Chapter 1

General introduction

Worldwide, the prevalence of overweight, defined as a body mass index (BMI) of 25 kg/m^2 or higher, has reached pandemic proportions. In 2013, an estimated 2.1 billion adults worldwide were overweight and among them were 671 million adults with obesity, defined as a BMI of 30 kg/m^2 or higher [1]. These numbers correspond to an age-standardized global prevalence of overweight of 36.9 % in men and 38.0% in women with the standard population based on the World Health Organization standard age structure [2]. Globally, the age-standardized prevalence of obesity was 10.1% in men and 13.9% in women, with a high variation between countries. Whereas the prevalence of obesity in the United States was 31.6% in men and 33.9% in women, in the Netherlands, in 2013, the prevalence of obese men and women was estimated to be around 9.2% and 11.2% [3]. The global prevalence of overweight is expected to increase further and by 2030, 3.3 billion adults could be either overweight or obese (57.8%) [4].

This rising prevalence of overweight is a major threat to global health, as overweight is a well-established risk factor for type 2 diabetes and cardiovascular disease. Cardiovascular disease is worldwide the leading cause of death, accounting for 14.1 million deaths in 2012 [5], of which 38,371 were in the Netherlands [6]. In 2014, an estimated 387 million adults (8.3%) worldwide had diabetes, of whom 887 thousand (national prevalence: 7.2%) were in the Netherlands [7]. Above a BMI of 25 kg/m^2, each 5 kg/m^2 higher BMI was associated with 40% increase in cardiovascular mortality, and more than 100% increase in diabetes-related mortality [8]. As a result, projections estimate that in the United States, when the number of obese adults will have risen with 65 million by 2030, this will add 5.4-6.8 million cases of coronary heart disease or stroke and 5.5-6.8 million cases of diabetes [9]. Therefore, the expected worldwide increase in the prevalence of overweight will have a huge impact on the incidence of type 2 diabetes and cardiovascular disease. This rise will not only affect mortality and morbidity, but will also impact on the increase of years lived with disability [10, 11].

GLUCOSE METABOLISM AND ATHEROSCLEROSIS

Large observational studies have shown that persons with type 2 diabetes have a 2- to 3-fold increased risk of cardiovascular disease [12, 13]. Overt cardiovascular disease is the end stage of atherosclerosis progression in which the arterial wall thickens gradually and eventually a plaque or thrombus is formed that obstructs the artery [14]. Likewise, type 2 diabetes is the result of insulin resistance progression. Insulin resistant cells have a diminished ability to transport glucose from the bloodstream into the cell, but normal plasma glucose concentrations can be maintained by increasing insulin secretion by beta-cells in the pancreas. Once insulin secretion cannot adequately compensate for tissue insulin resistance (i.e., beta-cell failure), hyperglycemia develops and this may progress to type 2 diabetes. A follow-up study in 3,145 individuals has shown that insulin resistance is present many years before diabetes is diagnosed [15], thereby already increasing insulin and glucose concentrations. Therefore, it is important

to know whether insulin resistance, hyperinsulinemia, and hyperglycemia are associated with atherosclerosis and cardiovascular disease before type 2 diabetes is diagnosed.

Animal studies have shown that high plasma glucose and insulin concentrations, as well as insulin resistance can induce atherosclerosis [16-18]. Insulin resistance may be involved in the development of early and advanced atherosclerosis via various mechanisms, including dyslipidemia and inflammation [16]. Hyperglycemia appears to be exclusively involved in early atherosclerosis and contributes to endothelial dysfunction by increased oxidative stress and production of advanced glycation endproducts [17]. In addition, insulin resistance appears to modify the effect of insulin on the vascular wall; insulin is anti-atherogenic in the insulin sensitive state and pro-atherogenic in the insulin resistant state [18]. Unfortunately, it is not clear to what extent these mechanisms contribute to the development of atherosclerosis in humans. Results from randomized controlled trials suggest that the contribution of hyperglycemia to the development of atherosclerosis are limited, since intensive glycemic control (i.e. lowering HbA_{1C} concentrations) did not result in a reduction of the incidence of cardiovascular disease in persons with pre-diabetes or type 2 diabetes.

Recent meta-analyses, however, have shown that high plasma glucose concentrations, and to a lesser extent, high insulin concentrations in persons without diabetes were associated with an increased risk of cardiovascular disease [13, 19]. Also on a subclinical level of atherosclerosis, previous studies reported that measures of insulin resistance [20, 21] and hyperglycemia [21-23] were associated with carotid intima-media thickness (cIMT), a marker of atherosclerosis, in persons without diabetes. However, insulin resistance precedes hyperglycemia and these observational studies have not distinguished their individual contributions to subclinical atherosclerosis. Therefore, atherosclerosis and cardiovascular disease may be caused by insulin resistance rather than being a consequence of the toxic effects of elevated glucose concentrations. This distinction is essential to understand the development of atherosclerosis and can thereby guide specific therapeutic interventions.

Furthermore, when studying associations between insulin resistance, hyperglycemia and atherosclerosis, it is important to take adiposity into account. Adiposity can lead to insulin resistance, and consequential hyperglycemia, and can contribute to atherosclerosis through various mechanisms which will be discussed in more detail in the following paragraphs. Hence, adiposity can be considered as a common cause of both insulin resistance and atherosclerosis [24] and, may be responsible for the observed associations between insulin resistance and atherosclerosis (**Figure 1**).

ABDOMINAL OBESITY

During the past fifty years, it has emerged that not only the degree of adiposity is a risk factor for type 2 diabetes and cardiovascular disease, but also the way the adipose tissue is distributed in

the body. Already in 1956 it was observed that persons with an accumulation of adipose tissue around the abdomen more often had type 2 diabetes and cardiovascular disease than those with adipose tissue elsewhere [25]. From 1980s onwards, large cohort studies have shown that anthropometric measures of abdominal obesity such as waist circumference and waist-to-hip ratio are associated with an increased risk of type 2 diabetes and cardiovascular disease, even after adjustment for overall adiposity [26-33]. Waist circumference and waist-to-hip ratio, however, cannot distinguish between abdominal subcutaneous and visceral adipose tissue. With the development of more advanced imaging techniques, such as computed tomography (CT) and magnetic resonance imaging (MRI), subcutaneous and visceral adipose tissue depots could be directly quantified. Small cross-sectional studies showed that the anthropometric measures of abdominal adiposity were more strongly associated with the amount of intra-abdominal adipose tissue (i.e., visceral adipose tissue) than with subcutaneous adipose tissue [34-36]. In 1987 it was first shown that in obese persons visceral fat accumulation was associated with disturbances in glucose and lipid metabolism [37]. These results ultimately led to the hypothesis that the excess risk of type 2 diabetes and cardiovascular disease associated with abdominal obesity is due to increased amounts of visceral adipose tissue [38]. Several mechanisms have been proposed to explain how excess adiposity and visceral fat accumulation specifically lead to type 2 diabetes and cardiovascular disease. Adipose tissue dysfunction may be the central mechanism linking obesity and visceral fat accumulation to insulin resistance and atherosclerosis.

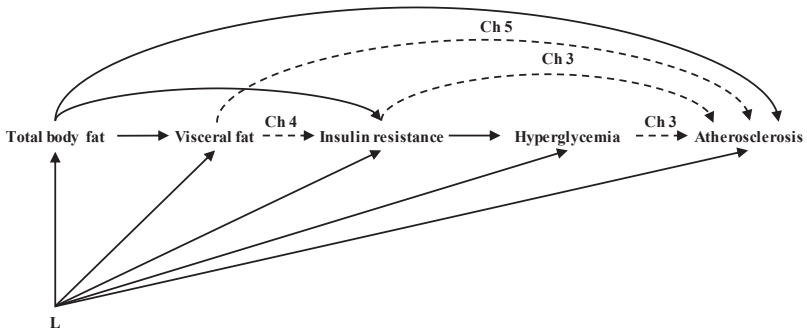

Figure 1. Hypothesis Path diagram
Figure represents disease progression from fat accumulation to atherosclerosis.
Ch, Chapter; L, known confounding factors including age, sex, ethnicity, education, tobacco smoking, alcohol consumption, and physical activity, but there may be unknown or unmeasured confounding factors.
⟶: evidence from literature
⇢: pathway under study in this thesis

ADIPOSE TISSUE DYSFUNCTION

Usually, excess energy is stored as triglycerides in subcutaneous adipose tissue. Subcutaneous adipose tissue on average accounts for 80 to 90% of total body fat and visceral fat for 6 to 20% [39] The ability to store excess energy predominantly in subcutaneous fat varies between individuals and probably has a multifactorial origin [39, 40]. Whereas on average women have more total body fat than men, men have more visceral fat than women [39]. Studies in identical twin pairs suggest that there is a strong genetic component of body fat distribution [41-43] and genome wide association studies have identified loci associated with visceral fat accumulation. Other factors that are associated with visceral fat accumulation are: age, ethnicity, sex hormones, increased concentrations of glucocorticoids, and physical activity [44]. The main mechanism of adipose tissue to expand is by increasing the size of adipocytes and probably to a lesser extent adipocyte number [45]. Hypertrophic adipocytes (cell diameter > 150 μm) release various stress factors and could induce hypoxia. Hypoxia in adipose tissue may trigger monocytes to migrate into adipose tissue and differentiate into macrophages [46]. The accumulation of macrophages in adipose tissue may lead to adipose tissue dysfunction. Adipocyte tissue dysfunction is characterized by hypertrophic adipocytes, impaired adipogenesis, an increased secretion of non-esterified fatty acids (NEFAs) and pro-inflammatory cytokines (adipokines) [46, 47], and a decreased ability to further store lipids in subcutaneous adipose tissue [44]. As a result, triglycerides will be stored viscerally and in and around organs such as the liver, heart, kidney, skeletal muscle, and pancreas. This is also referred to as the lipid overflow hypothesis (**Figure 2**) [38, 44, 48, 49].

Dysfunctional adipose tissue is thought to promote insulin resistance and atherosclerosis mainly via secretion of NEFAs and pro-inflammatory adipokines [46, 47, 49]. Increased concentrations of NEFAs induce fat accumulation in skeletal muscle and liver cells [50, 51]. This interferes with insulin signaling, leading to a decreased glucose uptake by skeletal muscle cells and an increased glucose production by the liver [50, 51]. Beta-cells are also susceptible to the toxic effect of NEFAs, which may induce beta-cell apoptosis [52]. Pro-inflammatory adipokines such as TNF-α and IL-6, and the hormone leptin may further suppress insulin signaling [53]. These pro-inflammatory adipokines may also be involved in several stages in the development of atherosclerosis. In the early stage, they can promote endothelial dysfunction and monocyte recruitment and adhesion [54, 55]. In the more advanced stage, they may stimulate smooth muscle cell proliferation and induce a pro-coagulant state, leading to plaque and thrombus formation [54, 55]. High concentrations of NEFAs promotes the formation of foam cells [55]. In addition, as described previously, dysfunctional adipose tissue may also lead to atherosclerosis through insulin resistance and consequential hyperglycemia [16].

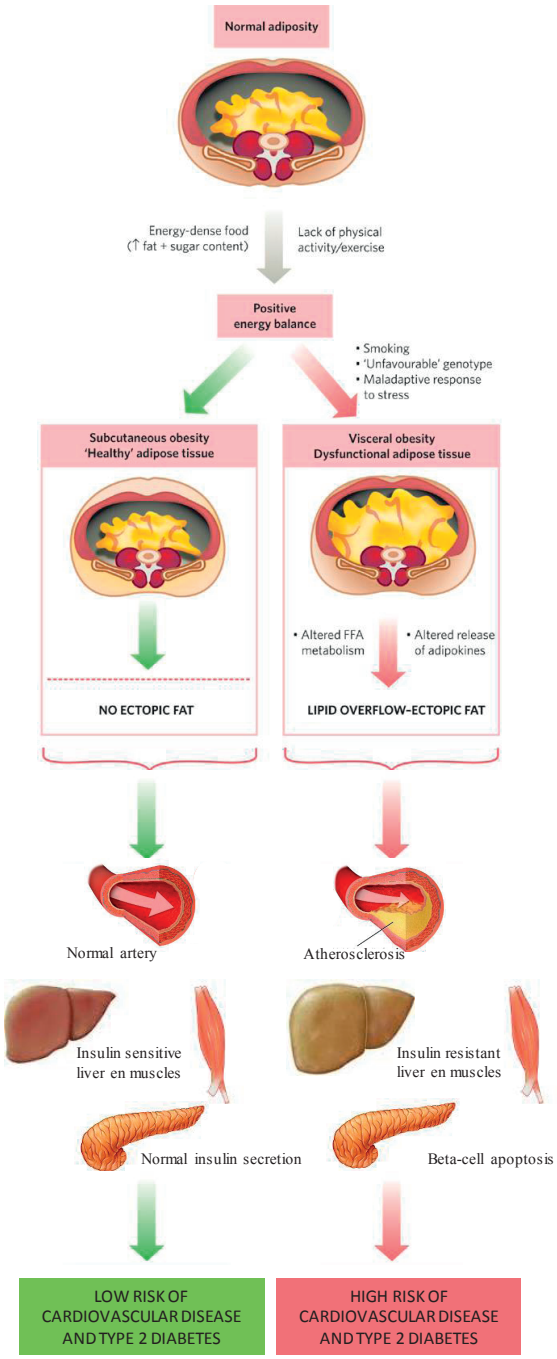

Figure 2. Adipose tissue dysfunction and its complications
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SPECIFIC ROLE FOR VISCERAL FAT IN THE DEVELOPMENT OF DISEASE?

Recent studies that directly assessed visceral fat amount by CT or MRI have shown that visceral fat accumulation is associated with measures of insulin resistance [56-62] and cIMT [57, 63-68]. However, whether visceral fat is specifically involved in the development of insulin resistance and atherosclerosis remains unclear.

One the one hand, visceral adipose tissue has a higher secretion of NEFAs [69] and pro-inflammatory adipokines [70] per mg tissue than subcutaneous adipose tissue. Furthermore, visceral adipose tissue products directly drain in the portal vein, exposing the liver to elevated concentrations of NEFAs and adipokines which may lead to hepatic insulin resistance [71, 72]. This is referred to as the portal vein hypothesis and may explain why specifically visceral fat seems related to insulin resistance and atherosclerosis. On the other hand, subcutaneous adipose tissue represents on average 80 to 90% of total body fat [73] and may therefore contribute to a larger extent to the pool of circulating NEFAs and adipokines than visceral adipose tissue [69, 74].

Visceral fat is strongly associated with total body fat [75]. Therefore, results of studies investigating relationships between visceral fat, insulin resistance, hyperglycemia and atherosclerosis should among others, be adjusted for total body fat (Figure 2) [76]. Most previous studies did not adjust for total body fat and consistently observed that an increased amount of visceral fat was associated with insulin resistance [56-58] and cIMT [57, 63, 65-67]. Studies that did adjust for total body fat observed that visceral fat remained associated with insulin resistance [59-62], but inconsistent results were reported for cIMT [64, 68]. In one study visceral fat contributed to cIMT above total body fat [64], whereas in another study visceral fat did not contribute and total body fat was therefore more important in the association with cIMT [68]. Therefore, the specific contribution of visceral fat to the development of insulin resistance and atherosclerosis remain unclear.

OBJECTIVE AND OUTLINE OF THIS THESIS

The main objective of this thesis was to unravel relationships between obesity, insulin resistance, hyperglycemia and atherosclerosis (**Figure 1**). We investigated whether insulin resistance and hyperglycemia are associated with subclinical atherosclerosis and incident cardiovascular disease, and to what extent the observed associations are explained by body fat. We further aimed to study the specific contribution of visceral fat accumulation to the development of insulin resistance and atherosclerosis, using direct assessment of visceral and subcutaneous adipose tissue while adjusting for total body fat.

In **Chapter 2**, we performed a meta-analysis of cohort studies on the associations of hyperglycemia, hyperinsulinemia, and insulin resistance with incident cardiovascular disease in persons without diabetes mellitus. We hypothesized that insulin resistance is most strongly associated with incident cardiovascular disease.

The studies included in this meta-analysis had not distinguished the contributions of hyperglycemia and insulin resistance in the development of incident cardiovascular disease. Furthermore, not all adjusted for adiposity. Therefore, in **Chapter 3**, we investigated the relative contributions of insulin resistance and hyperglycemia to subclinical atherosclerosis, and studied to what extent associations between insulin resistance, hyperglycemia and atherosclerosis could be explained by adiposity. For this purpose, we used BMI as a measure of overall adiposity and waist circumference as a measure of abdominal adiposity.

The anthropometric measure waist circumference does not distinguish between visceral and subcutaneous adipose tissue within the abdomen. We therefore used MRI techniques to directly assess abdominal subcutaneous and visceral adipose tissue and we investigated in **Chapter 4** the association of abdominal subcutaneous and visceral adipose tissue with insulin resistance and insulin secretion in men and women. In **Chapter 5** we investigated the relative contributions of abdominal subcutaneous and visceral adipose tissue, and their ratio to subclinical atherosclerosis, taking total body fat into account. Throughout this thesis we used the cIMT as a marker of subclinical atherosclerosis and we studied its reproducibility in persons with overweight in **Chapter 6**. Finally, **Chapter 7** summarizes the results of this thesis and discusses its strengths, limitations and implications.

THE NEO STUDY

To answer the research questions of chapters three to six, we used the baseline measurements of the Netherlands Epidemiology of Obesity (NEO) study. The NEO study is a population-based prospective cohort study in 6,673 individuals aged between 45 and 65 years, with an oversampling of persons with a BMI of 27 kg/m^2 or higher, who were recruited in the greater area of Leiden (in the West of The Netherlands) and included between September 2008 and October 2012. All participants underwent an extensive physical examination including anthropometric measurements, blood sampling, and a cIMT measurement. In a random subset of 2,581 participants without contraindications for undergoing MRI, abdominal subcutaneous and visceral adipose tissue were assessed by MRI [77].

ASSESSMENT OF BODY FAT

As mentioned earlier, several anthropometric and imaging techniques are available to assess total and regional body fat. The main methods to assess total body, subcutaneous and visceral fat are summarized in **Table 1**. The most accurate and precise methods to measure total body fat are: densitometry, hydrometry, neutron activation analysis and measurement of total body potassium. These methods are considered the "gold standards" for measurement of total body composition; however, they are expensive, time-consuming, and some expose persons to radiation. Therefore, these methods cannot be used in large population studies. In epidemiology, BMI is most frequently used as a measure of overall adiposity, but it cannot distinguish between fat mass and fat free mass and will, for example, misclassify particularly muscular persons as overweight, or even persons with large amounts of visceral fat but little subcutaneous fat as lean [78]. Furthermore, most methods that are used to assess total body fat do not indicate the location of the adipose tissue. Waist circumference and waist-to-hip ratio are frequently used

Table 1. Different methods to asses total body fat or fat distribution: advantages, disadvantages, validity, and feasibility in large studies

Method	Advantage (+)/ Disadvantage (–)	Validity	Feasibility in large studies
Whole body measurement			
Total body potassium *A small proportion of total body potassium is radioactive (^{40}K) and found in known proportions in fat-free mass. Gamma rays emitted by ^{40}K can be counted and fat-free mass can be estimated.*	+ accurate and precise (gold standard) – limited availability, very expensive, difficult	very high	very low
Neutron activation analysis *A neutron stream is projected on the body, resulting in a radioactive isotope of the element of interest and the gamma rays emitted by this element can be counted. Specific elements are used to estimate total body fat.*	+ accurate and precise (gold standard) – limited availability, very expensive, difficult, radiation dose	very high	very low
Hydrometry (Isotope dilution) *An isotopic tracer, such as deuterium, is ingested and distributed in body water. The dilution concentration of this isotope in excreted fluids (e.g. urine) can be measured and is used to estimate total body water and fat.*	+ accurate and precise (gold standard) – expensive, time-consuming, dependent on hydration status	very high	very low
Densitometry *This method determines body volume by the volume of water (underwater weighting) or air (Whole-Body Air Displacement Plethysmography) that is displaced when a person is respectively immersed in water or is sitting in a closed chamber. The body density is calculated from body volume and weight and subsequently body fat is estimated using standard equations.*	+ accurate and precise (gold standard) – limited availability, expensive, requires specific maneuvers, equations assume that densities of fat-free mass are constant [91]	very high	low

Table 1. (continued)

Method	Advantage (+)/ Disadvantage (–)	Validity	Feasibility in large studies
Bioelectrical Impendence Analysis *The electrical impedance is measured between two electrodes located at both feet (foot-to-foot) or hand and foot (hand-to-foot). This impedance is used to estimate total body water, and hence fat mass.*	+ non-invasive, quick – measured between two extremities, affected by hydration status	high	high
Body Mass Index *BMI is calculated by dividing weight in kilograms by the height in meters squared.*	+ non-invasive, quick, inexpensive – no distinction between fat mass and fat free mass	intermediate	very high
Whole body and regional measurement			
Computed Tomography *X-rays are projected on the body and the different ability of tissues to block these X-rays are used to create cross-sectional images of the body.*	+ able to discriminate between visceral and subcutaneous fat – radiation dose, expensive	high	low
Magnetic Resonance Imaging *The body is exposed to a magnetic field and radiofrequency waves are projected on the body. A radiofrequency signal emitted by activated atomic nuclei is used to create cross-sectional images. The volume of adipose tissue can be calculated from these images.*	+ able to discriminate between visceral and subcutaneous fat, non-invasive, – expensive	high	low
Dual Energy X-ray Absorptiometry *The body is scanned with two X-ray beams of different energy levels. Differences in attenuation of these X-rays by soft tissue and bone are used to create an image and to calculate body fat.*	– small radiation dose, small errors (<1%) with hydration changes [92]	high	intermediate
Regional measurement			
Ultrasound *An ultrasound beam is projected on the skin. Differences in reflection of ultrasound waves are used to create an image and the thickness of the adipose layer of interest can be measured.*	+ able to discriminate between visceral and subcutaneous fat, non-invasive, quick	high	Intermediate
Waist and hip circumference *Waist circumference is usually measured midway between the border of the lower costa margin and the iliac crest, above the umbilicus. Hip circumference is measured around the largest circumference of hips or buttocks.*	+ non-invasive, quick, inexpensive – moderate correlation with total body and visceral fat in cadavers [93]	intermediate	very high
Skinfold thickness *Skinfold thickness can be measured at different sites and represents subcutaneous adipose tissue.*	+ non-invasive, quick, inexpensive – high inter-observer variability [94]	intermediate	very high

References are listed in the reference list.
BMI, Body Mass Index

in epidemiological studies to reflect accumulation of adipose tissue around the abdomen [26, 27, 30, 32]. Imaging modalities such as echography, MRI, and CT, allow direct quantification of visceral and subcutaneous adipose tissue depots. In the NEO study, total body fat was assessed by bioelectrical impedance analysis in all participants and by Dual Energy X-ray Absorptiometry in a random subset of 916 participants. BMI, waist and hip circumference were measured and waist circumference was used as a measure of abdominal adiposity. Abdominal subcutaneous and visceral adipose tissue depots were quantified with MRI.

ASSESSMENT OF GLUCOSE METABOLISM

Insulin resistance and impaired insulin secretion, the two key features of type 2 diabetes, can be measured using direct and indirect methods. Direct methods to assess insulin sensitivity are the insulin suppression test and the hyperinsulinemic euglygemic glucose clamp. This euglycemic clamp is considered the gold standard for the direct assessment of insulin sensitivity. In this technique, insulin is infused at a constant rate and to maintain euglycemia, glucose is infused at a variable rate. Insulin sensitivity is estimated using the ratio of the mean glucose infusion to the mean insulin concentrations over the last 20 to 30 minutes of the clamp [79]. Its validity relies on several assumptions, namely achieving a steady state condition and complete suppression of hepatic gluconeogenesis. The glucose clamp technique is also time-consuming, expensive, and difficult to assess. Consequently, direct assessment of insulin sensitivity, is not feasible in large population studies. Therefore, in such studies insulin sensitivity is often assed by non-invasive, inexpensive indirect methods such as the Quantitative Insulin Sensitivity Check Index (QUICKI), Matsuda-index or the Homeostasis Model Assessment of insulin resistance (HOMA-IR). HOMA is a mathematical model which can be used to estimate insulin resistance and insulin secretion (HOMA-B) from fasting plasma glucose and insulin concentrations [80]. HOMA estimates of insulin resistance and insulin secretion correspond well to estimates derived from euglycemic and hyperglycemic glucose clamps (gold standards) [81, 82]. A limitation of HOMA is that insulin resistance and insulin secretion are estimated solely from fasting plasma glucose and insulin concentrations and can therefore not account for disturbances in non-fasting conditions. The Matsuda Insulin Sensitivity Index is calculated using fasting and non-fasting plasma glucose and insulin concentrations and therefore also accounts for disturbances in non-fasting conditions [83]. The Insulinogenic Index is the ratio of the increment in insulin concentration to glucose concentration 30 minutes after an oral glucose tolerance or mixed meal test. The Insulinogenic Index reflects first-phase insulin secretion and loss of this response may be the earliest sign of impaired insulin secretion in patients with type 2 diabetes [82]. In the NEO study, the Insulinogenic Index and Matsuda Insulin Sensitivity Index were calculated after an mixed meal and used as estimates of insulin secretion and insulin sensitivity respectively. In addition, both Indices were used to calculate the Disposition Index (Matsuda Insulin Sensitivity Index

* Insulinogenic Index) which is a measure of insulin secretion that accounts for variations in whole body insulin sensitivity [84]. HOMA was used to estimate both insulin sensitivity (HOMA-IR) and insulin secretion (HOMA-B).

ASSESSMENT OF ATHEROSCLEROSIS

Several non-invasive imaging techniques can be used to assess atherosclerosis, each reflecting different stages in the development of atherosclerosis. Traditionally, angiography is considered the gold standard for the detection of arterial stenosis. Disadvantages of angiography are its invasive nature and that it provides no information regarding the thickness or composition of the arterial wall. The thickness of the intima and media layer in the carotid artery can be visualized and measured by ultrasound. This so-called carotid intima-media thickness (cIMT) is considered a marker of early atherosclerosis [85]. Ultrasound can also be used to assess the presence of plaques in the carotid artery, which is considered a marker of more advanced atherosclerosis [85]. This can also be done with more expensive methods such as CT and MRI, and recently positron emission tomography (PET) imaging has been proposed as a method to measure arterial inflammation [86]. Other methods to assess early atherosclerosis are flow mediated dilatation or pulse wave velocity, but these are more time-consuming and thereby less feasible in large population studies. The carotid intima-media thickness corresponds to histology of the arterial wall [87] and is strongly associated with future risk of cardiovascular disease [88, 89]. For this reason, cIMT is frequently used as a marker of atherosclerosis to investigate the effect of potential cardiovascular risk factors on atherosclerosis.

Precision of the cIMT measurement is important, because observed variance of cIMT should reflect 'true differences' between individuals and not measurement error. Previous studies have shown that the reproducibility of the cIMT varies according to the measured artery, the equipment, number of assessors, and study population [90]. In overweight individuals excess fat in the neck region may render cIMT difficult to assess, though its reproducibility in a population of adults with overweight is unknown. In the NEO study, we studied the reproducibility of the cIMT in persons with overweight and used the cIMT as a measure of subclinical atherosclerosis.