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Discussion

The general aim of this thesis was to examine whether associations, as found in experimental and case-control studies[1,2,4,6,7,189,] between body fatness and microvascular function could be observed in population-based samples of apparently healthy subjects. The central underlying hypothesis is that body fatness is related to diabetes and hypertension, at least partly by affecting microvascular function. Although microvascular perfusion is clearly diminished in markedly obese subjects[4,6,189,] these case-control studies do not necessarily provide evidence for a linear relationship between body fatness and microvascular function in the general population.

Where case-control studies can contribute to unravel the aetiology or mechanism behind distinct clinical phenotypes, an epidemiological study can address the relationship between determinants and health outcomes in the general population. Prior to this thesis, there had been only two population-based studies addressing correlates of microvascular (dys) function, which both had to deal with some degree of subject selection (study populations selected on insulin sensitivity or blood pressure)[29,30]. Whether the relationships between adiposity and microvascular perfusion found in these studies could be translated to the general population, in which extremes make up a smaller proportion of the total, remained to be seen. Collateral aims of this thesis were to explore predictors of developmental patterns of body fatness, as well as novel associations with microvascular dysfunction.

In this chapter, the main findings of the chapters are summarized, followed by a discussion in the light of methodological considerations and a discussion in a broader perspective.

Main findings

The main finding of the studies conducted in both the AGHLS and the NHS cohort was the absence of a clear relationship between body fatness and microvascular perfusion. At first sight this appears in contrast with results from experimental and clinical case-control studies, in which a mediating role for microvascular function between body fatness and glucometabolic and cardiovascular disease was suggested[1,2,4,6,7,189].

In chapter 4, the cross-sectional and 6-year change in body fatness have been related to microvascular function. No relationship between microvascular function and body fatness could be detected. Of note, a gender difference

in levels of microvascular function (less baseline capillaries in men versus women) was revealed.

Chapter 5 and 6 discuss developmental patterns of body fatness over 30 years in relation to both adolescent predictors (chapter 5) and adult micro- and macrovascular function (chapter 6) at the age of 42 years. An unfavourable developmental pattern of body fatness was predicted by alcohol and physical inactivity, but the unfavourable pattern of body fatness did not relate to micro- nor macrovascular function. Also smoking in youth appeared to be associated with lower risk of an unfavourable developmental pattern of body fatness.

Chapter 7 also addressed the cross sectional relationship of body fatness with microvascular function, but this time older and metabolically perturbed subjects were examined. In the NHS study sample, 50% of the participants shown to have impaired glucose metabolism, 6 years before. Neither glucose metabolism status nor body fatness showed a relationship with microvascular function. The gender difference as found in the AGHLS cohort (chapter 4), was confirmed.

In chapter 8, a new possible predictor of microvascular dysfunction was examined. This new predictor is sleep quality and duration. Although sleep has previously been invoked in the pathobiology of body fatness and cardiovascular disease[269], it has never before been linked to microvascular dysfunction. In the AGHLS microvascular function showed an association with sleep.

To conclude, microvascular dysfunction seems not significantly affected by common variations in body fatness, in normal, apparently healthy, adults, nor in metabolically perturbed and slightly older adults. As novel findings, gender and sleep do show an association with microvascular function.

Methodological considerations

Microvascular function

Microvascular function is an important prerequisite of peripheral insulin-mediated glucose uptake, which mainly takes place in muscle tissue, but also in other target organs. To test microvascular function using a non-invasive procedure, capillary perfusion of the nailfold skin was visualized using capillaroscopy[1,29,30]. The skin is examined in rest and during peak reactive hyperaemia (PRH) caused by releasing a pressure cuff after an oxygen-shortage induced by 4 minutes of arterial occlusion. The baseline capillary density (BCD) represents the number of capillaries that are perfused for basal perfusion of the tissue. Post-occlusive capillaroscopy (PRH) enables visualization of inflow of blood in the capillaries triggered by acute oxygen demand.

A more invasive method to measure microvascular perfusion in the muscle is Contrast Enhanced Ultrasound Sonography(CEUS). This method uses

an intravenous contrast agent and ultrasound sonography. This method is mainly used in experimental studies[15]. Using this method, the actual target tissue in terms of glucose uptake, muscle is visualized instead of skin. During a hyperinsulinemic euglycemic clamp, insulin induced microvascular blood volume (MBV) measured by CEUS correlates moderately (r=0.57) with percentage skin capillary recruitment (PRH%)[15]. But MBV measured with CEUS correlates better (r=0.68 ($p<0.05$)) with absolute PRH during a hyperinsulinemic euglycemic clamp. Without a clamp (representing tissue in rest), MBV measured by CEUS correlates even better (r=0.73 ($p<0.05$)) with BCD using capillaroscopy [private communication: R.Meijer].

BCD is thought to represent the minimum of perfused capillaries required for metabolic activity in rest[1,4,6,28,168]. Overall, skin BCD is around 40 capillaries per mm2. Studies described in this thesis showed a difference in BCD between men and women, with about 5 capillaries per mm^2 more in women. This gender difference was not previously reported in literature, but is important when analysing relationships between microvascular function and for example insulin sensitivity. Despite the fact that women have higher levels of body fatness, and increased body fatness is hypothesized to be characterized by lower capillary perfusion, women show a higher BCD. Why women have a higher BCD, could not be examined using the current studies, but one of the obvious possible explanations is the sex hormone difference between men and women.

Differences in microvascular function have also been suggested between groups with different glucose tolerance status. The general hypothesis is that with decreased insulin sensitivity, BCD remains stable, but PRH decreases[81]. In the current studies however, BCD is increased in the group with impaired glucose metabolism. A linear assumption, 'the lower the inulin sensitivity, the lower the capillary density', does not seem to fit with our results. Moreover, PRH was not lower in patients with perturbed glucose metabolism.

Usually, the percentage of PRH is used as measure for the functional status of the capillaries. This percentage is measured as

(PRH-BCD)/
BCD

In this calculation BCD is used for standardisation. Subsequently, a decrease in what we define as functional status of the capillaries can be based on an increase in BCD rather than a decrease in PRH. An explanation for anticipated decrease in PRH versus the currently found increase in BCD, could be the healthiness of the used study populations. When actual microvascular dysfunction has developed, in for example severely obese subject, PRH may be decreased because capillaries have been damaged and are no longer able to appear during a stimulus. When capillaries are, or have been, moderately exposed to unfavourable conditions, such as increased body fatness, but have not yet been damaged or disappeared, the

first response could theoretically be an increase in BCD to meet increased demands of the tissue.

In this thesis, microvascular function is measured using capillaroscopy which is a measure of tissue perfusion in the skin, whereas muscle is the actual target tissue. Given the good reproducibility of the capillaroscopy measures as presented in chapter 4 together with the correlations of microvascular function measured with capillaroscopy (skin) and CEUS (muscle) in rest as well as during a stimulus[15], there is no reason to believe that the relationship in the current study populations, would have looked much different, when using for example muscle perfusion measures, such as CEUS.

Body fatness

There is an ongoing discussion in the literature regarding measures of body fatness. For epidemiological and clinical purposes, anthropometrics such as BMI, skinfold thickness and waist and hip circumference are widely used[16,22,23]. Both body fatness and body fatness distribution are used with the rationale that a high waist circumference or waist-to-hip ratio with a given BMI or total amount of body fatness (central pattern of body fatness regardless of total body fatness), represent those subjects who are characterized by high visceral fatness[24]. But in fact it does only represent those who have high abdominal fatness and it can't exclude nor define the proportion of ectopic fatness[24,25]. Despite the fact that amounts of ectopic and visceral fatness are not fully captured in anthropometric measures, body fatness and body fatness distribution are known to have different roles in risk predition[22,270].

These anthropometric measures are still widely used, and their validity has been strengthened by results obtained with the less observer-dependent, method of dual energy X-ray absorptiometry (DEXA). A DEXA scan generates objective measures of both total body fatness and body fatness distributions[22,54]. Despite different techniques in the field of body fatness, anthropometrics such as BMI and waist circumference are still widely used in the clinical studies and guidelines around the world, mainly for practical reasons[187].

Which of the fatness measures is best to use when it comes to studies involving microvascular function, diabetes and hypertension is difficult and cannot be concluded from our studies. Choices for measures of body fatness have been made based on availability and specific focus of the chapters. In the AGHLS, anthropometrics including skinfold thickness and BMI have been measured from the age of 13 onwards. Only in the last two rounds of measurement, at the age of 36 and 40, a DEXA scan has been performed. In the analyses using cross-sectional and 6-years- changes in body fatness, the DEXA scan was used (chapter 4). In the life course analyses (chapter 6) skinfold measurements were used. Skinfolds were preferred over measurements such as BMI or waist for the simple reason that growth affects skinfold thickness

to a lesser extent. The use of Ponderal Index, has been suggested in youth as a measure of obesity rather than the body mass index or waist circumference and waist-to-hip ratio because it is less closely correlated with height[271]. We did not use Ponderal Index, because it is not suitable for adulthood, and the analyses performed in chapter 6 require the same measures over time.

In the NHS only the round of measurement in 2010 consisted of a DEXA scan measurement, thus no longitudinal changes could be used. Cross sectional analysis involved both DEXA measures and measures of BMI (for comparison with other studies).

Results as presented in the current thesis could have been different when measures of body fatness enabled differentiation between ectopic and visceral fatness rather than body fatness and body fatness distribution. Both measures of body fatness and body fatness distribution have been used. However, if true ectopic fat could have been measured, effects of body fatness on the microvasculature may have been more pronounced.

Insulin sensitivity

A widely discussed subject is how to obtain information on insulin sensitivity or glucose tolerance status in humans. With decreased insulin sensitivity, a concomitant change in glucose tolerance will appear, but the degree to which this occurs is critically dependent on other factors, such as pancreatic beta-cell function. In general however, decreasing insulin sensitivity predisposes to higher glucose levels in blood. How exactly this change in both insulin and glucose are measured differs between studies and each of the methods have their limitations.

Circulating levels of glucose can change from minute to minute. Also, it is only in part a measure of the actions of insulin. Concerning changes between points in time, levels of glycated hemoglobin, HbA1c, are much more stable[27]. However, HbA1c levels do not directly represent actions of insulin either. A third measure of glucose metabolism, and one that does start to involve insulin action, is HOMA-R score. This is calculated using both glucose and insulin levels, but again deals with the variability of measures in serum samples over time. Moreover, the origin (hepatic versus muscle) of the changes in glucose and insulin are not clear and probably vary between subjects. An experimental tool to estimate muscle insulin sensitivity is using a hyperinsulinemic euglycemic clamp. During this procedure, insulin sensitivity is measured as the rate of glucose infusion needed to keep the plasma glucose at a constant level during hyperinsulinemia. However, this technique is not suitable for observational population research for financial and time reasons.

Previous reports from the NHS studies have proposed the use of HbA1c in general practice instead of circulating glucose levels, but stressed that urgent questions needed answering before changing from glucose to HbA1c in general practice. Specifically subjects that are 'at risk' according to one

of the measures, but score 'normal' on the other, for example 'high and low glycators', are characterized by different cardiovascular outcome patterns[27]. In the current round of measurement of the NHS, no relationship between body fatness and HOMA-R could be detected. Based on WHO glucose-tolerance group (based on fasting and 2hr glucose levels) there has solely been a small difference between NGT and IGM subjects in baseline capillary density. In the AGHLS, HbA1c and HOMA-R have been used to predict microvascular function. Also, HOMA-R has shown to be second important mediator in the relationship between body fatness and macrovascular function, but has not been shown to be of any influence when it comes to microvascular function.

Regarding glucometabolic measures in the AGHLS and NHS, only fasting serum samples of glucose, insulin and HbA1c and the calculated HOMA-R have been used. No such extensive and invasive measures as a hyperinsulinemic euglycemic clamp test were performed. When using a clamp test, the challenge that is provided to the microcirculation is one that is very different from the fasting state. Under the steady-state conditions of the eugclycemic clamp, the assumption is that glucose disposal reflects glucose uptake by the peripheral tissue, and is a measure of insulin sensitivity. HOMA-R is based on fasting glucose and insulin concentrations, both of which are measures to reflect insulin metabolism in the fasting state[245]. But in this fasting state the majority of glucose uptake occurs in insulin-dependent tissues, and concentrations of glucose and insulin more likely reflect hepatic rather than peripheral phenomena.

If, in the currently used study populations, a euglycemic clamp would have been used instead of markers of insulin sensitivity from fasting serum samples we would have had a better estimation of insulin sensitivity of the peripheral tissue rather than hepatic insulin sensitivity[86,245]. Also assuming that there is a better correlation between measures of microvascular function (skin perfusion) and peripheral insulin sensitivity (amongst others: skin). Results of relationships between parameters of insulin sensitivity and microvascular function could have been more pronounced.

In conclusion, none of the employed techniques to measure insulin sensitivity shows a relationship with microvascular function, nor have they shown a significant influence on the relationship between body fatness and microvascular function. Currently used measures of insulin sensitivity may have masked effects that are pronounced when using measures of actual peripheral insulin sensitivity.

Statistical methods

In the current thesis, both cross-section and longitudinal statistical methods were used to describe relationships between physical characteristics and possible determinants of diabetes and/or hypertension. Specifically the longitudinal approaches were relatively new in the field of microvascular

function and has shed light on the development of obesity induced microvascular dysfunction[29,30].

Both, the AGHLS[26] and the NHS[27], are observational studies and, with that, indicate that exposure to a determinant is randomly distributed, as is obviously not the case in most case-control studies, in which extreme phenotype populations can be selected. These extreme phenotype populations are, to lesser extent, part of the study sample when using a population based sample. Although it can be seen as an advantage of population based research, in the currently presented studies the lack of extreme phenotype subjects could also have masked effects that would have appeared when more subjects with a suboptimal health status, or actual disease, were included in the study population.

In epidemiology large cohorts are examined which allows for the use of more sophisticated statistical methods than 'mean difference tests' such as χ^2 and a t- tests. The methods that have been used in chapter 4, 7 and 8 are relatively simple and concern cross sectional differences (one time point) and longitudinal changes (difference between two time points) using regression analyses with continuous dependent variables. Chapter 5 is an example of logistic regression analyses in which a dichotomous dependent variables has been used. The Latent Class Growth Analysis (LCGA) that was used in chapter 6 is a sophisticated method to divide the population under study into subgroups not based on common knowledge or cut-off points[213,228,] but it can be seen as a data driven approach. The LCGA combines slope and average values into one growth pattern.

Besides using more sophisticated techniques, all articles partly focused on confirmation of findings from previous (experimental) research and have therefore also used techniques that were used by others. These techniques have not shown other or contradictory results of the more uncommon, sophisticated tests in the same study samples. For example, we could confirm a relationship of (life-course) body fatness with macrovascular function in the AGHLS[57]. Also a decrease in percentage capillary recruitment in IGM subjects in the NHS population based on WHO glucose tolerance cut-off's has been confirmed[11]. In both cases, currently used techniques have added valuable information to the knowledge on body fatness induced microvascular dysfunction. In the first case, this concerns life course patterns of body fatness in a normal healthy population. And in the second case, the non-linearity ánd the BCD-increase based decrease in capillary recruitment of the IGM group. Large prospective cohort studies also enable analysis of confounding, moderating and mediating factors. Regarding confounding, both the results of crude and (fully) adjusted analyses were presented throughout all the papers presented in this thesis. Regarding moderation, to the best of our knowledge chapter 4 has been the first to find a difference between male and female subjects on measures of microvascular capillary density. Regarding

mediation, on a technical level, methods to examine confounding and mediation are the same, however, the interpretation is different[241].

Where a mediator is involved in the causal pathway, a confounder is not. I.e. confounders and mediators both are obtained measuring the difference in regression coefficients when adding the variable of interest(confounder or mediator) to the (univariate) model with a dependent and independent variable. In chapter 7, the mediating role for microvascular function in the relationship between body fatness and insulin sensitivity is examined. The absence of a relationship between body fatness and insulin sensitivity as well as assumed relationships between microvascular function and both body fatness and insulin sensitivity (possibly caused by techniques used to obtain insulin sensitivity) makes it difficult to examine the mediating role.

Future perspective

In conclusion, the hypothesis that originated from experimental and case-control studies, namely that an increase in body fatness induces microvascular dysfunction in a linear fashion, could not been confirmed in the population-based studies in this thesis. A relationship between sleep and microvascular function has been found, as well as a difference in capillary density between males and females.

Taking into account that the methods that have been used may, in terms of precision and responsiveness, be imperfect measures of microcirculation, body fatness and insulin sensitivity, current results do not fully disqualify the hypothesis of body fatness induced microvascular dysfunction.

Some suggestions can be made for future research. Fist, microvascular function may be more influenced by perivascular adipose tissue than systemic adipose tissue depots[183]. Measuring perivascular adipose tissue in healthy subjects on a large scale is difficult, but can be of great importance in revealing missing links in the onset of body fatness induced microvascular dysfunction. Second, healthy subjects may have an adequate buffer capacity, to overcome the challenges that we have provided (i.e. PRH) when measuring microvascular function. Also, when using more extreme measures such as meal tests or clamp situations, we could possibly reveal the first signs of damage in peripheral glucose uptake we have been looking for. Fourth, many experimental studies use the percentage increase in PRH. With the recently found increase in BCD, future studies should present either actual PRH or provide information on the BCD. Finally, the difference in capillary density for males and females needs attention in future research as it can explain part of the increase in obese (more women are obese).

Despite these limitations and recommendations for future research, in terms of precision and responsiveness, studies in this thesis have suggested an effect of sleep on microvascular function. The explanation for this finding may, at least partly, be stress or low grade inflammation[257–259]. Spiegel et

al.[253,254] describe a stress-related mechanism which causes raised cortisol levels and lower glucose tolerance. On the other hand, Tochikubo et al.[255] and Bansil et al.[256] describe a more metabolic syndrome-related mechanism being responsible for increased blood pressure in disturbed sleep. Confirmation of the association between sleep and microvascular function and further examination of the role of stress and low grade inflammation in microvascular dysfunction is needed.

For clinical practice and a framework for mechanistic understanding, it remains important to know that severely obese subjects tend to have microvascular dysfunction. The same however, chapter 4, 6 and 7 show that this may not be true for the varying levels of body fatness as they occur more commonly in the general population.