were unaffected in our study [27]. Also, the change in IHL did not contribute to the association between weight loss intervention and changes in plasma C3, independent of changes in VAT. A reduction in plasma C3 could result in a reduced potential for activation of the alternative complement pathway and of the common amplification

loop. There may, however, also be effects that are independent of canonical complement activation. As an example, C3 can directly interact with fibrinogen [28]. Incorporation of C3 into fibrin clots can contribute to hypofibrinolysis and hence to enhanced thrombosis risk [28].

In our study, the diet-induced change in C3 partly explained the beneficial effect of weight loss intervention on the endothelial markers sE-selectin and sICAM. A cross-sectional association of C3 with these markers was reported before [26]. Our results suggest that a weight loss-induced reduction in C3 reduces endothelial dysfunction in apparently healthy, men with abdominal obesity. These results may have potential relevance in light of the worldwide SARS-CoV-2 epidemic. Severe COVID-19 disease is characterized by, among others, inflammation, endothelial dysfunction, and thrombotic microangiopathy [29, 30], and men with obesity have a high risk to develop severe coronavirus disease-2019 (COVID-19) upon infection [31]. Inhibition of C3 activation improved outcomes in severe COVID-19 [32] and we speculate that reduction in plasma C3 via diet-induced weight loss might be beneficial in the prevention of severe COVID-19 disease in men with obesity.

In contrast to plasma C3, the concentration of the other complement factor that were studies, factor D and C3a, did not change with weight loss intervention. At baseline, factor D was slightly higher in participants with obesity than in one with normal weight, which is in line with previous publications [15, 33] although not always significant[22]. Given its profound expression in adipose tissue, one might intuitively expect the plasma concentration of factor D to be responsive to weight loss, but our current data do not support that premise. In fact, , the change in factor D concentration with weight loss intervention was small and not significant, which is agreement with what was found before [22].

It was previously reported that the expression of factor D in adipose tissue may differ between men and women. In women, factor D was inversely correlated with BMI in SAT but not in VAT, while in men, a similar inverse correlation with SAT was present

but a positive correlation was observed in VAT [34]. Based on these cross-sectional observations, we speculate that in men with obesity, weight loss intervention would decrease the expression (and production) of factor D by adipocytes in VAT, but this may be counteracted by a concomitant increase in factor D expression (and production) by SAT, the total result of which would be the observed, non-significant effect of weight loss intervention on circulating factor D in men with obesity. Also, *in vitro* studies showed that factor D, as well as C3a, may be active contributors to lipid accumulation in adipocytes, rather than merely products of adipose tissue, which would provide an additional explanation for the fact that they are not, or less, responsive to diet-induced weight loss [35].

Although baseline C3a was approximately 9% higher in men with abdominal obesity compared to lean men, this difference was not statistically significant. Previous publications did report higher C3a in people with obesity than in lean individuals, but these reports often concerned studies in persons with extreme levels of obesity, with obesity-related comorbidities such as metabolic syndrome, and/or with larger numbers of participants [15, 36-38]. This may explain why our current findings that C3a did not change upon weight loss intervention in men with abdominal obesity contrasts with a previous report that in women plasma C3a was reduced upon extreme weight loss resulting from bariatric surgery [38]. Our current observations are, however, in agreement with the observation that C3a was neither reduced in women with anorexia, nor increased upon weight gain [22]. In addition, we previously reported that in our current weight loss intervention study the concentration of several plasma biomarkers of systemic inflammation were not altered by the weight loss intervention [25]. C3a is an inflammatory factor [39] and our observation that C3a was not changed is therefore in line with these previous findings.

This study has several strengths. Firstly, it is a randomized and controlled weight loss intervention. Second, the intervention is combined with a cross-sectional comparison of lean participants and participants with abdominal obesity, allowing for

a comprehensive evaluation. Third, participants were apparently healthy and moderately obese, thus representing a large group of persons in our society. Fourth, the MRI data allowed us to look beyond abdominal obesity per se, and to evaluate the independent effects of distinct fat depots. Our study also has limitations. First, no measurements were available between baseline and follow up, therefore we cannot determine the order of events that result from the weight loss intervention. Also, our observation that the concentration of C3a was not altered in plasma does not necessarily exclude the possibility that the weight loss intervention did, to some extent, affect local generation of C3a. (Local) C3 activation can induce endothelial dysfunction [12, 40] but C3 may also be produced by endothelial cells, at least *in vitro* [41, 42]. Hence, reverse causation cannot be fully excluded. Lastly, our study involved Caucasian men, which prohibits extension of the findings to women and to other ethnicities.

Conclusions

In conclusion, we showed that diet-induced weight loss intervention reduced the plasma concentrations of complement C3 in men with abdominal obesity. This reduction in C3 was mainly explained by the reduction in VAT. In turn, the reduction in C3 partly explained the weight loss-associated improvement of plasma biomarkers of endothelial dysfunction, in particular sE-selectin and sICAM. Reduction in C3 can be one of the mechanisms via which diet-induced weight loss intervention could reduce the risk of obesity-associated diseases such as cardiovascular disease and type 2 diabetes.

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Supplemental material

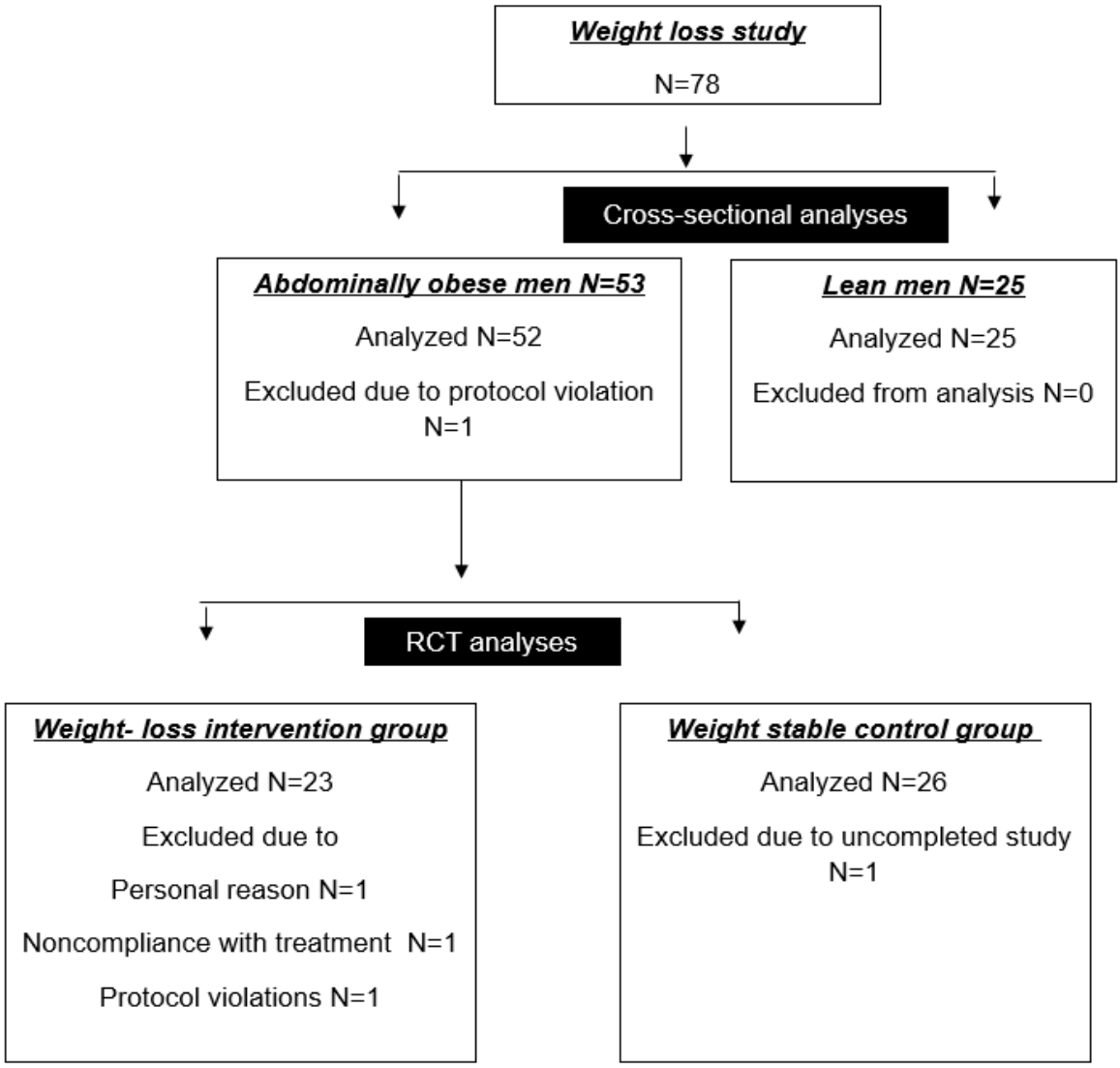
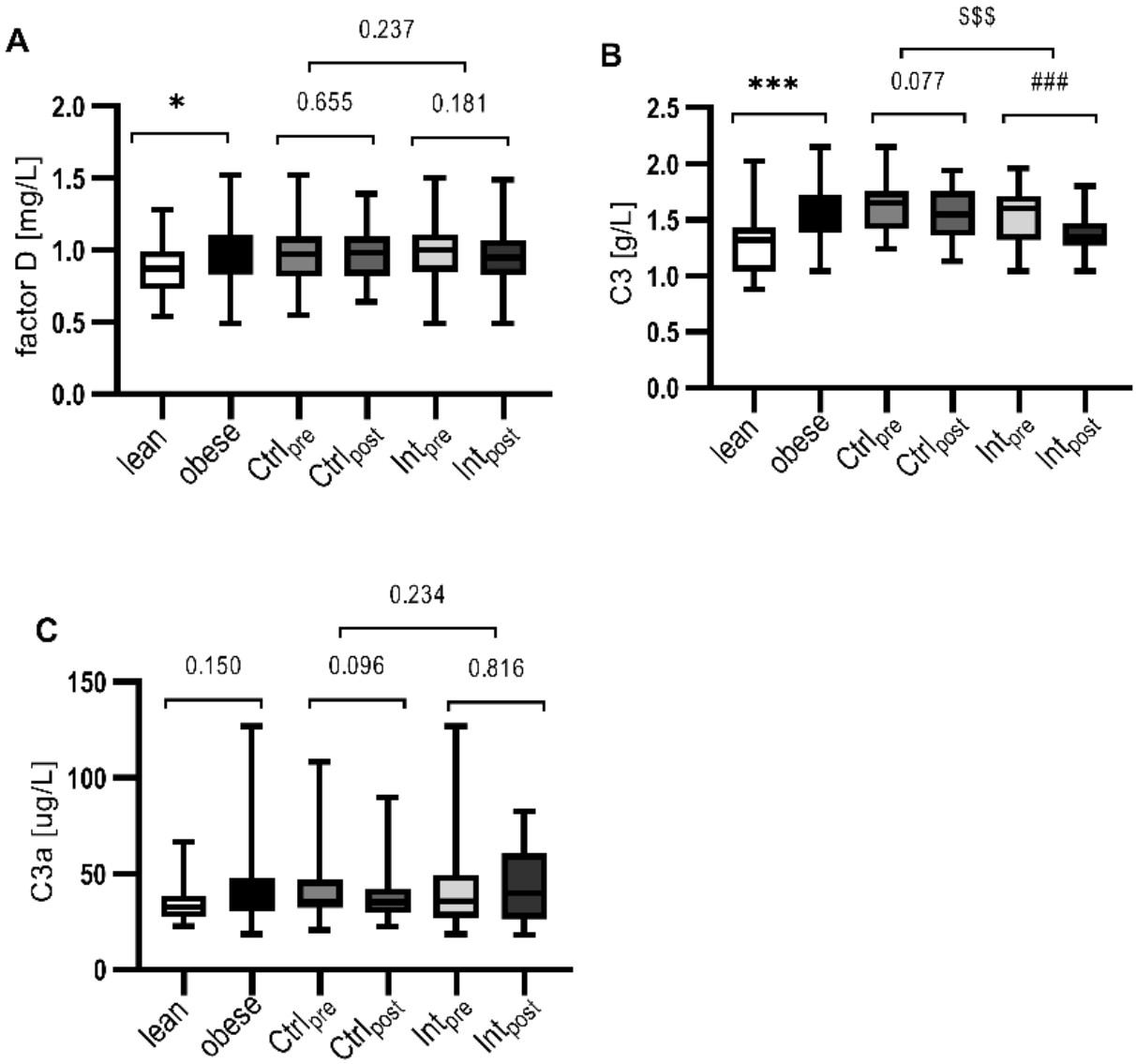


Figure S5-1. Derivation of final study population [24]



Legend to figure S5-2: Characteristics of the study population and effects of weight loss intervention on plasma concentration of complement components. (**A**) Factor D, (**B**) Complement C3, (**C**) Complement C3a. Box plots are as follows: black line, median; box edges, 1st and 3rd quartiles; whiskers, minimum and maximum of all data. Cross-sectional differences were obtained by independent Student's *t* tests (lean vs. obese; * P<0.05, ***P<0.001), differences over time by paired *t* test (baseline vs. follow-up; ### P<0.001), and differences between groups over time by means of 1-factor ANCOVA with baseline value as covariate (control group vs. intervention group; $$$ P<0.001). C3a concentrations at baseline and follow-up were skewed distributed, while the change of C3a concentration showed a normal distribution. When C3a was ln-transformed C3 in a sensitivity analysis, the effect of weight loss intervention was comparable.

Supplemental table S1: *Linear association between change in fat measures and change in plasma C3 concentration in intervention study*

	β [95% CI]	*P value*
	Δ factor D (mg/L)	
Δ BMI (kg/m^2)	0.010 [-0.008; 0.027]	*0.287*
Δ Subcutaneous fat (L)	0.028 [-0.035; 0.091]	*0.376*
Δ Visceral fat volume (L)	0.055 [-0.002; 0.111]	*0.058*
Δ intrahepatic lipid (%)	0.002 [-0.013; 0.016]	*0.789*
	Δ complement C3 (g/L)	
Δ BMI (kg/m^2)	**0.042 [0.016; 0.069]**	***0.002***
Δ Subcutaneous fat (L)	**0.107 [0.011; 0.203]**	***0.030***
Δ Visceral fat volume (L)	**0.156 [0.075; 0.238]**	***<0.001***
Δ intrahepatic lipid (%)	0.020 [-0.003; 0.042]	*0.085*
	Δ complement C3a (µg/L)	
Δ BMI (kg/m^2)	-0.541 [-2.805; 1.723]	*0.633*
Δ Subcutaneous fat (L)	-1.478 [-9.416; 6.461]	*0.710*
Δ Visceral fat volume (L)	-2.389 [-9.724; 4.946]	*0.515*
Δ intrahepatic lipid (%)	-1.498 [-3.256; 0.261]	*0.093*

Legend to Table S1: Crude unstandardized associations between changes in BMI or fat depot and changes in plasma concentration of the complement factors. Results are shown for all obese men who participated in the intervention study (n=49).

Supplemental table S2: *The mediating effect of C3 on the association of waist (cm) or VAT (L) with markers of endothelial dysfunction (dependent variables) in simple mediator models*

Dependent	**Independent: waist (cm)** [1]				**Dependent**	**Independent: VAT (L)** [2]			
	path c	β	95% CI	% [b]		path [d]	β	95% CI	% [b]
EndDys score (SD)	c	**0.01**	**[0.00; 0.03]**		EndDys score (SD)	c	**0.32**	**[0.06; 0.58]**	
	c'	0.00	[-0.01; 0.02]			c'	0.10	[-0.23; 0.42]	
	a*b C3	**0.01**	**[0.00; 0.02]**	***100%***		a*b C3	**0.22**	**[0.00; 0.51]**	***69%***
sE-selectin (ng/ml)	c	**1.45**	**[0.61; 2.28]**		sE-selectin (ng/ml)	c	**22.83**	**[11.93; 33.74]**	
	c'	**1.06**	**[0.13; 1.99]**			c'	**19.57**	**[5.54; 33.61]**	
	a*b C3	0.39	[-0.05; 1.01]	27%		a*b C3	3.26	[-6.95; 13.21]	14%
sICAM (ng/ml)	c	0.82	[-0.15; 1.80]		sICAM (ng/ml)	**c**	**15.14**	**[2.25; 28.03]**	
	c'	0.11	[-0.95; 1.16]			c'	4.44	[-11.72; 20.61]	
	a*b C3	0.72	[0.16; 1.68]			a*b C3	10.69	[-1.67; 25.60]	*71%*
vWF (ng/ml)	c	-0.027	[-0.89; 0.84]		vWF (ng/ml)	c	-1.90	[-12.56; 8.76]	
	c'	-0.28	[-1.26; 0.70]			c'	-8.76	[-22.3; 4.76]	
	a*b C3	0.25	[-0.44; 0.82]			a*b C3	6.86	[-1.27; 16.45]	
sVCAM (ng/ml)	c	0.18	[-1.47; 1.83]		sVCAM (ng/ml)	c	7.68	[-12.55; 27.91]	
	c'	-0.98	[-2.77; 0.81]			c'	-4.90	[-30.60; 20.80]	
	a*b C3	1.16	[0.027; 2.17]			a*b C3	12.57	[-2.65; 30.58]	

Legend to table S2: [1] N=77; [2] N=76, [c] is total effect i.e. the regression coefficient of the association of waist or VAT as independent and the respective marker of endothelial dysfunction as outcome, c' is the direct effect, a*b is the indirect affect via plasma C3. [d] The proportion mediated effect [a*b/c] was only calculated when the total effect (c path) was significant

Chapter 6

Summary and general discussion

Obesity affects not only adults, but also children as well as adolescents all over the world. The number of persons with overweight approaches approximately 2 billion worldwide, and persons with obesity are around 700 million [1, 2]. Persons with obesity are more easily affected by life-threatening medical complications, such as dyslipidemia, type 2 diabetes (T2D), cardiovascular disease (CVD), and metabolic syndrome [3, 4]. Obesity-associated medical complications, especially CVD, are the leading cause of the morbidity and mortality globally [5]. The higher presence of obesity, overweight and associated medical complications worsen medical and economic burdens [5, 6]. Therefore, more insight in the aetiology of obesity and its related complications is needed.

The pathways that underlie the link between obesity and cardiometabolic diseases are not yet fully elucidated. The complement system may be involved because complement is produced in adipose tissue, is higher in obesity and has been implicated in cardiometabolic disease. The studies in this thesis focus on components of the alternative pathway because this pathway has been most consistently implicated in cardiometabolic diseases. For these studies we have measured C3, which is the central component of the alternative complement pathway; C3a, which is the cleaved product of C3; and factor D, the rate-limiting protease in the activation of the alternative complement pathway, in ~3700 participants of the Maastricht Study and in 75 participants who participated in a weight loss intervention.

1. Main findings

In **chapter 2**, we investigated the association of factor D with vascular dysfunction and CVD in The Maastricht Study. We found that a greater plasma concentration of factor D significantly associated with multiple markers of low-grade inflammation and endothelial dysfunction, as well as with more CVD, and particularly with more cerebral CVD in men. In contrast, factor D was associated with neither ankle-brachial index (ABI), a marker of subclinical peripheral atherosclerosis, nor carotid intima-media thickness

(carotid IMT), a marker of arterial injury. Overall, these findings imply that factor D is involved in CVD which may manifest via low-grade-inflammation and endothelial dysfunction, possibly accompanied by a higher tendency to develop atherothrombosis, rather than via enhanced atherosclerosis.

In **chapter 3**, we investigated the association of factor D and C3 with arterial stiffness, as represented by carotid-femoral pulse wave velocity (PWV), carotid distensibility coefficient (DC) and carotid Young's elastic modulus (YEM), in The Maastricht Study. We found that concentrations of factor D and C3 were positively associated with greater arterial stiffness, but not independently of age, sex, education status, heart rate (HR), mean arterial pressure (MAP), and presence of T2D. The association of factor D with arterial stiffness was for a large part explained by age, while the association of C3 with arterial stiffness was primarily explained by HR and MAP. Overall, these findings imply that a small part of the observed associations of factor D and C3 with arterial stiffness might be attributed to a causal path leading from alternative complement activation, via hypertension and T2D, to arterial stiffness.

In **chapter 4**, we investigated whether complement factors (complement C3, factor D and C3a) explained (parts of) the association of measures of obesity [BMI (body mass index), waist, visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT) with disturbed metabolism homeostasis (fasting glucose, insulin resistance, prevalence of T2D) in The Maastricht Study. We found that the measures of obesity were significantly and positively associated with a disturbed metabolism homeostasis: fasting glucose, insulin resistance, and prevalence of T2D. We also found that C3 explained a substantial part of the relationship of obesity with those measures of disturbed metabolism homeostasis. Consistent with these observations for C3, C3a also explained significant, albeit very small, parts of the association of adiposity with disturbed glucose homeostasis. We also found that factor D was a minor mediator in the association of VAT and SAT with insulin resistance while, in contrast, factor D was a significant suppressor in the associations of obesity with fasting glucose and T2D. The

mediating effects of C3 and C3a in the associations between adiposity and T2D were more pronounced in women, and the suppressor effect of factor D was more pronounced in men. Overall, these findings imply that direct or indirect effects of C3 and C3a on insulin resistance and glucose metabolism, both contributing to T2D, can start from expanded and dysfunctional fat depots. They also concur with accumulating evidence that factor D and C3 may play different roles in the association of adiposity with T2D.

Finally, in **chapter 5,** we investigated the effects of a weight loss intervention in abdominally obese men on the plasma concentration of complement C3, factor D and C3a. We found that the weight loss intervention reduced C3, but not factor D or C3a. We also observed that the effect of the intervention on plasma C3 was explained by the reduction in VAT. We additionally showed that the effect of the weight loss intervention on plasma markers of endothelial dysfunction was mediated by complement C3 since the reduction in C3 partly explained the weight loss-associated improvement of plasma biomarkers of endothelial dysfunction, in particular soluble endothelial selectin (sE-selectin) and soluble intercellular adhesion molecule-1 (sICAM-1). Overall, these findings imply that one of possible mechanisms by which the diet-induced weight loss intervention could improve obesity-associated diseases such as CVD and T2D, may be via reduction in circulating complement C3.

2. Methodological considerations

2.1 Internal validity

The internal validity refers to how well the inference represents the studied population. The internal validity of a study could be affected by a systematic error, also called bias. Bias distorts the true association between the main independent variable(s) and dependent variable(s). In the coming paragraph I will discuss three forms of bias:

selection bias, information bias and confounding as well as how they may have influenced the main findings presented in this thesis.

2.1.1 Selection bias

In an observational cohort study, *selection bias* occurs when the selected population is not able to represent the source population. Two kinds of selection bias may have been introduced in the analyses in this thesis: sampling bias and attrition bias

Sampling bias occurs when procedures performed for participant recruitment affect the inclusion of study participants. In The Maastricht Study, participants were recruited via self-selection, and then were given 3.5-day measurements. Therefore, this indicates that those who were interested in the study were more likely to participate, and may have higher education level, healthier condition, and a healthier lifestyle. Such a bias may underestimate the real associations.

Complete-case analysis may introduce *attrition bias* due to non-random factors affecting the study participants. For instance some participants could not be included in the analysis because not all measurements were conducted. All main analyses in this thesis were conducted based on the complete-case analysis approach, in which participants were excluded from analysis if one or more than one variables are missing, no matter whether the missing variable is an exposure, outcome, or a confounder in cross-sectional analysis, or if the participants cannot be followed up in longitudinal study. If the exclusion of participants in a complete-case analysis is non-random, attrition bias occurs. We therefore compared the characteristics of included and excluded participants to estimate if missing data were random or not. In the cross-sectional analyses done in The Maastricht Study, individuals with and without data on exposures (complement C3 and factor D) (n=136), outcomes (arterial stiffness) (n=84) and confounders (n=525) had a comparable cardiometabolic profile (**chapter 3**). In **Chapter 4**, excluded participants were less healthy, which means part of relatively larger data was missing. These missing data were expected to underestimate the

association between exposures and outcomes. In the longitudinal study (**chapter 5**), around 7.5% of the participants were excluded due to failure to follow-up and/or violating the protocol. Weight loss reduced complement C3, but this was not the case for factor D, which was corroborated by previous publications [7, 8]. We therefore thought it posed only a limited harm on our analysis.

2.1.2 Information bias

Information bias is caused by erroneous information on exposures (independent variables), outcomes (dependent variables), and/or both [9]. Erroneous information includes measurement error, when continuous data is not measured well, and/or misclassification error, when categorical data is not well classified [9]. Information bias is divided into *random error,* which mainly affects precision. It introduces variability among different measurements since some values are lower and others may be higher than true score. The effect of random error will become less as the sample size becomes larger [9]. And *systematic error* that, when it occurs in exposures and/or outcomes, may lead to overestimation and/or underestimation of the association [9].

Random error may be introduced in measurements. In this thesis, the exposures and outcomes were obtained using several methods, such as plasma measurements (complement factor D, C3, C3a, markers of low-grade inflammation and endothelial dysfunction, fasting glucose, insulin resistance, diagnosis T2D) and physical measurements (BMI, waist, VAT, SAT, cfPWV, carotid YEM, carotid IMT, ABI). Well-trained researchers conducted measurements. Part of the measurements (such as complement factors) in duplicate in order to reduce random error. Errors in the measurements are therefore likely to be randomly distributed across the study population. Random errors in exposures bias results may toward null as regression dilute bias, random errors in outcomes may widen the confidence intervals [10]. Systematic error occurs when consistent difference exists between observed value and true value of variables. Systematic error influences the accuracy of one measurement.

Systematic error would make the observed values larger or smaller than the true value in the specific direction. In this thesis, we used several ways to reduce systematic error: Firstly, we use multiple measures to record observations, for instance we use BMI, waist, VAT, SAT to represent obesity **(chapter 4, 5)**, therefore we do not have to depend on only one method. Secondly, participants were randomly allocated in weight-stable group and weight-loss group **(chapter 5).** Thirdly, considering that participants' behaviors and observed values could be affected by researcher's expectancies, the researchers are blind for the conditions of participants before participants were allocated and/or measured **(chapter 2-5)** Taken together, The Maastricht Study (**chapter 2-4**) and the weight loss study (**chapter 5**) are well-designed, and the standardized protocols were conducted by well-trained researchers to minimize information error.

2.1.3 Confounding and overadjustment

A *confounder* is a factor that associates with the exposure and at the same time is a risk factor for outcomes, but it does not involve in causal path between the exposures and outcomes [9, 11] . The effect of confounders distorting the true association between exposures and outcomes is called confounding [9]. In this thesis, in case the true association between exposures and outcomes was distorted by confounders, we used certain statistical analysis to correct for confounding such as multiple linear regression and multiple logistic regression. Based on the extensive phenotypes of the Maastricht study [12], we were able to correct for a substantial number of potential confounders to reduce confounding bias, including demographics, lifestyle factors, CVD risk factors, etc.

However, residual confounding, which may have been caused by imperfect measurement of a confounder or misclassification [13], may occur. In **chapter 2-5**, energy intake, alcohol consumption, smoking, and exercise were acquired by

participant's self-reposted questionnaires. These kinds of information likely made recall bias.

Overadjustment may exist in our fully adjusted models if the potential confounders lie in the causal path from exposures to outcomes. Given that the potential confounders, such as measures of obesity, blood pressure and T2D may mediate the association between complement factors and arterial stiffness, we thought overadjustment may have occurred in **chapter 3**. We conservatively interpreted the association of complement factors and arterial stiffness, which was non-significant. However, this conservative interpretation in chapter 3 may hide the real or significant association. Likewise, in **chapter 2**, we may have overadjusted for blood pressure, lipid profile, measures of glucose metabolism in the additional analysis, since blood pressure, lipid profile, and measures of glucose metabolism may lie in the causal pathway between complement factors and CVD.

2.2 External validity

External validity refers to the *generalizability* of our findings to other populations that were not included in the current studies [9]. Our findings from **chapter 2-4** were based on The Maastricht Study [12], a large observational cohort study that consists of middle-aged to elderly Caucasian individuals and oversampled with T2D. The findings from **chapter 5** were based on an intervention study [14] that consisted of 18 to 65-year-old and abdominally obese Caucasian men, without T2D and CVD. The generalizability of our findings to other population groups with different ethnicities, gender, age, health status, would require further study. However, based on the aetiological role of complement in metabolic disorders, our findings of consequences of complement factors could be generalizable to other populations. Indeed, the association between complement factors and metabolic disorders have been found in non-whites as well [15].

2.3 Causality

In longitudinal analyses the investigator can be sure about the order of exposures and outcomes and can build a relatively solid case for a causal link. This is for instance the case in **chapter 5**, in which we showed that weight loss reduced the plasma C3 concentration. Chapter 5 also showed that C3 reduction partly explained the weight loss improved endothelial dysfunction. For that part of the data we have to take into consideration that C3 can also be produced by vascular endothelium [16], hence improved endothelial dysfunction may also affect C3 concentration, which might have introduced reverse causation. Cross-sectional analyses (**chapter 2-4**) cannot build a solid causal link between exposures and outcomes, because the exposures and outcomes were estimated at the same time. The causal inference of our observations is based on numerous experimental studies, such as mouse models and cell work. As an example, in mice on high fat diet, factor D deficiency lowered TNF-alpha and hepatic inflammation [17], which is in line with our observation **(chapter 2)** that factor D positively associated with LGI. In our analysis the observed associations were comprehensively adjusted for potential confounders, which strengthens the possibility that the relationships were causal.

3. Implications and future perspectives

Implications

Obese people are vulnerable to various metabolic diseases, such as T2D and CVD [3, 4], and the current obesity epidemic and related diseases imposes a health and economic burden on society [5, 6]. In this thesis, we explored the potential aetiology of complement factors on these diseases. Cross-sectional studies in this thesis were conducted in The Maastricht Study, an observational study oversampled with T2D, while longitudinal studies were conducted in a weight loss intervention study. We showed that complement factors, in particular C3, factor D and C3a, were associated

with obesity and weight loss, as well as with T2D and CVD, and additionally showed that in particular complement C3, could explain a part of the association of obesity with CVD and T2D (Figure 6.1). The associations of obesity with complement factors [18-21] have been studied widely. However, the investigation for the effect of complement on metabolic disease are much less. Investigations on mediating effects of complement factors on the association between obesity and metabolic disease are limited.

In this thesis we showed some unexpected observations. For example, based on positive association of factor D with endothelial dysfunction and low-grade inflammation [22], the involvement of complement activation in atherosclerotic plaques [23], as well as the implication of complement-mediated inflammation in CVD in humans [24, 25], we expected to find that factor D have a positive strong association with carotid IMT. In **chapter 2**, however, we found that factor D inversely and significantly associated with carotid IMT in individuals without T2D, and non-significantly in those with T2D. It did surprise us, but the result from one recent publication was in line with our observation, which showed factor D inversely associated with carotid IMT in a Chinese cohort of obese individuals [15]. Moreover, experimental data showed that factor D attenuated progression of atherosclerosis [26].

In **chapter 4** we showed that factor D suppressed association between obesity measures and disturbed glucose metabolism. This was to some extent an unexpected finding, given that factor D is the rate limiting serine-protease of alternative pathway activation and its positive role in inflammation [20, 22]. Some existing publications may explain our findings. Factor D positively affects beta cell function and survival [27, 28], stimulation of insulin secretion by fat-derived factor D may lead to better control of the blood glucose level and thereby reduce the risk of T2D. Factor D was also confirmed to be lower in newly diagnosed diabetes than in those without T2D [29]. Hence, more production of factor D in obesity may be a way to downregulate the glucose level resulted from obesity-related insulin resistance.

In **Chapter 5**, we found that weight loss reduced plasma C3 concentration but, unexpectedly, not factor D. A possible explanation for this may be that factor D was shown to correlate inversely with SAT but positively with VAT [30]. We speculated that the weight loss intervention decreased the obesity, which then decreased the expression and production of factor D by in the VAT depot, while at the same time increasing the expression and production of factor D by the SAT depot. When both of these effects counteract each other, then the total result observed is non-significant, or even non-existing. In chapter 5, we also observed that VAT, but not liver fat, explained the reduction of weight loss on C3, even though the liver is main source of plasma C3 [31, 32]. Circulating inflammatory factors are the most likely mechanism via which a reduction VAT would reduce hepatic C3 production [33]. However, there was no improvement in low-grade inflammation in our study [27], as also reflected by our observation that C3a was not decreased, and even non-significantly increased, after weight loss. We therefore speculated that the weight loss intervention reduced the production of C3 in the VAT depot instead of reducing the production of C3 in the liver.

In all analyses included in this thesis interactions with sex and with diabetes status were evaluated, if appropriate. This was possible because The Maastricht Study has sufficient power for such interaction analyses. Some of the associations of complement factors with metabolic disease indeed differed according to either sex or T2D.

Interaction with diabetes: In **chapter 2**, factor D was more strongly associated with endothelial dysfunction in individuals *without* T2D than in those with T2D. In this chapter, the inverse association of factor D with carotid IMT was also stronger in participants *without* T2D, whereas no significant association was seen in those with T2D. In contrast, in **chapter 3** factor D was positively associated with cfPWV in individuals *with* T2D, but not in non-diabetic individuals. While in **chapter 4**, factor D was a stronger suppressor in individuals *with* T2D than those without T2D. This

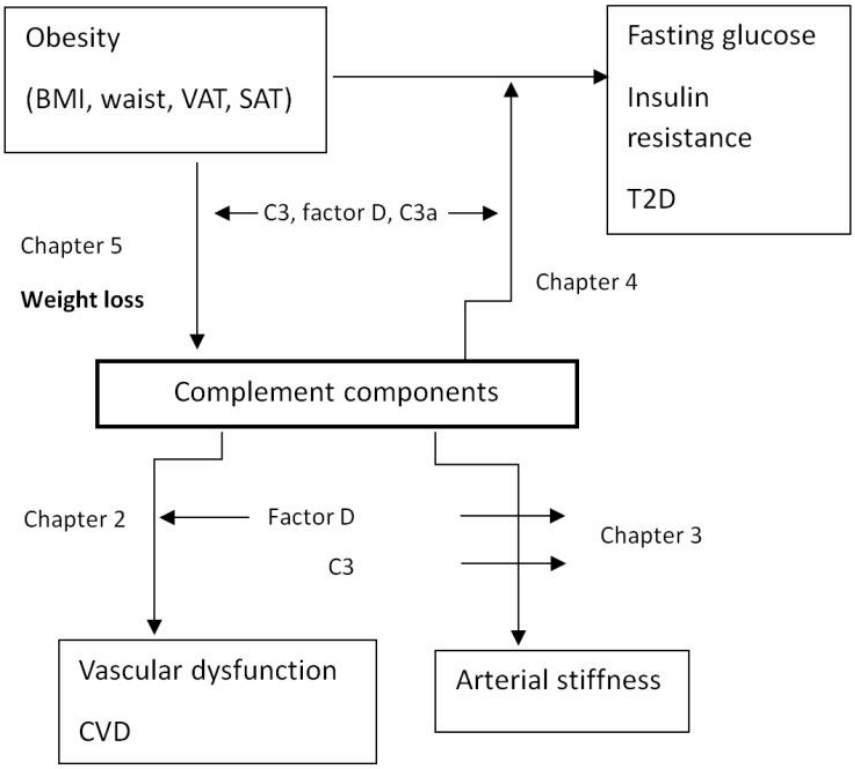
indicated the biological role of factor D in CVD is very complex and factor D may play different roles in participants with and without T2D.

Interactions with sex: in **chapter 4** we observed that C3 and C3a explained a larger part of the association of obesity measures with T2D in women than men. This may be explained by the fact that more C3a were observed in women in our study, which suggested women had higher activity of alternative pathway.

These are interesting observations that imply that in future (intervention) studies designed to evaluate the role of complement factors in metabolic diseases, sex and presence of T2D should be taken into consideration.

Future perspectives

Further evaluations are still required. ❶ The findings from cross-sectional analysis need longitudinal studies to validate the causality. For instance, the association of complement factor with vascular damage and adverse CVD (**chapter 2**) need to be validated. ❷ All the findings in these analyses (**chapter 2-5**) need to be replicated in studies with non-Caucasian ethnicities and need to be expanded to all age groups. ❸ Findings in **chapter 5** also need to be replicated in women.



Legend to fig 6.1: associations of complement components with cardiometabolic disease The long solid arrows show the association found in this thesis, short solid arrows show the complement factors involved in specific chapter.

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Chapter 7

Impact and concluding remarks

Social and economic relevance

Obesity, a global epidemic, nowadays affects over 650 million adults, and over 1.9 billion adults at the previous stage, overweight, tend to be affected all over the world [1, 2]. Obese persons are at higher risk to suffer from obesity-related diseases such as cardiovascular disease (CVD) and type 2 diabetes (T2D). [3, 4]. People with obesity, CVD and/or T2D suffer from more physical issues and have a shorter life-span. CVD is the major challenge for global health, and the leading cause of death worldwide [5]. According to the World Health Organization, CVD took an estimated number of 18 million lives in 2019, an estimated 32% of deaths, globally. T2D, a chronic metabolic disease, is another one of the top four major causes of mortality all over the world. According to World Health Organization, over 400 million adults aged 18 years and older have T2D, and an estimated 2 million deaths were caused by diabetes in 2019. These metabolic diseases also increase economic burden for individuals and society as a result of higher medical expenses and productivity losses.

In this thesis, we add more knowledge to the academic research field. We obtained a clearer understanding of the aetiological role of complement factors in cardiometabolic disease and its explaining role on how obesity contributes to cardiometabolic disease. In addition, one of our studies also showed that weight loss-induced changes in complement components may contribute to better vascular function. Based on our findings, further longitudinal studies, experimental studies in animals and/or intervention studies may be designed. These further studies may concentrate on the value of individual complement components as markers to predict these metabolic diseases. In order to study their value in prediction, the associations of (the change in) concentration of complement factors with incident cardiometabolic disease should be evaluated. Alternatively, these studies may focus on the value of individual complement factors as a target to treat these related metabolic diseases. If further evaluations show that these complement factors have added values in prediction and/or treatment of these metabolic disease, the use of complement

factors as risk predictor or target may reduce mortality and the medical burden that results from these metabolic diseases.

Target group

Participants in The Maastricht Study are Caucasian and aged between 40 and 75 years old, and those in weight loss study are abdominally obese male Caucasian aged 18-65 years old. In general, our findings show the possible aetiological role of complement factors in cardiovascular disease, how much of the association between obesity and type 2 diabetes could be explained by complement factors, as well as the reduction effect of weight loss on complement factor, which improve endothelial dysfunction marker. Our results hence support the concept that complement may be the part of the path via which obesity contributes to cardiometabolic disease.

The potential of C3, C3a and factor D in risk-prediction for risk obesity-associated cardiometabolic diseases: In the future, the use of complement factors in risk-prediction for risk obesity-associated cardiometabolic diseases is likely. Complement factors are associated with metabolic disease, and these associations were confirmed in longitudinal study. Although longitudinal analyses make the case for a role of complement stronger, these analyses still cannot prove causality. Nevertheless, complement factors could be regarded as a predictor.

C3, C3a and factor D as potential targets in treatment of obesity-associated cardiometabolic diseases: In the future, intervention trials aiming to reduce complement factors, e.g. by changes in lifestyle, may improve cardiometabolic disease. In addition, novel therapeutic drugs may be developed promisingly to improve cardiovascular disease by targeting complement. However, because, complement is part of immune system, inhibiting complement factors and/or its activation may lower the ability of the immune system to defense against pathogen infection. Therefore, a potential intervention with therapeutic drugs that affect complement activation may make patients more vulnerable to infectious disease. One possibility to prevent this is

combination of such a potential intervention with therapeutic drug boosting the immune system to compensate the loss of protection from immune system that is weakened by the complement inhibitor. Moreover, an intervention that inhibits factor D may also worsen fasting glucose levels and T2D status, since factor D suppressed the association between obesity and disturbed glucose metabolism. Therefore, the interventions are supposed to be applied cautiously, and attention should be paid how to compensate for the loss of the ability of complement factors to defense against pathogen.

Summary of the main findings reported in this thesis and concluding remarks

Our current findings confirmed and expanded results reported in previous publications and fill part of the knowledge gap in this field. Many previous studies on the role of complement in CVD focused on clinically diagnosed disease. The deep-phenotyping information of The Maastricht Study [6] allowed evaluation of several aspects of the underlying (subclinical) processes that may lead to CVD (**chapter 2**). In the large observational population-based cohort, we showed factor D was associated with low-grade inflammation, endothelial dysfunction which is in line with previous publication conducted in a middle-sized cohort [7]. We also showed factor D has a positive association with CVD, which has a comparable odds ratio with the results from the middle-sized cohort [7] that had a non-significant P-value. We showed a non-significant association between factor D and carotid IMT, which was in line with previous publication. The observed association with ABI added new knowledge to this field. The large number of participants and enrichment for T2D in this cohort allowed us to investigate whether T2D status influenced the associations of factor D with adverse vascular disease. We found in people without T2D has a stronger positive association of factor D with ED, and a stronger reverse association with carotid IMT, which adds relevant knowledge to the field.

In chapter 3, we improved the study design compared to previous studies by analyzing the associations of C3 and factor D with arterial stiffness within the same study: The Maastricht Study. Previous studies about complement factors were relatively small. The Maastricht Study is a large population-based cohort study, which give our analysis more power. We were able to study whether T2D status influenced the associations of complement factors with arterial stiffness, based on the large number of participants and enrichment for T2D in this cohort. We found that factor D was positively associated with cf-PWV in individuals with T2D instead of C3 or non-diabetic individuals. These findings show us relative comprehensive knowledge of associations of C3 and factor D with arterial stiffness.

In **Chapter 4**, This study consisted of various phenotyping, for instance, main exposures (BMI, waist, VAT, SAT), outcome (T2D, fasting glucose level, insulin resistance), and plasma concentration of complement factors, and this allowed us to investigate the explaining effect of complement C3, C3a and factor D on the association of various obesity measures with disturbed glucose metabolism in the same one study. Novel findings in this chapter were that C3 explained 12.7%-41.4% of the association between obesity measures and fasting glucose, C3a explained 0.31%-1.92% of the association of obesity measures with fasting glucose and T2D, however factor D suppressed the association of obesity with fasting glucose and T2D. These findings suggest that complement C3 and factor D may work on these metabolic diseases via different mechanisms.

In **chapter 5**, we are the first to show, in abdominally obese men, that weight-loss induced a decrease in plasma C3 concentration which was explained by reduction of VAT. This intervention also improved endothelial function, which was partly explained by the achieved reduction in C3. Our results suggest that a reduction in complement C3 with subsequent improvement in endothelial dysfunction may be part of the mechanism by which diet-induced weight loss improve cardiovascular disease risk. These findings show us the potential of reduction of C3 on improving vascular

disease, which may provide a new target for further study focusing on predicting and/or improving cardiometabolic diseases.

In summary, the investigations that are presented in this thesis show a potential effect of key components of the alternative complement pathway in cardiovascular disease and how much of the association between obesity and these cardiometabolic disease could be explained by these complement factors. This thesis may provide a clue for prediction, prevention, perhaps even treatment of cardiometabolic disease. However, rationale and validation of further investigation in potential clinical practice must be critically evaluated.

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Curriculum Vitae

Shunxin Jin was born in 5th of May, 1990 in Shandong, China. She obtained bachelor degree of bioscience in 2013. In 2013, she obtained Teacher's Qualification Certificate granted by Ministry of Education of People's Republic of China. In the same year, she started her master study in Central South University. In 2016, she graduated with master degree of Biochemistry and Molecular Biology. In the same year, she would like to find out more possibilities. Therefore, she worked in Beijing for 2 years. In October of 2018, with the funding of China Scholarship council, she started her PhD research in the Faculty of Health, Medicine, and Life, Maastricht University, Netherlands. She actively participated in academic conferences and projects.

Scientific output

List of Publications

1. **Jin S**, Kusters Y, Houben A, Plat J, Joris PJ, Mensink RP, Schalkwijk CG, Stehouwer CDA, and van Greevenbroek MMJ. *A randomized diet-induced weight-loss intervention reduces plasma complement C3: Possible implication for endothelial dysfunction.* Obesity (Silver Spring), 2022. 30(7): p. 1401-1410.
2. **Jin S**, Reesink KD, Kroon AA, de Galan B, van der Kallen CJH, Wesselius A, Schalkwijk CG, Stehouwer CDA, and van Greevenbroek MMJ. *Complement factors D and C3 cross-sectionally associate with arterial stiffness, but not independently of metabolic risk factors: The Maastricht Study.* J Hypertens, 2022. 40(11): p. 2161-2170.
3. **Jin S**, Eussen SJPM, Schalkwijk CG, Stehouwer CDA, and van Greevenbroek MMJ. *The cross-sectional associations of complement factor D with vascular dysfunction and cardiovascular disease: The Maastricht Study*. (under second-time review)
4. **Jin S,** Wang S, Schalkwijk CG, Eussen SJPM, van der Kallen CJH, Koster A, Kooi E, Brouwers MCGJ, Stehouwer CDA, and van Greevenbroek MMJ. *Plasma concentrations of complement C3 explain part of the associations of adiposity with insulin resistance, fasting glucose and type 2 diabetes: The Maastricht Study.* (in preparation)

Oral presentations

1. Annual meeting of the European Association for the Study of Diabetes 2019 (Barcelona, Spain): The alternative complement pathway is associated with an adverse lipid profile: The CODAM study
2. Annual Dutch Diabetes Research Meeting 2020 (Netherlands): Complement factor D (adipsin) is positively associated with cfPWV in individuals with T2D: The Maastricht study
3. Meeting of the European Council for Cardiovascular Research 2021(Monastier di Treviso, Italy): Complement factor D and C3 are associated with arterial stiffness,

independent of age, sex, heart rate and blood pressure but not of cardiometabolic factors: The Maastricht Study

4. Annual Dutch Diabetes Research Meeting 2021 (Netherlands): Diet-induced Weight Loss Lowers Plasma Complement C3 via Reduction of Visceral Adipose Tissue: a Randomized Controlled Trial in Abdominally Obese Men
5. Meeting of the European Council for Cardiovascular Research 2021(Monastier di Treviso, Italy): The association of factor D with cardiovascular disease: The Maastricht Study
6. Annual meeting of the European Association for the Study of Diabetes 2022 (Stockholm, Sweden): Plasma concentrations of complement C3 partly explain the associations of measures of adiposity with type 2 diabetes: The Maastricht Study

Poster presentations

1. International Complement Workshop 2021(Germany): Complement factor D and C3 are associated with arterial stiffness, independent of age, sex, heart rate and blood pressure but not of cardiometabolic factors: The Maastricht Study
2. International Complement Workshop 2021 (Germany): Diet-induced Weight Loss Lowers Plasma Complement C3 via Reduction of Visceral Adipose Tissue: a Randomized Controlled Trial in Abdominally Obese Men
3. European Meeting on Complement in Human Disease 2022 (Bern Switzerland): Plasma concentrations of complement C3 partly explain the associations of measures of adiposity with type 2 diabetes: The Maastricht Study