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

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

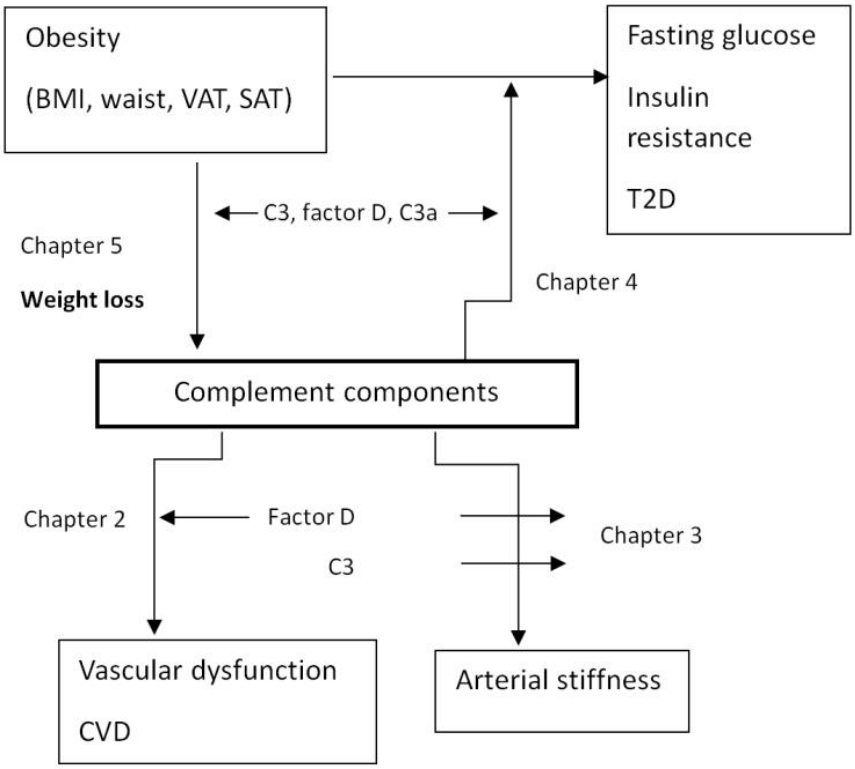
indicated the biological role of factor D in CVD is very complex and factor D may play different roles in participants with and without T2D.

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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