BIRTH WEIGHT, MICROVASCULAR FUNCTION AND CARDIOVASCULAR RISK FACTORS

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BIRTH WEIGHT, MICROVASCULAR FUNCTION AND CARDIOVASCULAR RISK FACTORS

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"Only a fool is sure of anything, a wise man keeps on guessing."
MacGyver quoting his grandfather Harry

Contents

1

General introduction and objectives of this thesis

Background

Cardiovascular disease continues to be the most common cause of death in Western countries and its incidence is now rising in other parts of the world, such as Asia and Eastern Europe. In the Netherlands, almost 50,000 people die of cardiovascular disease every year. This explains approximately 35% of the total mortality. In addition, many other individuals suffer from loss of quality of life due to cardiovascular disease. For many decades, efforts have been made to elucidate the pathophysiological mechanisms of cardiovascular disease in order to develop specific therapeutic interventions. Several risk factors have been identified (so-called classical risk factors, such as dyslipidaemia, hypertension, obesity, smoking and diabetes), and several therapeutic interventions aimed at these risk factors have been developed. However, the pathophysiological origins of these risk factors are often not clear, making it difficult to develop preventive strategies. In addition, these risk factors can only partly explain the occurrence of cardiovascular disease. Therefore, research now focuses on gaining more insights into the pathophysiological origins of known cardiovascular risk factors as well as on the search for new cardiovascular risk factors. Recent studies have suggested that an impaired microvascular function and low birth weight, as well as the relationship between these two factors, may play an important role in the development of cardiovascular disease. Both factors may be potential targets for early prevention of cardiovascular disease.

The aim of the present thesis is to gain more insight into the relationships among birth weight, microvascular function and cardiovascular risk factors. A simplified scheme of the postulated relations among birth weight, microvascular function and cardiovascular risk factors is shown in figure 1. A better understanding of the relationships among these variables may contribute to prevention of cardiovascular disease and reveal new therapeutic targets.

1 Microvascular function and cardiovascular risk factors

Microcirculation is the collective name for the smallest components of the cardiovascular system – the arterioles, capillaries and venules. The microcirculation fulfils several important functions.[1] A primary function of the microcirculation is to optimise nutrient and oxygen supply within the tissue in response to variations in demand. Furthermore, a substantial proportion of the drop in hydrostatic pressure occurs in the microcirculation. Therefore, the microcirculation is important in determining the peripheral vascular resistance as well as the delivery of nutrients to the tissues. In the following section, the role of the microcirculation with respect to the regulation of blood pressure and the determination of insulin sensitivity will be discussed. In addition, possible determinants of microcirculatory function will be reviewed.

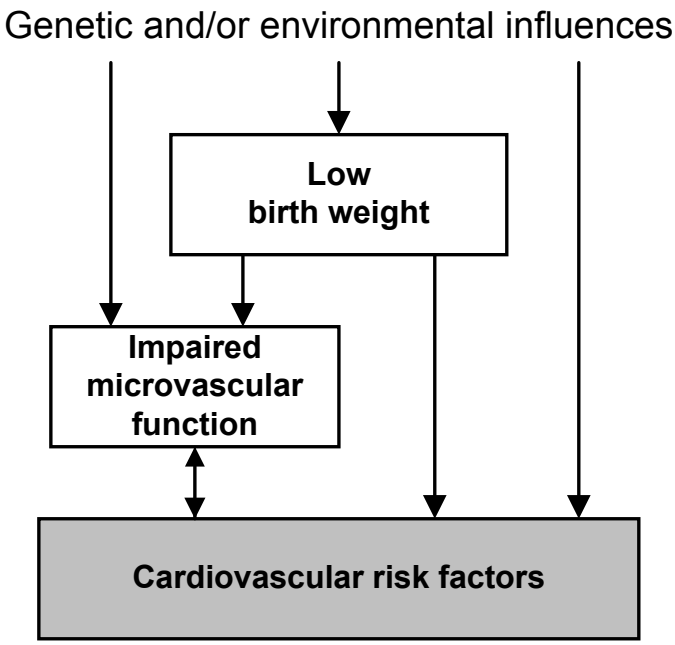

Figure 1. The postulated relations among birth weight, microvascular function and cardiovascular risk factors. Both birth weight and microvascular function are related to cardiovascular risk factors, and microvascular function may link birth weight with cardiovascular risk. All of these variables may be influenced by genetic and/or environmental influences.

1.1 Microvascular function and the regulation of blood pressure

Established essential hypertension is characterised by a normal cardiac output and an elevated peripheral vascular resistance as a result of changes in the microcirculation.[2 3] Already in 1974, experimental studies suggested that a decreased microvascular density (rarefaction) is an important mechanism in essential hypertension.[4] Indeed, in patients with hypertension, a reduction in the number of perfused capillaries and arterioles can be observed in muscle,[5] skin,[6-9] conjunctivae[10] and retina.[11,12] Several findings suggest that rarefaction is not secondary to high blood pressure, but may precede the development of hypertension. Firstly, microvascular rarefaction is an early abnormality in hypertension. Rarefaction of capillaries and small arterioles have been found in cremaster muscles of young spontaneously hypertensive rats with a marginally increased blood pressure.[13] Similarly, in humans, a decreased capillary density can already be demonstrated in subjects with borderline hypertension.[14,15] Also, in individuals with a familial predisposition for hypertension, but a normal blood pressure, a decreased capillary density has been observed in the skin.[16] This observation has been confirmed in the muscle.[17] Secondly, capillary rarefaction in hypertensive subjects predicts the increase in blood pressure over two decades.[18] Thirdly, mathematical modelling predicts an exponential relationship between capillary and arteriolar number and vascular resistance.[19,20] Taken together, microvascular dysfunction, in particular

rarefaction, may have important consequences for peripheral vascular resistance and blood pressure.

1.2 Microvascular function and the determination of insulin sensitivity

Insulin resistance is the inability of insulin to stimulate glucose uptake in glucose-requiring tissues. Before binding to its receptor on the plasma membrane, insulin must be delivered to the tissue in normal amount and time course. Disturbances in these precellular steps, in particular disturbances in insulin-mediated changes in muscle perfusion, may contribute to insulin resistance of glucose uptake.[21] This hypothesis is supported by the associations between insulin-mediated increases in total limb blood flow and whole-body glucose uptake.[22-24] Recently, however, there is increasing evidence that it is not total limb blood flow, but nutritive capillary flow that is important for the development of insulin resistance. Studies using the perfused rat hindlimb have shown that insulin induces an increase in capillary perfusion, measured as the metabolism of 1-methyl xanthine. This insulin-induced increase in capillary perfusion increased skeletal muscle glucose metabolism, even if total blood flow to the muscle remained constant.[25] In addition, acute vasoconstriction without changing total flow caused impaired muscle glucose uptake, with a strong relation between changes in capillary perfusion and insulin-mediated glucose uptake.[26] Furthermore, insulin was found to increase capillary perfusion as measured with a laser Doppler probe positioned on the muscle surface in the rat hindlimb, without a change of total limb blood flow.[27] In humans, there is some indirect evidence for insulin-induced capillary recruitment in muscle.[28,29] These findings and the association between basal and insulin-stimulated muscle blood volume and glucose uptake[30] suggest that factors regulating muscle blood volume, e.g. muscle capillary density and capillary recruitment, contribute to insulin sensitivity. In support of this concept, an association between insulin sensitivity and muscle capillary density has been shown.[31] In addition, the number of capillaries per fibre ratio is significantly associated with basal and insulin-stimulated blood flow.[32] Moreover, skin capillary recruitment during post-occlusive reactive hyperaemia is associated with insulin-mediated glucose uptake and vasodilation in normotensive and hypertensive subjects.[9,33] Taken together, microvascular dysfunction, in particular an impaired capillary recruitment, may contribute to the defects in insulin's vascular and metabolic actions.

1.3 Determinants of microvascular function

An impaired microcirculatory function, as discussed above, may be important in the development of hypertension and insulin resistance. Through various mechanisms, several classical and new risk factors for cardiovascular disease may underlie disturbances in microcirculatory function. None of these determinants is incompatible with the others, and they may be strongly interrelated.

Age and sex

A reduced microvasodilator capacity with aging has been reported in muscle[34] and skin.[35] A higher skin microvascular reactivity in women compared to men has also been described.[35,36]

Hypertension

Although there is evidence that abnormalities in the microcirculation may cause or contribute to high blood pressure (see above), there is also evidence from experimental studies that microvascular abnormalities are a response to an increased vascular pressure. It has been shown that arteriolar diameters are decreased in experimental secondary hypertension.[3] In addition, a reduction in the number or density of microvessels has been reported in many forms of clinical and experimental hypertension.[3] Therefore, a "vicious cycle" may exist in which the microcirculation initiates and/or amplifies an increase in blood pressure.[1]

Diabetes and insulin resistance

Diabetes has been shown to be associated with abnormalities in the microcirculation in humans. A reduction in microvascular vasodilatory capacity has been shown in individuals with diabetes[37] and individuals at risk for diabetes.[38,39] These abnormalities may result from and contribute to changes in glucose metabolism. As described above, microvascular dysfunction, in particular an impaired capillary recruitment, may result in defects in glucose metabolism. However, several experimental and human studies have demonstrated that impairments in glucose metabolism may also contribute to a decreased microvascular function. Experimental[40-42] and human[43,44] studies suggest that glucose impairs microvascular function. Several mechanisms may link hyperglycaemia with an impaired microvascular function, including the activation of protein kinase C, increased activity of the polyol pathway, non-enzymatic glycation and oxidative stress.[45]

Insulin resistance at a vascular level has also been hypothesized to be a mechanistic determinant of microvascular function.[46] The effects of insulin on nitric oxide production and subsequent vasorelaxation appear to be mediated, in part, through the insulin receptor utilizing a phosphatidylinositol 3 (PI3)-kinase -dependent pathway.[47] Interestingly, the insulin signalling pathway through PI3-kinase is a necessary effector of insulin-mediated glucose transport.[48] Therefore, insulin resistance at the level of PI3-kinase may link the diminished vasodilation induced by insulin with a decreased insulin-mediated glucose uptake. The diminished vasodilation may then contribute to a higher blood pressure or diminished insulin-mediated glucose uptake in insulin-resistant individuals. Whether or not the PI3-kinase pathway is also involved in the vasodilation induced by other vasodilatory stimuli is not clear, but a recent study suggested that part of this pathway is likely to play a role in acetylcholine-mediated vasodilation.[49]

Dyslipidaemia

Lipid levels have also been related to microvascular function. In men with familial combined hyperlipidaemia, a reduced capillary density at rest and during reactive hyperaemia has been reported.[50] In addition, in women, serum lipids and lipoproteins were correlated with skin vessel reactivity.[51] The impaired responses of the microcirculation may be reversed after cholesterol-lowering therapy.[52,53] However, although dyslipidaemia may cause microvascular abnormalities, it has also been suggested that the microcirculation may contribute to the development of an atherogenic lipid profile through impaired action of endothelial-bound lipoprotein lipase (LPL).[54,55] LPL is the rate-limiting enzyme for triglyceride utilisation and its physiological site of action is the capillary endothelial surface. LPL dysfunction may lead to increased plasma triglycerides and reduced concentration of HDL cholesterol.[54]

Obesity

Growing evidence suggests that obesity plays an important role in the development of hypertension, diabetes, and dyslipidaemia. These disorders may explain the association between obesity and an impaired microvascular function, but there is also evidence for other mediators. Free fatty acids are increased in obesity. Free fatty acids can impair endothelium-dependent and insulin-induced vasodilation in resistance vessels.[56,57] In addition, elevation of free fatty acids causes insulin resistance.[58] The effect of free fatty acids on insulin resistance may be mediated by defects in microvascular function. The effects of free fatty acids on the microcirculation have not been reported. Adipocytes also produce pro-inflammatory cytokines. Several animal and human studies have demonstrated that high levels of TNF-α mRNA and protein are associated with insulin resistance.[59-63] In addition, administration of TNF-α to animals can induce insulin resistance,[64,65] whereas neutralisation of TNF-α can improve insulin sensitivity.[59,66] In animals, it has been demonstrated that TNF-α causes defects in capillary function, with a decreased access of insulin and glucose to tissues, resulting in insulin resistance.[67] However, it is not known whether TNF-α is associated with microcirculatory function in humans. Obesity is also related to an increased sympathetic activation. It has been demonstrated that heart rate, as a crude measure of sympathetic activity, is inversely associated with capillary density in muscle.[18] Furthermore, an acute increase in sympathetic activation is associated with an impairment in total forearm blood flow[68] and laser Doppler skin blood flow.[69] The converse also appears to be true: experimental studies found that blocking the α1-adrenoreceptor (which are predominantly found in capillaries) with prazosin increased capillary density and blood flow.[70]

Cigarette smoking

One of the major risk factors for cardiovascular disease is cigarette smoking.[71,72] Cigarette smoking is associated with an acute increase in arterial wall stiffness [73,74] and an immediate endothelial dysfunction of the large arteries,[75,76] which are recognized to

be important early phenomena in the pathogenesis of atherosclerosis. Animal studies have demonstrated that cigarette smoking is associated with acute changes in microvascular perfusion.[77-79] Several human studies have shown that chronic[80,81] and acute[79,82,83] smoking are associated with detrimental effects on the microcirculation. However, the acute effects of smoking on capillary function and microvascular endothelium-dependent vasodilation have not been investigated, and in most studies[79,82] a control experiment with sham smoking was not performed.

Vascular development

Recently, evidence has accumulated that cardiovascular diseases are related to growth in utero. An adverse intrauterine environment and/or a genetic predisposition have been proposed to impair fetal growth and cause disease in later life. The outgrowth of the microvascular bed in different tissues may also be impaired. This early abnormality in the microcirculation may be compounded by well-known cardiovascular risk factors in later life. The association of size at birth with microvascular characteristics and adult cardiovascular risk is discussed in more detail in the next section.

2 Birth weight and cardiovascular risk factors

2.1 Epidemiological studies

In the last 10 years, evidence has accumulated that size at birth is associated with cardiovascular disease. The majority of the studies have used birth weight as a measure of intrauterine growth. Babies born with a low birth weight (figure 2) have an increased risk of cardiovascular disease. For example, among 16,000 men and women in Hertfordshire (United Kingdom), death rates from coronary heart disease fell twofold between those at the lower and upper ends of the birth weight distribution.[84] This association between birth weight and coronary heart disease has been confirmed in studies in Sweden,[85] Wales,[86] the United States[87] and India.[88] In addition, numerous epidemiological studies have demonstrated a link between small size at birth and risk factors for cardiovascular disease, such as blood pressure, insulin resistance, plasma lipids and fibrinogen.

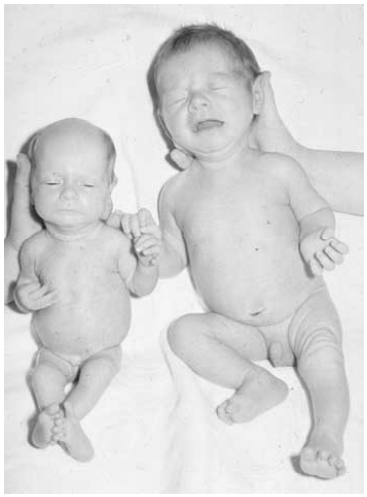

Figure 2. Baby born with a low birth weight (left) and baby born with a normal birth weight (right).

Birth weight and blood pressure

Evidence from 80 studies included in a recent systemic review demonstrates that, in children, adolescents and adults, there is an inverse association between birth weight and systolic blood pressure.[89] Figure 3 shows the result of the studies reporting multiple

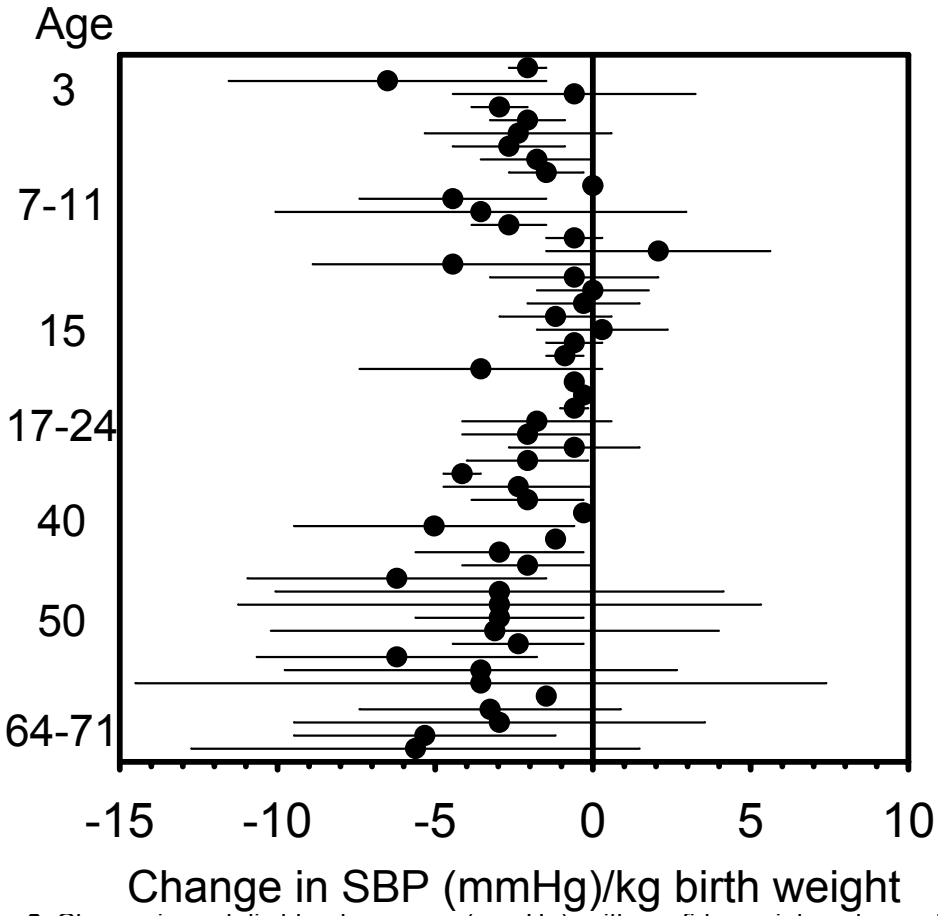

Figure 3. Change in systolic blood pressure (mm Hg), with confidence intervals, per kg increase in birth weight.[89]

regression analysis of the birth weight-blood pressure relationship.[89] Each point represents a study population and these populations are ordered by age. The horizontal position of each population with its confidence interval describes the change in blood pressure that was associated with a 1 kg increase in birth weight. Although it has recently been suggested that the size of the association between birth weight and blood pressure may be overestimated due to publication bias,[90] the published studies demonstrate that, on average, a 1 kg higher birth weight is associated with a 2 mmHg lower blood pressure.[89] In clinical practice this may seem a small difference, but these are relevant differences between the mean values of populations.[85] For example, lowering mean systolic blood pressure in a population by 2 mmHg corresponds to a 8% reduction in the risk of stroke.[91] As can be seen in figure 3, the birth weight-blood pressure association tends to be stronger in older than in younger individuals. Longitudinal studies have confirmed that the association of birth weight with blood pressure is weak in children, but tends to become progressively stronger with ageing.[92-95]

Gestational age is also inversely associated with blood pressure[96-98] and several studies have shown that the birth weight-blood pressure association is somewhat

diminished after adjustment for gestational age.[95,96,98] Nevertheless, the association between birth weight and blood pressure is largely independent of gestational age.[99,100]

Birth weight and insulin resistance

Epidemiological studies have suggested that low birth weight is associated with insulin resistance in later life. Among 308 Swedish elderly men born at term, lower birth weight was associated with lower insulin sensitivity as measured with the euglycaemic hyperinsulinaemic clamp.[101] This association has been confirmed in other studies that investigated the association between birth weight and indicators of insulin sensitivity.[102-107] Insulin resistance is an early metabolic defect that both precedes and predicts impaired glucose tolerance and diabetes. Indeed, several studies have demonstrated that lower birth weight is associated with an increased risk for non-insulin-dependent diabetes and/or glucose intolerance.[108-111] Similar to the association between birth weight and blood pressure, longitudinal studies in children[112] and adults[113] have suggested that the relationship of insulin resistance with birth weight is strengthened with increasing age.

Birth weight, serum lipids and plasma fibrinogen

Although the evidence is less clear than for blood pressure and insulin resistance, several studies have suggested that total cholesterol,[114-116] low density lipoprotein cholesterol[114,115] and apolipoprotein B,[102,114,117,118] which are known risk factors for cardiovascular disease, are inversely related to size at birth. In addition, there is some evidence that small size at birth is associated with decreased levels of high density lipoprotein (HDL) cholesterol[113,119] and apolipoprotein A1.[120]

Fibrinogen is an independent predictor of cardiovascular disease.[121] It has been suggested that increased plasma concentrations of fibrinogen in later life are related to lower birth weight. An association between size at birth and fibrinogen could be found in some studies,[122-124] but not in others.[102,119,125]

Birth weight and height

A short height has practical and psychological consequences. In addition, short height is an independent risk factor for cardiovascular disease.[126-128] Many studies have shown that a shorter height is associated with a lower weight at birth.[112,129-137] A recent study of approximately 40,000 young men showed that there was a mean difference of more than 7 cm in height between men with a low and a high birth weight (<2500 and >4500 g, respectively), and a mean difference of almost 10 cm in height between men who were short and those who were long at birth (<48 and >55 cm, respectively).[138]

2.2 The origins and mechanisms of the association between birth weight and cardiovascular risk factors

The epidemiological studies investigating the association between birth weight and cardiovascular risk factors leave two important issues unresolved. Firstly, it is not known whether the origin of these associations is intrauterine and/or genetic. Secondly, the mechanisms underlying these associations are unclear.

The origin of the association between birth weight and cardiovascular risk factors

The leading hypothesis suggests that intrauterine malnutrition impairs intrauterine growth and causes disease in adult life,[139] and that improvements in intrauterine nutrition may prevent disease in later life. This would have major implications for public health, especially in developing countries where low birth weight is common. However, both birth weight and cardiovascular disease are influenced by genetic factors. Therefore, the alternative view is that genetic factors influencing both birth weight and adult disease are responsible for the association.[125,140,141] If genetic factors are responsible, improvements in intrauterine nutrition may not prevent disease in later life.

Twin studies offer a unique opportunity to distinguish between intrauterine and genetic causes.[142] Differences within dizygotic twin pairs are a function of both genetic and nongenetic factors, whereas differences within monozygotic (identical) pairs are almost completely caused by nongenetic factors. If genetic factors do not play a role in the association between birth weight and cardiovascular risk factors, one would expect that, both for dizygotic and for monozygotic twins, the twin with the lowest birth weight from each pair will also have the highest level of the cardiovascular risk factor compared to the cotwin with the highest birth weight. In addition, negative associations between intrapair differences in birth weight and intrapair differences in the risk factor should exist both in dizygotic and in monozygotic twins. If, however, genetic factors do play a role, these associations would hold true only for dizygotic twins, and not for monozygotic twins.

It has also been proposed that the relationship between birth weight and cardiovascular disease is due to confounding by an adverse environment that is related to small size at birth and later cardiovascular disease. Socioeconomic status in particular may have an important impact on lifestyle choices that are related to both birth weight[143] and cardiovascular risk (factors).[144-146] Although a few studies have shown that adjustment for lifestyle factors, such as smoking, employment, alcohol consumption and exercise, had little effect on the association between birth weight and cardiovascular disease, [85-87] influences of other (unknown) factors cannot be excluded. In this respect, intrapair analyses in twins allow the elimination of parental environmental factors that may be related to their offspring's cardiovascular disease and birth weight. However, it should be noted that influences in adult life may modify the association between size at birth and cardiovascular disease and its risk factors. Higher

blood pressure,[92,95,100] diabetes[109] and coronary heart disease[147] may be more prevalent in individuals that were small at birth but became obese in later life.

The mechanisms underlying the association between birth weight and cardiovascular risk factors

Regardless of whether or not the origin of the relationship of birth weight with adult disease is genetic or environmental, there are a number of possible mechanisms that may link low birth weight to higher blood pressure, insulin resistance and other cardiovascular risk factors. None of these determinants is incompatible with the others, and they may be strongly interrelated.

Several studies have suggested that birth weight is related to properties of blood vessels. Pulse wave velocity, as a measure of the elasticity of large vessels, in middle age, but not in young adults,[148] was inversely correlated with birth weight.[149 150] Flow-mediated vasodilation in the brachial artery during post-occlusive hyperaemia has been related to birth weight in a study in children[151] and young adults.[152,153] Martin et al. have reported an impaired acetylcholine-mediated vasodilation in small-for-gestational-age neonates at 3 days of age[154] and in low-birth-weight children at 9 years of age.[155] However, other studies did not show a significant association between birth weight and acetylcholine-mediated vasodilation in adults[156] and 3-month-old infants.[157] Two studies have reported the association between birth weight and characteristics of the capillary network in adults. Chapman et al. have demonstrated structural changes in the retinal microvascular network of low-birth-weight men, suggesting lower than normal microvascular density,[158] and Serné et al. have demonstrated an association between birth weight and capillary recruitment in healthy adults.[156] However, it could not be resolved whether the impaired capillary recruitment in individuals with a low birth weight was a cause or a consequence of higher blood pressure and/or insulin resistance, as these variables were strongly interrelated.[156]

Changes in autonomic nervous system activity are involved in the development of high blood pressure.[159] In addition, although the mechanisms are not clear, sympathetic activation is related to insulin resistance[68] and cardiovascular risk.[160] Several experimental studies have demonstrated associations between size at birth and an increased sympathetic activity,[161,162] and low birth weight has been related to high resting heart rate in middle-aged individuals.[163] However, it is not known whether birth weight is associated with the activity of the sympathetic (or parasympathetic) nervous systems, nor whether any such associations can explain the relationship of birth weight with blood pressure, insulin resistance and cardiovascular disease.

Low birth weight may be related to a reduced number of nephrons which in turn leads to increased hydrostatic pressure in the glomerular capillaries, glomerular hyperfiltration and the development of glomerular sclerosis. This may lead to hypertension and progressive glomerular injury.[164] In Aborigines, low birth weight was associated with a higher urinary albumin-creatinine ratio.[165] Several studies have shown

that lower birth weight is associated with a reduced glomerular number,[166,167] but other studies did not find this association.[168,169]

It has been hypothesised that changes in hormonal axes explain the link between birth weight and subsequent development of cardiovascular risk factors and disease. Recent studies suggest that abnormalities of the hypothalamic-pituitary-adrenal axis may be of particular importance. It has been shown that plasma concentrations of cortisol are related to birth weight,[170,171] blood pressure[170,171] and insulin resistance.[170,172] In addition, enhanced responses of plasma cortisol to ACTH were associated with lower birth weight, higher blood pressure and glucose intolerance.[173] In a study of 9-year old children, those who had been small at birth had increased urinary adrenal androgen and glucocorticoid metabolite excretion. The growth hormone insulin like growth factor-1 (IGF-1) axis may also be important for the development of cardiovascular disease in individuals with a low birth weight. A shortage of IGF-1 may be related to type 2 diabetes,[174] hypertension[175] and cardiovascular disease.[176] In umbilical cord blood, plasma IGF-1 is lower in babies with a low than in those with a high birth weight.[177,178] However, 9-year old children who had low birth weight had higher, not lower, plasma IGF-1 concentrations.[179]

Taken together, multiple interrelated mechanisms may be potentially responsible for the association between low birth weight and increased cardiovascular risk factors (figure 4).

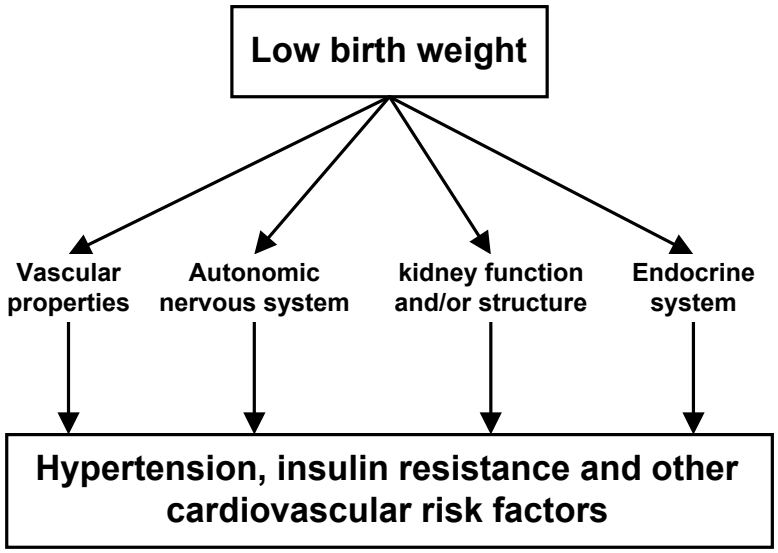

Figure 4. Multiple interrelated mechanisms may be responsible for the association between low birth weight and increased cardiovascular risk factors

Outline of the thesis

Against this background, the following studies were performed to gain more insight into the relationships among birth weight, microvascular function and cardiovascular risk factors.

Part 1 Birth weight and microvascular function
Several studies in adults have demonstrated that low birth weight was associated with an impaired microvascular function. However, it could not be resolved whether the impaired microvascular function in individuals with a low birth weight was a cause or a consequence of a higher blood pressure and/or insulin resistance. Studies of younger individuals may elucidate important aspects of the temporal relationships among birth weight, blood pressure, insulin sensitivity and microvascular function. We therefore investigated ambulatory blood pressure, insulin sensitivity and microvascular function in a group of prepubertal healthy children with a wide range in birth weight (Chapter 2).

Part 2 Microvascular function and cardiovascular risk factors
It has been proposed that insulin-mediated changes in muscle perfusion modulate insulin-mediated glucose uptake. However, the putative effects of insulin on the microcirculation which may permit such modulation have not been studied in humans. We, therefore, examined the effects of prolonged systemic hyperinsulinaemia on (micro)vascular function (Chapter 3). In addition, the effects of locally administered insulin on skin blood flow were assessed.

Microvascular function has been proposed as a possible mechanism explaining the association of acute smoking with an increased blood pressure and a decreased insulin sensitivity. However, the acute effects of smoking on microvascular function have not been studied. We have investigated the acute effects of smoking on microvascular function in healthy smokers (Chapter 4).

The inflammatory cytokine tumour necrosis factor α (TNF-α) has been reported to play an important role in insulin resistance. The mechanism by which TNF-α may cause insulin resistance is not clear. It has been suggested that TNF-α causes defects in capillary function, with a decreased access of insulin and glucose to tissues. To test this hypothesis, we assessed TNF-α, skin capillary recruitment during post-occlusive reactive hyperaemia and insulin sensitivity in healthy individuals (Chapter 5). In addition, to investigate whether these associations are already present in prepubertal children, we measured these variables in 21 of their children.

Coronary microvascular disease may explain the occurrence of myocardial ischaemia without overt coronary artery blockage, as well as heart failure and mortality after myocardial infarction. However, methods to assess the coronary microcirculation are invasive and applicable only in experimental settings. The skin microcirculation offers an opportunity to noninvasively explore the relation of systemic microvascular

dysfunction to (risk factors for) coronary heart disease. To investigate whether microvascular function in skin is a valid model for the study of the relationships between cardiovascular risk factors and microvascular function, we investigated whether the coronary heart disease risk score according to the Framingham Study is associated with microvascular function in skin (Chapter 6).

Part 3 Birth weight and cardiovascular risk factors in twins

Twin studies offer a unique opportunity to distinguish between intrauterine and genetic origins of the association between birth weight and cardiovascular risk factors in later life. We make several introductory remarks on the use of twin studies to investigate the influence of intrauterine and genetic factors (Chapter 7). We also discuss whether the comparison of within-pair analyses with unpaired analyses can be used to identify maternal influences on the association between birth weight and cardiovascular risk factors.[180]

Many epidemiological studies have shown an inverse association between birth weight and blood pressure. To examine whether this association is explained by intrauterine or genetic factors, we investigated birth weight and blood pressure in dizygotic and monozygotic adolescent twin pairs (Chapter 8). Alterations in sympathetic and parasympathetic activity may be important mechanisms explaining this association. Therefore, we also examined the association of birth weight with cardiac pre-ejection period and respiratory sinus arrhythmia (indicators of cardiac sympathetic and parasympathetic activity, respectively), and with blood pressure in these twin pairs (Chapter 9).

To examine whether the association between birth weight and insulin sensitivity is explained by intrauterine or genetic factors, we investigated the association of birth weight with insulin sensitivity, calculated from fasting insulin and glucose levels in dizygotic and monozygotic twin pairs (Chapter 10).

We also investigated whether the association of birth weight with serum lipids (Chapter 11) and plasma fibrinogen (Chapter 13) could be explained by genetic or intrauterine factors. The metabolic alterations in cholesterol metabolism that underlie these changes in plasma lipids are not known. To examine the association between birth weight and cholesterol metabolism and the possible influence of genetic factors, we investigated birth weight and markers of cholesterol synthesis and absorption in these dizygotic and monozygotic twin pairs (Chapter 12).

Finally, we investigated the genetic and intrauterine influences on the association between size at birth and height in later life in adolescent twins (Chapter 14). A short height is associated with practical and psychological consequences. In addition, short height is an independent risk factor for cardiovascular disease. To investigate whether these associations persisted into adulthood, we also analysed follow-up data on adult height in a subgroup of these twin pairs.

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2

The association between birth weight and capillary recruitment is independent of blood pressure and insulin sensitivity
A study in prepubertal children

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Abstract

Background Alterations in microvascular function have been hypothesised as a possible mechanism explaining the negative association of weight at birth with blood pressure and insulin resistance in adult life. However, these variables are closely associated, so that it has been difficult to establish whether microvascular dysfunction is a cause or a consequence of increased blood pressure or insulin resistance.

Methods Twenty-one prepubertal healthy children showing a wide range in birth weight. Birth weight data were obtained from hospital records. Blood pressure was measured with an ambulatory 24-h blood pressure monitor and insulin sensitivity was assessed with the hyperinsulinaemic euglycaemic clamp technique. Microvascular function (i.e. capillary recruitment during post-occlusive reactive hyperaemia and endothelium (in)dependent vasodilatation of the skin) was evaluated by videomicroscopy and iontophoresis of acetylcholine and sodium nitroprusside.

Results Birth weight was positively and significantly associated with capillary recruitment (slope: 22% [95%-CI: 0.1-43%] per kg birth weight; $P<0.05$). Birth weight was not associated with systolic blood pressure and insulin sensitivity (slope: -0.11 mg/kg/min per pmol/l [-2.4-2.2] per kg birth weight, $P=0.9$; and 1.4 mmHg [-5.0-7.7] per kg birth weight, $P=0.7$, respectively). The association between low birth weight and impaired capillary recruitment was not affected by adjustment for blood pressure and insulin sensitivity. Birth weight was not associated with endothelium-(in)dependent vasodilatation.

Conclusions These results suggest that the association between birth weight and capillary recruitment is independent of blood pressure and insulin sensitivity. These findings are consistent with the hypothesis that an impaired capillary recruitment plays a mechanistic role in the association of birth weight with blood pressure and insulin resistance in adult life.

Introduction

Epidemiological studies have consistently demonstrated that weight at birth is negatively associated with blood pressure and insulin resistance in adult life.[1;2] Regardless of whether or not the origin of these relationships is genetic[3;4] or environmental,[5] alterations in microvascular function have been proposed as a possible mechanism explaining these associations.[3;6;7] Impaired microvascular function in individuals with a low birth weight may increase vascular resistance and reduce insulin action.[3;6;7] In support of this concept, we have previously demonstrated that capillary recruitment during post-occlusive reactive hyperaemia (PRH) was related to birth weight, blood pressure and insulin sensitivity in adults.[8] However, it could not be resolved whether the impaired capillary recruitment in individuals with a low birth weight was a cause or a consequence of the higher blood pressure and/or insulin resistance.

Studies of younger individuals may elucidate important aspects of the temporal relationships among birth weight, blood pressure, insulin sensitivity and microvascular function. Specifically, studying children offers the possibility to identify factors present in subjects with a lower birth weight before overt high blood pressure, insulin resistance or other disturbances have emerged.

We therefore assessed ambulatory blood pressure, insulin sensitivity and microvascular function (i.e. capillary recruitment and endothelium (in)dependent vasodilatation) in a group of prepubertal healthy children with a wide range in birth weight.

Methods

Subjects

Twenty one healthy, prepubertal children participated in this study. All children were of Caucasian origin. They were recruited from the same catchment area and had been born at the VU University Medical Center in Amsterdam. Only singleton children born at term (i.e. gestational age >= 37 weeks) were recruited. The exclusion criteria were a complicated delivery, an Apgar score < 6 at 5 minutes post partum and major congenital abnormalities. Children were also excluded if they had been born after IVF-induced pregnancy, and when the pregnancy had been complicated by any serious infection, pre-eclampsia or maternal use of medication that may have affected birth weight. In addition, children were excluded if they had any serious chronic or acute illness or familial hypercholesterolaemia. With these exclusion criteria, we examined the local database from 1990-1993. In order to create a wide range in birth weight values, subjects were recruited from four strata of birth weight values (<P_{10}, P_{10-34}, P_{35-64} and P_{65-}

$_{90}$ according to Kloosterman[9]). Subjects with a birth weight above the 90th percentile were excluded because of the reported inverse J-shaped association between birth weight and cardiovascular disease.[10] Families still living at the same address were contacted by letter and phone. Overall, approximately 15% of eligible children complied. Two children had a parent with essential hypertension. Characteristics of the children are summarised in Table 1. The investigation conforms with the principles outlined in the Declaration of Helsinki. The study protocol was approved by the local ethical committee. Written informed consent was obtained from both parents and verbal informed consent was obtained from the children.

Measurements

The microvascular measurements were conducted in the morning after 30 minutes of acclimatisation in a quiet, temperature-controlled room (T=23.4±0.4ºC), with the subjects in the sitting position and the investigated, non-dominant hand at heart level. All subjects had abstained from caffeine-containing drinks overnight. Nailfold and iontophoresis studies were performed on the same day. The microvascular and metabolic studies (see below) were carried out on separate days approximately one week apart, and performed by two different investigators (RGIJ and JJV, respectively). Both investigators were cross-blinded to the microvascular reactivity and insulin sensitivity results. The results of the 24-h ambulatory blood pressure monitoring were also not available to these investigators.

Perfused nailfold capillaries in the dorsal skin of the third finger were visualised by a capillary microscope, as described previously.[8;11;12] Two separate visual fields of 1 mm^2 were recorded before and after four minutes of arterial occlusion with a digital cuff, and the images were stored on videotape. The number of capillaries at baseline and directly after release of the cuff were counted off-line for respectively 15 and 30 seconds by a single experienced investigator (RGIJ) from a freeze-framed reproduction of the videotape and from the running videotape when it was uncertain whether a capillary was present or not. (The major part of the increase in capillary number occurs within a few seconds.) Capillary density was defined as the number of erythrocyte-perfused capillaries per square millimeter of nailfold skin. PRH after four minutes of arterial occlusion with a digital cuff was used to assess functional recruitment of capillaries.[8;11;12] The number of capillaries in the resting state was counted during a 15-second period, only counting continuously perfused capillaries, as previously described.[13] Directly after release of the cuff, the number of perfused capillaries was counted. Percentage capillary recruitment during PRH was assessed by dividing the increase in capillary density during PRH by the capillary density in the resting state. Intrasubject coefficient of variation (CV) was 17.2±12.1% (measured on two occasions in 7 subjects). Capillary densities could not be determined in one child due to colouring of the skin.

Endothelium-dependent and -independent vasodilatation of skin microcirculation was evaluated by iontophoresis of acetylcholine and sodium nitroprusside in combination with laser Doppler fluxmetry as previously described in more detail.[11;12] A protocol of multiple fixed doses (current intensity X delivery time) was employed resulting in an incremental dose-response curve. Skin temperature was monitored. Acetylcholine (1%; Miochol, Bournonville Pharma, The Netherlands) was delivered using an anodal current; 7 doses (0.1 milliamps (mA) for 20 s) were delivered, with a 60-s interval between each dose. Sodium nitroprusside (0.1%; Nipride, Roche, The Netherlands) was delivered using a cathodal current; 9 doses (0.2 mA for 20 s) were delivered, with a 90-s interval between each dose. Acetylcholine-dependent laser Doppler flux was measured on the middle phalanx of the third finger, whereas nitroprusside-dependent laser Doppler flux was measured on the middle phalanx of the fourth finger. Approximately 15 minutes elapsed between these two measurements. Intrasubject coefficients of variation (CV) of the percentage increase from baseline to the plateau phase (final two iontophoretic deliveries) was 13.5±7.7% for acetylcholine and 18.7±23.4% for sodium nitroprusside (measured on two occasions in 7 subjects).

Ambulatory monitoring (Spacelabs 90207, Redmond, Washington, USA) was used to obtain 24-h recordings of blood pressure and heart rate. The non-dominant arm was used with an appropriately sized cuff. The monitors were programmed to take blood pressure and heart rate readings every 20 minutes from 7.00 - 22.00 and every 30 minutes from 22.00 - 7.00.[14] The subjects completed an activity diary. The readings were downloaded onto a computer spreadsheet and individually edited into daytime and night time periods from the subjects' diaries.[15] For logistical reasons, 24-hour blood pressure monitoring could not be performed in one subject.

Sensitivity to insulin-mediated glucose uptake was assessed by the hyperinsulinaemic, euglycaemic clamp technique, as described previously.[12] Briefly, insulin (Velosulin; Novo Nordisk, Bagsvaerd, Denmark) was infused in a primed continuous manner at a rate of 60 mU kg^{-1} $hour^{-1}$ for 2 h. Normoglycaemia was maintained by adjusting the rate of a 20% D-glucose infusion based on plasma glucose measurements performed at 5-min intervals. Whole body glucose uptake (M) was calculated from the glucose infusion rate during the last 60 minutes and expressed per unit of plasma insulin concentration (M/I).[16] Plasma insulin concentrations were measured by radioimmunoassay techniques (Immunoradiometric Assay, Medgenix Diagnostics, Fleurus, Belgium). For convenience the M/I ratio was multiplied by 100.

Anthropometric measurements (which included weight, height, waist circumference and hip circumference) were performed on all participants by one trained investigator (JJV) as described previously.[12] The body mass index was calculated by dividing weight in kilograms by height in meters squared. The waist-to-hip ratio was calculated as a measure of body fat distribution.

Table 1. Characteristics of the children

n (male/female)	21 (11/10)
Age (years)	8.6±1.2
Birth weight (gram)	3335±490
Gestational age (weeks)	39.8±1.1
Waist (cm)	57.9 ±8.8
Hip (cm)	70.3±8.6
Waist-to-hip ratio	0.82±0.04
Height (cm)	136.6±8.0
Weight (kg)	30.7±7.2
Body mass index (kg/m^2)	16.3±2.9
Ambulatory systolic blood pressure (mmHg)	
24-h	111±6
daytime	115±8
nighttime	106±5
Ambulatory diastolic blood pressure (mmHg)	
24-h	66±4
daytime	71±6
nighttime	59±4
Insulin sensitivity (mg/kg/min per pmol/l) x 100	3.4±2.0
Fasting plasma glucose (mmol/l)	4.6±0.2
Fasting plasma insulin (pmol/l)*	49 (35-67)
Fasting serum total cholesterol (mmol/l)	3.9±0.6

Data are presented as mean±SD, or as
*median (interquartile range)

Statistical analysis

Variables are presented as mean ± standard deviation (SD), or, in case of a skewed distribution, median and interquartile range (IQR). The paired Student's t-test was used to compare capillary densities before and after arterial occlusion, and to compare vasodilatatory responses before and following administration of acetylcholine and sodium nitroprusside. Pearson's correlation was used to investigate the relationships among birth weight, blood pressure, insulin sensitivity and microvascular function. Subsequently, a multiple regression analysis was used to analyse whether the associations observed between birth weight and capillary recruitment remained when allowing for age, sex, blood pressure insulin sensitivity, current weight, body mass index, waist-to-hip ratio, waist or gestational age. To investigate the possible influence of the presence of a familial predisposition for hypertension[17] we also adjusted for parental hypertension. In addition, we did analyses with birth weight standard deviation scores independent of sex, gestational age and parity for each infant using the Dutch growth reference charts.[9] Interaction analysis was performed to investigate whether sex, current weight, body mass index, waist-to-hip ratio or waist influenced the association between birth weight and capillary recruitment. A two-tailed P-value of < 0.05 was considered significant. All analyses were performed on a personal computer using the statistical software package SPSS version 9.0 (SPSS, Chicago, IL, USA).

Table 2. Microvascular measurements

Variable	mean±SD
Capillary recruitment during PRH	
Baseline capillary density (per mm^2)	39.8±6.6
Peak capillary density (per mm^2)	52.5±11.7***
Capillary recruitment (%)	33±24
Acetylcholine-mediated vasodilatation	
Skin temperature (ºC)	30.3±1.1
Baseline skin perfusion (PU)	31.6±11.0
Plateau after acetylcholine (PU)	146.4±67.0***
Acetylcholine-mediated vasodilatation (%)	423.6±270.0
Sodium nitroprusside-mediated vasodilatation	
Skin temperature (ºC)	31.3±0.9
Baseline skin perfusion (PU)	38.9±11.2
Plateau after sodium nitroprusside (PU)	154.9±61.0***
Sodium nitroprusside-mediated vasodilatation (%)	318.9±207.5

****P*<0.001

PRH indicates post-occlusive reactive hyperaemia; PU, perfusion units

Results

Birth data

Birth weight averaged 3345±501g (Table 1), and ranged from 2560 to 4240 g. Gestational age averaged 39.8±1.2 weeks (Table 1), and ranged from 38 to 42 weeks.

Blood pressure, heart rate and whole body glucose uptake

Ambulatory blood pressure and heart rate data are shown in Table 1. During the hyperinsulinaemic euglycaemic clamp, glucose levels were maintained at 5.1±0.3 mmol/l. Attained serum free insulin concentrations averaged 337±166 pmol/l. The rate of glucose uptake, expressed per kg body weight, was 10.1±4.2 $mg.kg^{-1}.min^{-1}$.

Microvascular function

Table 2 shows the microvascular measurements. After four minutes of arterial occlusion, capillary density increased significantly compared to baseline (P<0.0001). In addition, blood flow increased significantly after iontophoresis of acetylcholine and sodium nitroprusside compared to baseline (P<0.0001).

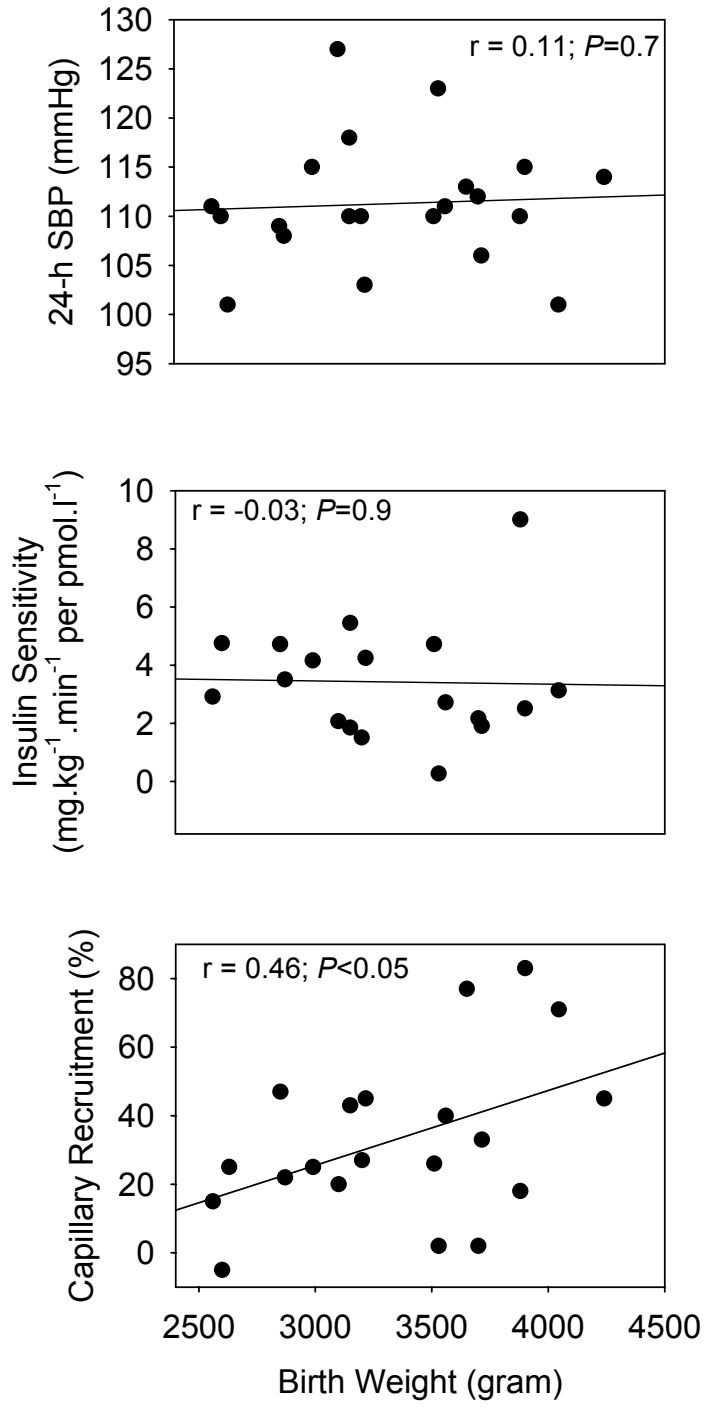

Figure 1 Associations between birth weight and 24h-systolic blood pressure, insulin sensitivity and capillary recruitment during post-occlusive reactive hyperaemia.

Associations of birth weight with microvascular function, blood pressure and insulin sensitivity

Birth weight was positively and significantly associated with capillary recruitment during PRH (figure 1). Linear regression analysis demonstrated that an increase in birth weight of 1 kg was associated with an increase in capillary recruitment of 22% (95%-CI: 0.1-43%; $P<0.05$).

Birth weight was not associated with systolic blood pressure or insulin sensitivity (1.4 mmHg [-5.0-7.7] per kg birth weight $P=0.7$; and -0.11 mg/kg/min per pmol/l [-2.4-2.2] per kg birth weight, $P=0.9$, respectively; figure 1). Birth weight was also not associated with systolic blood pressure or insulin sensitivity after adjustment for current weight (1.7 mmHg [-3.6-7.0] per kg birth weight, $P=0.5$; and -0.42 mg/kg/min per pmol/l [-3.5-2.1] per kg birth weight, $P=0.6$, respectively). The association between birth weight and capillary recruitment was not affected by adjustment for blood pressure or insulin sensitivity (24% [95%-CI: 3-43%] per kg birth weight, $P<0.05$; and 22% [95%-CI: -4-48%] per kg birth weight, $P=0.09$, respectively). Capillary recruitment was not associated with blood pressure or insulin sensitivity (-0.07 mmHg [95%-CI: -0.2-0.07mmHg] per 1% capillary recruitment, $P=0.3$; and 0.002 mg/kg/min per pmol/l [95%-CI: -0.05-0.05 mg/kg/min per pmol/l] per 1% capillary recruitment, $P=0.9$, respectively).

The association between birth weight and capillary recruitment was similar after adjustment for current weight, BMI or waist-to-hip ratio (slope: 22% [95%-CI: 1-43%] per kg birth weight, $P<0.05$; 23% [95%-CI: 1-44%] per kg birth weight, $P<0.05$; and 24% [95%-CI: 4-45%] per kg birth weight, $P<0.05$, respectively). Adjustment for sex did not influence the associations. In addition, interaction analysis indicated that the association between birth weight and capillary recruitment was similar in boys and girls, and similar subjects with a high and in subjects with a low current weight or waist-to-hip ratio (data not shown).

Birth weight was not associated with acetylcholine- and sodium-nitroprusside-mediated vasodilatation (-142% [-397-114] per kg birth weight, $P=0.3$, and 7.4% [-198-213] per kg birth weight, $P=0.9$).

Gestational age (range: 38-42 weeks) was associated with birth weight (32.9 g [8.3-57.5] per day, $P=0.01$) and capillary recruitment (1.3% [0.6-2.6] per day, $P=0.04$). After adjustment for gestational age, the association between birth weight and capillary recruitment was diminished (14% [95%-CI: -12-39%] per kg birth weight, $P=0.25$). Similarly, the association between gestational age and capillary recruitment was diminished after adjustment for birth weight (0.9% [-0.7-2.4] per day, $P=0.25$). Gestational age was not associated with blood pressure, insulin sensitivity or endothelium-(in)dependent vasodilatation. Adjustment for parity did not influence the association between birth weight and capillary recruitment (data not shown). In an additional analysis, birth weight standard deviation scores independent of sex,

gestational age and parity were significantly associated with capillary recruitment (11% [95%-CI: 1-21%] per birth weight standard deviation score, P<0.05).

Adjustment for familial predisposition to hypertension did not influence the results. The results were also similar if the M-value was used instead of the M/I value, if systolic blood pressure during day time or night time was used instead of 24-hour systolic blood pressure, if current body mass index was used instead of weight, and if waist was used instead of waist-to-hip ratio (data not shown).

Discussion

We have previously demonstrated that capillary recruitment during PRH was related to birth weight, blood pressure and insulin sensitivity in adults.[8] However, it could not be resolved whether the impaired capillary recruitment in subjects with a low birth weight was a cause or a consequence of the higher blood pressure and/or insulin resistance. In this study of prepubertal children, birth weight was associated with capillary recruitment, but not with blood pressure or insulin sensitivity. In addition, the association between birth weight and capillary recruitment was independent of changes in blood pressure and insulin sensitivity. The strength of the association between birth weight and capillary recruitment in children was similar to the strength of the association in adults.[8] Taken together, the previous[8] and current data suggest that an impairment in capillary recruitment is a primary disturbance in individuals with a lower birth weight, and is not secondary to higher blood pressure and/or insulin resistance. Indeed, experimental[18;19] and human studies[7;20;21] suggest that a decreased microvascular function contributes to an increase in vascular resistance and antedates hypertension. In addition, an impaired microvascular function has been suggested to reduce insulin sensitivity by decreasing the delivery of insulin and glucose.[22;23] Therefore, microvascular function may play a mechanistic role in the association of low birth weight with increased blood pressure and diminished insulin sensitivity.

To our knowledge, we are the first to report on the association between birth weight and capillary recruitment during PRH in children. Studies investigating the association between birth weight and other measures of microvascular function are conflicting. Martin et al. have reported an impaired acetylcholine-mediated vasodilatation in small-for-gestational-age neonates at 3 days of age[24] and in low-birth-weight children at 9 years of age.[25] However, these findings should be interpreted with caution. The results in neonates may have been influenced by the dramatic changes that occur at that time as an adaptation to the extra-uterine environment. The results in 9-year-old children may have been influenced by the selection of children with a very low birth weight (< -2 SD) only.[25] We,[8, present study] and others[26] did not find a significant association between birth weight and acetylcholine-mediated vasodilatation in adults, 7-10 year old children and 3-month-old infants. Two studies have reported the association between birth weight

and characteristics of the capillary network in adults. Chapman et al. have demonstrated structural changes in the retinal microvascular network of low-birth-weight men, suggesting lower than normal microvascular density,[27] and we have previously demonstrated an association between birth weight and capillary recruitment in healthy adults.[8] This decrease in capillary recruitment may be a consequence of a reduced endothelium-dependent vasodilation at the precapillary level.[13] Our findings of an association of birth weight with capillary recruitment, but not with endothelium-dependent vasodilation do not support this hypothesis, at least not in children. Therefore, we suggest that there is a direct association between birth weight and characteristics of the capillary network, as assessed by capillary recruitment during PRH. Previous studies suggest that an impairment in capillary recruitment may be determined by both functional and structural rarefaction.[13] In accordance, the skin maximal hyperaemic response to local heating which is considered to be determined at the structural microvascular level was found to be impaired in infants with a low birth weight.[28]

In previous studies on the relationship between birth weight and the microcirculation in children, insulin sensitivity was not measured and blood pressure was measured with conventional methods only.[24-26] Therefore, these studies could not rule out the possibility that changes in microvascular function were due to changes in blood pressure and/or insulin sensitivity. We have used 24-h ambulatory blood pressure monitoring and the hyperinsulinaemic euglycaemic clamp technique, which are considered to be the gold standard for the measurement of blood pressure and insulin sensitivity, respectively. Birth weight was not significantly associated with blood pressure and insulin sensitivity at the age of 7-10 years, and the association between birth weight and capillary recruitment was not affected by adjustment for these variables. The lack of a significant association of birth weight with blood pressure and insulin resistance in children is not surprising. Several longitudinal studies have suggested that the association of birth weight with blood pressure is weak or absent in children of this age group, but tends to become progressively greater in older groups of individuals.[29-32] Similarly, longitudinal studies in children[33] and adults[34] demonstrated that the relationship of insulin resistance with birth weight is strengthened with increasing age.

Several studies in adults demonstrated that the association between birth weight and cardiovascular risk is strengthened after adjustment for measures of current size (i.e. body mass index or weight)[29;35] and is stronger in subjects with a high than in subjects with a low current body size.[5] However, in our study of prepubertal children, the association between birth weight and capillary recruitment was not influenced by body mass index, current weight or waist-to-hip ratio. Taken together, these findings suggest that body size and body fat distribution do not play a central role in the *initial* association between birth weight and capillary recruitment in children**,** but may influence the association between birth weight and cardiovascular risk at a later age.

After adjustment of birth weight for gestational age using standard deviation scores according to the Dutch growth reference charts, the association between birth weight and capillary recruitment remained statistically significant. However, after adjustment for gestational age using linear regression analysis the association between birth weight and capillary recruitment was diminished. Similarly, the association between gestational age and capillary recruitment was diminished after adjustment for birth weight. These findings are in line with several recent studies that suggested an important role for gestational age in the association between birth weight and cardiovascular disease. Gestational age is inversely associated with blood pressure[36-38] and the association between birth weight and blood pressure is diminished after adjustment for gestational age.[32;36;38] In addition, Irving et al. demonstrated that prematurity was independently related to higher fasting levels of glucose and insulin, which are indicative of insulin resistance,[37] and Martyn et al. demonstrated that prematurity was associated with an increased death rate from coronary heart disease.[39] Therefore, birth weight and gestational age may both play an important role in the development of cardiovascular disease.

In conclusion, low birth weight is associated with impaired capillary recruitment in prepubertal children who were born at term. In these children, low birth weight was not associated with increased blood pressure or diminished insulin sensitivity, and the association between birth weight and capillary recruitment was independent of blood pressure and insulin sensitivity. These findings are consistent with the hypothesis that capillary recruitment plays a mechanistic role in the association of birth weight with blood pressure and insulin resistance.

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3

Direct evidence for insulin-induced capillary recruitment in skin of healthy subjects during physiological hyperinsulinemia

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Abstract

Background Insulin-mediated changes in muscle perfusion have been proposed to modulate insulin-mediated glucose uptake. However, the putative effects of insulin on the microcirculation which may permit such modulation have not been studied in humans. We have examined the effects of systemic hyperinsulinemia on skin (micro)vascular function in 8 healthy non-diabetic subjects. In addition, the effects of locally administered insulin on skin blood flow were assessed in 10 healthy subjects.

Methods During a hyperinsulinemic clamp, we measured leg blood flow with venous occlusion plethysmography; skin capillary density with capillaroscopy; endothelium-(in)dependent vasodilatation of skin microcirculation with iontophoresis of acetylcholine and sodium nitroprusside combined with laser Doppler fluxmetry; and skin vasomotion by Fourier-analysis of microcirculatory blood flow. To exclude non-specific changes in the hemodynamic variables, a time-volume control study was performed. Insulin iontophoresis was used to study the local effects of insulin on skin blood flow.

Results Compared to the control study, systemic hyperinsulinemia caused an increase in leg blood flow (-0.54±0.93 vs. +1.97±1.1 ml•min-1•dl-1, $P<0.01$); an increase in the number of perfused capillaries in the resting state (-3.7±3.0 vs. +3.4±1.4 per mm2, $P<0.001$) and during post-occlusive reactive hyperemia (-0.8±2.2 vs. +5.1±3.7 per mm2, $P<0.001$); an augmentation of the vasodilatation caused by acetylcholine (722±206 vs. 989±495%, $P<0.05$) and sodium nitroprusside (618±159 vs. 788±276%, $P<0.05$); and a change in vasomotion by increasing the relative contribution of the 0.01-0.02 Hz and 0.4-1.6 Hz spectral components ($P<0.05$). Compared to the control substance, locally administered insulin caused a rapid increase (~13.5 min) in skin microcirculatory blood flow (34.4±42.5 vs. 82.8±85.7%, $P<0.05$).

Conclusions Systemic hyperinsulinemia in skin, 1) induces recruitment of capillaries; 2) augments nitric oxide-mediated vasodilatation; and 3) influences vasomotion. In addition, locally administered insulin 4) induces a rapid increase in total skin blood flow, independent of systemic effects.

Introduction

Insulin increases skeletal muscle blood flow in a time- and concentration-dependent fashion through a mechanism that can be abolished by inhibiting nitric oxide synthase and/or Na+-K+-ATPase.[1,2] Recently, it has been hypothesized that insulin's metabolic and vasodilatory actions are functionally coupled[2,3] with an important role for microcirculatory function.[2,4-7] Specifically, it has been proposed that insulin, by reducing precapillary arteriolar tone and/or altering arteriolar vasomotion, redirects blood flow from non-nutritive vessels to nutritive capillary beds with a resultant increase in the overall number of perfused capillaries termed "functional capillary recruitment".[2,4] The latter would enhance the access of insulin and glucose to a greater mass of muscle cells for metabolism.[2,4,8]

Both experimental[5-7] and human studies[4,8] suggest that insulin-mediated changes in muscle perfusion can modulate insulin-mediated glucose uptake. However, studies in which glucose uptake has been measured during hyperinsulinaemia and manipulation of total limb blood flow have shown conflicting results.[1,9] This has been ascribed to the fact that various vasoactive agents may change total flow but have distinct effects on the microcirculation and on the distribution of blood flow in nutritive compared to non-nutritive vessels.[1,6,7,9] Therefore, a better understanding of the putative effects of insulin on the microcirculation which may permit modulation of glucose uptake is necessary.

At present, there is some evidence consistent with insulin-induced capillary recruitment.[4,6-8] In the isolated perfused rat hindlimb, insulin increased the metabolism of 1-methylxanthine, an indirect indicator of capillary recruitment.[6,7] In humans, an increase in the distribution volume of glucose has been demonstrated in parallel with the increase in blood flow during supraphysiological hyperinsulinaemia, suggesting capillary recruitment.[8] However, these studies provide only indirect evidence of insulin-mediated capillary recruitment.

Assessment of skin microcirculation with capillary microscopy enables direct visualization of capillary density and capillary recruitment. By use of this technique we recently demonstrated that capillary recruitment during post-occlusive reactive hyperaemia is associated with insulin's metabolic and vascular actions in both normal and hypertensive subjects.[10,11] Human skin also allows the continuous assessment of changes in the frequency of vasomotion.[12]

The current study examined the effects of systemic physiological hyperinsulinaemia on skin capillary density and capillary recruitment during post-occlusive reactive hyperaemia as assessed with capillary microscopy. The effects of insulin on vasomotion and endothelium-(in)dependent vasodilatation of skin microcirculation were measured with laser Doppler fluxmetry. In addition, iontophoresis of insulin was used to investigate the local effects on total skin blood flow independent of its systemic actions.

Methods

Subjects

Characteristics of the study subjects in each study are given in Table 1. All subjects were healthy as judged by medical history, non-diabetic according to ADA criteria,[13] and normotensive as determined by triplicate office blood pressure measurement. They did not use medication and all were non-smokers. None had a first-degree relative with diabetes mellitus type 2. The study protocol was approved by the local Ethics Committee and conformed with the principles outlined in the Declaration of Helsinki.

Methods

Study 1

Study Design

All subjects underwent the experimental protocol as shown in Figure 1.

The measurements were conducted in a quiet, temperature-controlled room (T=23.4±0.4ºC) at 8.00 AM, after a 12-hour fast, with the subjects in the supine position and after the subject had emptied his or her bladder. The subjects abstained from caffeine- and alcohol-containing drinks overnight. All microcirculatory measurements were performed with the investigated, non-dominant hand at heart level. Baseline measurements were obtained after allowing 30 min of rest and acclimatization after the

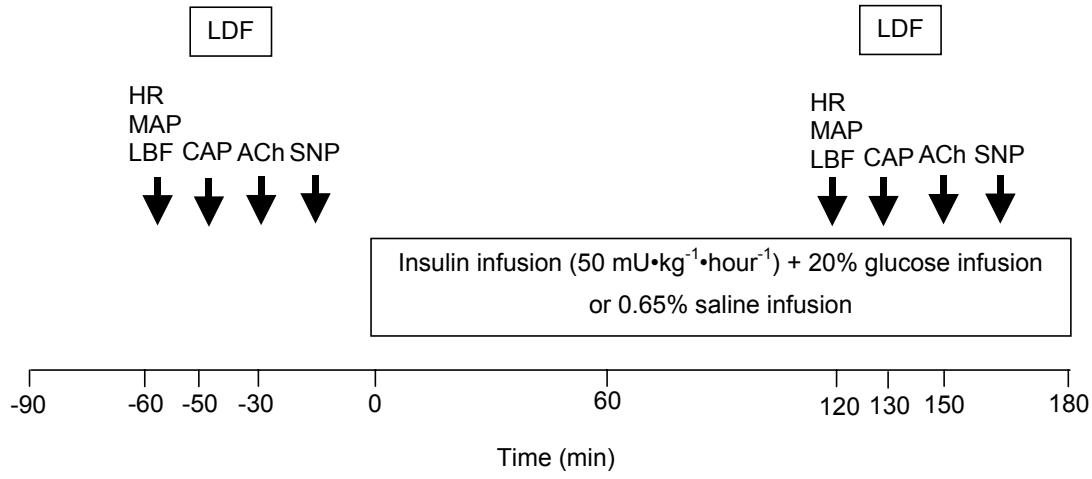

Figure 1. Design of the study. Measurements started after 30 min of acclimatization. LDF = laser Doppler flow measurement used to assess vasomotion; HR = heart rate; MAP = mean arterial pressure; LBF = leg blood flow assessed with venous occlusion plethysmography; CAP = intravital capillaroscopy; ACh = iontophoresis of acetylcholine; SNP = iontophoresis of sodium nitroprusside. A time- and volume-control study was performed one week after the clamp procedure in an identical fashion, but without insulin and glucose infusion.

Table 1. Characteristics of the healthy volunteers

	Study 1	Study 2
	mean±SD	mean±SD
n (male/female)	8 (6/2)	10 (10/0)
Age (years)	23±3.6	25±3.3
WHR	0.90±0.10	0.89±0.04
BMI (kg/m^2)	22.5±2.5	23.1±3.1
Systolic blood pressure (mmHg)	123±9	114±10
Diastolic blood pressure (mmHg)	71±9	78±9
M/I-value (mg•kg^{-1}•min^{-1} per pmol•l^{-1}) x 100	2.2±0.6	
Fasting plasma glucose (mmol/l)	4.9±0.3	4.8±0.5
Fasting serum total cholesterol (mmol/l)	4.5±0.7	4.8±0.9
Fasting HDL-cholesterol (mmol/l)	1.5±0.3	1.4±0.3
Fasting serum triglycerides (mmol/l)	1.0±0.4	1.0±0.3

WHR = waist-to-hip-ratio; BMI = body mass index; M/I-value = glucose infusion rate during a hyperinsulinemic clamp, expressed per unit of plasma insulin concentration.

insertion of two polytetrafluoroethylene catheters (Venflon; Viggo, Sweden): one in the right antecubital vein and one in a vein of the opposite forearm. To exclude non-specific changes in leg blood flow and microcirculatory function, a time- and volume-control study was performed one week after the clamp procedure in an identical fashion with infusion of the same amounts of fluid (0.65% saline) and with blood sampling at the same time intervals, however, without insulin and glucose infusion. The control experiments and hyperinsulinaemic clamp experiments were not randomised, because the amount of fluid to be infused during the control study depended on the volume of glucose infused during the hyperinsulinaemic clamp study.

Whole body glucose uptake

Sensitivity to insulin-mediated glucose uptake was assessed by a hyperinsulinaemic euglycaemic clamp technique with the subjects in the post-absorptive state. Insulin (Velosulin; Novo Nordisk, Denmark) was infused in a primed continuous manner at a rate of 50 mU kg-1 hour-1 for 180 minutes. Fasting glucose levels were maintained by adjusting the rate of a 20% glucose infusion based on plasma glucose measurements performed at 5-min intervals. Whole body glucose uptake (M) was calculated from the glucose infusion rate during the last 40 minutes and expressed per unit of plasma insulin concentration (M/I), thereby correcting for differences in steady-state plasma insulin levels (14). For convenience, the M/I ratio was multiplied by 100.

Leg blood flow

At t = - 60 min and at t = 120 min, leg blood flow was measured by mercury-in-silastic strain gauge venous occlusion plethysmography (EC6, Hokanson, Bellevue, USA) with the subjects in the supine position (11,15). An occlusive cuff was placed proximally

around the right leg, and the strain gauge was placed around the calf at the largest circumference. A pediatric cuff inflated to suprasystolic pressure was placed at the ankle to exclude the foot circulation. Hokanson dedicated software was used (NIVP3) to automatically balance the plethysmograph, inflate the cuff to 50 mmHg using a E20 Rapid Cuff Inflator and an AG101 Air Source, capture the inflow waveform, and calculate blood flow. Nine separate recordings of leg blood flow were made once every fifteen seconds. Flow measurements were expressed in terms of ml flow per dl, and represent the average of 7-9 separate recordings. In our hands, this technique has a CV of 10 to 14% (present study, 15). We non-invasively determined systolic blood pressure, diastolic blood pressure, mean arterial pressure, and heart rate (Colin Press-Mate BP-8800, Colin, Japan) with every leg blood flow determination. The average of four consecutive blood pressure and heart rate readings during each period were used for further analysis.

Capillary microscopy

The capillaroscopy studies were conducted at t = - 50 min and t = 130 min. Skin temperature was monitored. Nailfold capillaries in the dorsal skin of the third finger were visualized by an epi-illuminated microscope.[10,11,16] Capillaries were made visible in a standardised manner, making it possible to visualize the same microscopic field in the hyperinsulinaemic and time volume control studies. Capillaries were visualised approximately 1.5 mm proximal to the terminal row of capillaries in the middle of the nailfold of the third finger. This distance was the width of one visual field of the microscope. At this spot capillaries appear mostly as dots with only a small part of the arteriolar and venular limb also being visible. Subsequently, a characteristic capillary (i.e. a capillary that was constantly perfused and had an eye-catching morphological feature) was kept on the same spot exactly in the centre of the visual field (marked by a dot on the monitor), to ensure that capillary density was measured in the exact same visual field during the entire experiment and the control studies. Two adjacent visual fields of 1 mm^2 were recorded before and after four minutes of arterial occlusion and the images were stored on videotape. The number of capillaries was counted off-line by two experienced investigators (EHS, GDV) from a freeze-framed reproduction of the videotape and from the running videotape when it was uncertain whether a capillary was present or not. The investigators counting the capillaries were unaware of whether the videotapes were from the hyperinsulinemic clamp or the control study. Capillary density was defined as the number of erythrocyte-perfused capillaries per square millimetre of nailfold skin. During direct intravital microscopy without dyes, some capillaries seem continuously filled with erythrocytes while others are intermittently perfused. Capillary density in the resting state was counted during a 3-minute period, counting all, continuously and intermittently, perfused capillaries. Post-occlusive reactive hyperaemia (PRH) after 4 minutes of arterial occlusion with a digital cuff (Hokanson, USA), was used to assess functional recruitment of capillaries.[10,11,16] In this

case the number of capillaries in the resting state was counted during a 15-second period, only counting continuously perfused capillaries, as previously described.[16] Directly after release of the cuff, the number of perfused capillaries was counted. (The increase in capillary number occurs within a few seconds.) The absolute capillary recruitment during PRH was assessed by subtracting the capillary density in the resting state from the peak capillary density during PRH. The procedure was then repeated using a visual field adjacent to the first visual field. Data concerning capillary densities are the mean of two measurements. Intrasubject coefficients of variation (CV) of the capillary density in the resting state (15-s count vs. 3-min count) and the absolute capillary recruitment during PRH were 2.3±1.8% vs. 5.7±4.3% and 6.2±4.3%, respectively (present study; measured on two separate days in 8 subjects; data not shown).

Endothelium-(in)dependent vasodilatation of skin microcirculation

This was evaluated at t = - 30 min and t = 150 min by iontophoresis of acetylcholine and sodium nitroprusside in combination with laser Doppler fluxmetry as previously described in more detail.[10,11] A protocol of multiple fixed doses (current intensity X delivery time) was employed resulting in an incremental dose-response curve.[10,11] During the iontophoresis procedure a thermostatic laser Doppler probe was heated to 30 ºC in order to prevent a rapid decrease in skin temperature. Acetylcholine (1%; Miochol, Bournonville Pharma, The Netherlands) was delivered using an anodal current; 7 doses (0.1 milliamps (mA) for 20 s) were delivered, with a 60-s interval between each dose. Sodium nitroprusside (0.1%; Nipride, Roche, The Netherlands) was delivered using a cathodal current; 9 doses (0.2 mA for 20 s) were delivered, with a 90-s interval between each dose. Acetylcholine-dependent laser Doppler flux was measured on dorsal skin of the middle phalanx of the third finger, whereas nitroprusside-dependent laser Doppler flux was measured on the middle phalanx of the fourth finger. Dorsal skin of the middle phalanx was chosen because we were interested in the nutritive function of skin microcirculation as opposed to its thermoregulatory function. Dorsal skin is considered devoid of arteriovenous anastomoses, which serve the thermoregulatory function of skin microcirculation.[17] During hyperinsulinaemia and the control study, the same fingers were used as during the first baseline measurement. The increase from baseline to the final two and three minutes of the plateau phase were used for further analyses of the blood flow responses to acetylcholine and sodium nitroprusside, respectively. To exclude possible nonspecific microcirculatory reactivity, responses to the vehicles of both acetylcholine (mannitol 3%) and sodium nitroprusside (water for injection) were tested in four healthy subjects before and during hyperinsulinaemia using identical protocols to those used for both drugs.

Skin microcirculatory vasomotion

Blood flow was recorded at t = - 50 min and at t = 130 min using the Periflux 4000 laser Doppler system (Perimed, Sweden) in combination with a Periflux tissue heater set to 30 ºC (PF 4005 PeriTemp) to prevent a rapid decrease of skin temperature. The light, emitted from a near-infra-red laser diode with a power of 1.0 mW at a wavelength of 780 nm, was delivered by an optical-fibre probe (PF 408). The probe, combined with a thermostatic probeholder (PF450), was positioned on the dorsal side of the wrist of the non-dominant arm and was kept in place during the entire study period. A bandpass filter with cut-off frequencies at 20 Hz and 20 kHz, and a time constant of 0.2 s was selected. Perisoft dedicated software (PSW, Perimed, Sweden) was used for data acquisition on a personal computer. The signal was sampled for 15 min at 32 Hz.

Study 2.

Insulin iontophoresis

All subjects underwent iontophoresis of regular insulin (0.18 ml Actrapid 100IU/ml; Novo Nordisk, Denmark) and a control substance (0.18 ml diluting medium for soluble insulin injection; Novo Nordisk, Denmark) in a double-blind randomised order. The diluting medium (control substance) had the same composition as Actrapid, but did not contain insulin molecules. Approximately 10 minutes elapsed between the two measurements. The first measurement was performed on dorsal skin of the middle phalanx of the third finger, whereas the second measurement was performed on the middle phalanx of the fourth finger. The substances were delivered using a cathodal current.[18,19] To minimize nonspecific current-induced vasoreactivity, 9 doses (0.2 mA for 20 s) were delivered, with a 90-s interval between each dose resulting in an incremental dose-response curve. Blood flow was recorded continuously using the Periflux 4000 laser Doppler system (Perimed, Stockholm, Sweden) with the thermostatic probe (PF 481-2; recently modified by Perimed in order to improve the backscatter/flux ratio) set to 30 ºC. The increase from baseline to the final three minutes of the plateau phase were used for further analyses of the blood flow responses to insulin. Although systemic glucose-lowering effects of iontophoresed insulin could not be demonstrated in humans,[20] plasma glucose levels were measured before and after the iontophoresis procedures. The measurements were conducted under the same conditions as study 1, however, with the subjects in the sitting position.

Analytical methods

In study 1, all serum samples were directly centrifuged at -4°C and stored at –80 °C. Insulin levels were measured with an immunoradiometric assay (Medgenix Diagnostics, Fleurus, Belgium). Glucose levels during the hyperinsulinemic clamp were determined by the glucose oxidase method with a glucose analyser YSI2300 (Yellow Springs, OH, USA). In study 2, glucose levels were measured with the HemoCue Glucose Analyser (HemoCue AB, Angelholm, Sweden).

Table 2. Metabolic and hemodynamic variables before and during hyperinsulinemic clamp and time-volume control studies

	t=-60		t=120 min	
Variable	Insulin study	Control study	Insulin study	Control study
Plasma insulin (pmol/l)	55±12	56±20	409±61†	48±11‡
Blood glucose (mmol/l)	4.6±0.3	4.9±0.3	4.6±0.4	4.8±0.3
Heart rate (bpm)	56±6	57±8	63±11	56±11
Systolic blood pressure (mmHg)	127±10	125±11	124±10	123±11
Mean arterial pressure (mmHg)	87±6	85±9	84±8*	84±10
Diastolic blood pressure (mmHg)	66±6	65±9	63±8*	64±10
Leg blood flow (ml•min^{-1}•dl^{-1})	2.8±1.6	2.9±1.1	4.7±2.7*	2.4±1.0§

Values are expressed as means±SD; bpm = beats per minute; PU = arbitrary perfusion units; *P<0.05 and †P<0.001 vs. t=-60; ‡P<0.05 and §P<0.01: change during insulin study vs. change during control study.

Data analysis

Data are expressed as mean ± SD, unless stated otherwise. Comparison of microvascular measurements before and during the insulin and control study was performed with a paired t-test. In addition an analysis of variance (ANOVA) for repeated measurements was used to compare the dose-response curves. Fourier transformation was used for determination of frequency components within the laser Doppler signal.[12] The frequency band between 0.01 and 1.6 Hz was studied, which was divided into a set of intervals. For each interval, a short-time Fourier transform with a different window length was used. On the basis of a recently published study,[12] we chose five frequency intervals: 1) 0.01-0.02 Hz, which is thought to contain local endothelial activity; 2) 0.02-0.06 Hz, which is thought to contain neurogenic activity; 3) 0.06-0.15 Hz, which is associated with the myogenic response of the smooth muscle cells in the vessel wall; 4) 0.15-0.4 Hz, which is the frequency interval of respiratory function; and 5) 0.4-1.6 Hz, which contains the heart beat frequency. The Mann-Whitney (Wilcoxon) paired statistical test was used to compare differences in energy densities obtained with Fourier analysis. A two-tailed P-value of < 0.05 was considered significant. All analyses were performed on a personal computer using the statistical software package SPSS version 9.0.

Results

Study 1.

Metabolic and haemodynamic variables

Table 2 shows the metabolic and haemodynamic variables before and during the hyperinsulinemic clamp and the time-volume control studies.

Both mean arterial pressure (MAP) and diastolic blood pressure (DBP) decreased significantly during hyperinsulinaemia (P<0.05); however, this decrease was not significantly different (P=0.75) from the non-significant decrease in MAP and DBP observed during the control study. Heart rate increased during insulin infusion (P=0.07), but not during the control study (P=0.55). The increase in leg blood flow during hyperinsulinaemia was significantly different from the change in leg blood flow observed during the control study (+1.97±1.1 vs. –0.54±0.93 ml•min-1•dl-1, P<0.01).

Capillary microscopy

Capillaroscopy data are shown in Figure 2. At t=-50 baseline (15-s count) and peak capillary density during PRH were not significantly different between the insulin and control study (37.7±5.4 vs. 37.9±5.4 and 53.0±6.3 vs. 53.1±6.5 per mm^2). At t=130 baseline capillary density (both 15-s and 3-min count) significantly higher during the insulin study (37.7±5.4 vs. 41.1±5.0, P<0.001 and 52.4±5.8 vs. 57.1±5.8 per mm^2, respectively). Peak capillary density during PRH was also significantly higher during the insulin study (53.0±6.3 vs. 58.1±6.1 per mm^2, p<0.01). The increase in capillary number during post-occlusive reactive hyperaemia in the resting state (before insulin infusion) was associated with the increase in baseline capillary density during hyperinsulinaemia (r=+0.71;P<0.05 (15-s count) and r=+0.66;P=0.08 (3-min count)). During the control study, baseline capillary density (both 15-s and 3-min count) decreased significantly (37.9±5.4 vs. 34.2±6.8 per mm^2, P<0.05 and 52.5±5.4 vs. 47.6±5.3 per mm^2, P<0.05, respectively). Peak capillary density decreased non-significantly (53.1±6.5 vs. 52.3±5.5 per mm^2). The observed increase in capillary numbers during the insulin study was significantly different from the decrease observed

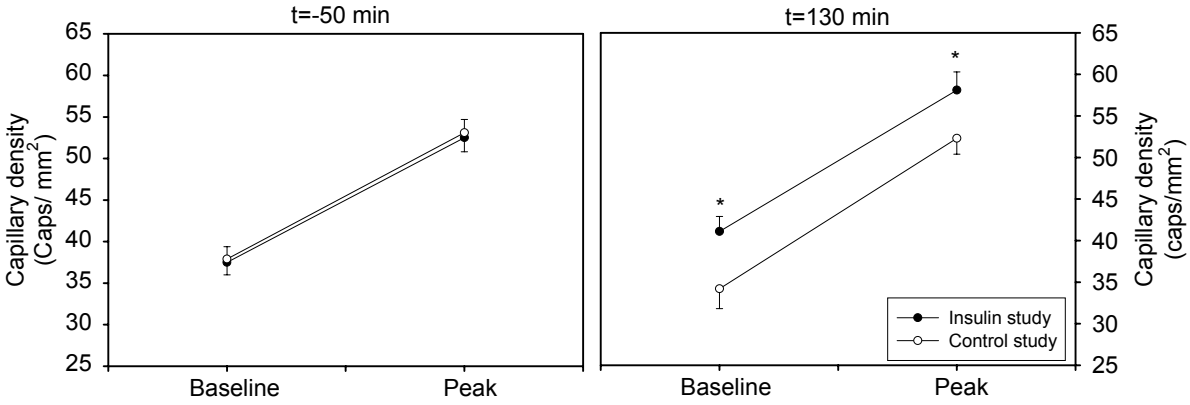

Figure 2. Capillary densities before (baseline) and during post-occlusive reactive hyperemia (peak) at t= -50 min and t=130 min during the hyperinsulinemic clamp study (closed circles) and time-volume control study (open circles). *P<0.05 versus control study. The results are expressed as means±SEM.

during the control study (P<0.01). Skin temperature was not significantly different during the experiments (32.7±0.9 ºC).

Endothelium-(in)dependent vasodilatation of skin microcirculation

Results are shown in Table 3 and Figure 3. The increase in baseline skin perfusion during hyperinsulinaemia was significantly different from the decrease in baseline perfusion observed during the control study (+4.5±4.9 vs. –1.0±2.6 PU, P<0.05 for the baseline measurements preceding the acetylcholine procedure, and +1.7±3.8 vs. –3.1±3.1 PU, P=0.05 for the baseline measurement preceding the sodium nitroprusside procedure). In addition, hyperinsulinaemia significantly increased the vasodilatation induced by both acetylcholine and sodium nitroprusside. The vehicle responses were unaltered during hyperinsulinaemia (absolute changes in microcirculatory flow before and during hyperinsulinaemia were: -2.1±7.4 vs. +1.0±5.0 PU, P=0.6, and +6.9±16.3 vs. +4.6±13.8, P=0.9 during iontophoretically applied acetylcholine vehicle and sodium

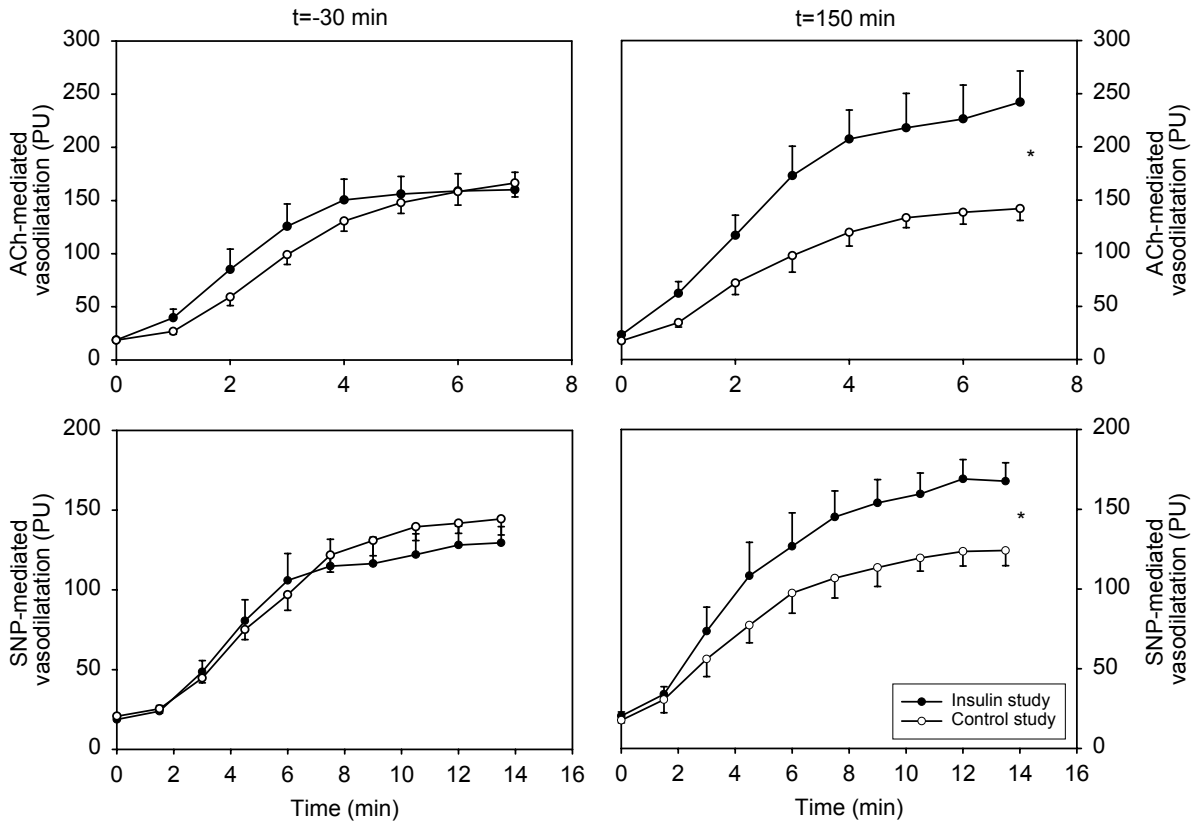

Figure 3. Blood flow responses to iontophoresis of acetylcholine and sodium nitroprusside at t=-30 min and t=150 min during the insulin study (closed circles) and control study (open circles). Blood flow is expressed in arbitrary perfusion units (PU). *P<0.05 versus control study. The results are expressed as means±SEM.

Table 3. Endothelium-dependent and endothelium-independent vasodilatation of skin microcirculation before and during hyperinsulinemic clamp and time-volume control studies

	t=-30 min		t= 150 min	
	Insulin study	Control study	Insulin study	Control study
ACh-mediated vasodilatation				
Skin temperature (ºC)	31.0±0.9	31.6±0.7	31.2±0.7	31.2±0.7
Baseline skin perfusion (PU)	18.8±5.2	18.6±3.6	23.7±7.8*	17.5±4.3‡
Plateau after ACh (PU)	159.6±45.7	162±36.0	234.2±84.7*	140.2±31.3‡
ACh-mediated vasodilatation (%)	780±304	811±319	989±495*	722±206‡
SNP-mediated vasodilatation				
Skin temperature (ºC)	31.2±1.0	31.4±0.8	31.3±0.7	31.6±0.9
Baseline skin perfusion (PU)	18.7±6.9	20.8±4.7	20.4±7.0	17.7±4.7*
Plateau after SNP (PU)	128.8±30.9	143.1±22.6	168.3±33.2†	123.8±25.7†‡
SNP-mediated vasodilatation (%)	629±186	611±155	788±276†	618±159‡

Values are expressed as means±SD; ACh = acetylcholine; SNP = sodium nitroprusside; PU = arbitrary perfusion units; *P<0.05 and †P<0.01 vs. t=-30; ‡P<0.05: change during insulin study vs. change during control study.

nitroprusside vehicle, respectively). Comparison of the total dose-response curves with ANOVA for repeated measures demonstrated no significant differences between the insulin and control study at t=-30 for both acetylcholine and sodium nitroprusside (Figure 3, left panel). In contrast, the same analysis at t=150 demonstrated significant differences between the insulin and control study ($F=8.108$, $p<0.05$ and $F=6.949$, $P<0.05$ for acetylcholine and sodium nitroprusside, respectively; Figure 3, right panel).

Skin microcirculatory vasomotion.

Table 4 summarizes the energy densities within each frequency interval and the calculated total energy density within the interval from 0.01 to 1.6 Hz. Hyperinsulinaemia increased the total spectral energy density of the blood flow signal. Compared to the control study, insulin significantly ($P<0.05$) increased the relative energy contribution of the frequency synchronized with the heart rate (from 0.4 to 1.6 Hz). In addition, the relative contribution of spectral components around 0.01 to 0.02 Hz, thought to result from local endothelial activity, was also significantly increased ($P<0.05$).

Study 2.

Insulin iontophoresis

Both insulin and the control substance increased skin blood flow significantly (33.2±7.9 to 59.7±26.7 PU, $P<0.01$ and 35.0±13.5 to 46.7±20.4, $P<0.05$, respectively; Figure 4). The absolute increase observed during iontophoresis of insulin was significantly larger than that observed during iontophoresis of the control substance (26.5±25.3 vs. 11.7±13.4 PU, $P<0.05$; ANOVA for repeated measures $F=6.115$, $P<0.05$). The relative increase from baseline was significantly higher during iontophoresis of insulin

Table 4. Energy densities of skin microcirculatory blood flow within each of the five frequency intervals before and during hyperinsulinemic clamp and time-volume control studies

	t=-50 min		t=130 min	
Frequency domain	Insulin study	Control study	Insulin study	Control study
0.01 – 0.02 Hz	0.49	0.53	1.54†	0.59‡
	(0.11-1.30)	(0.51-1.38)	(0.44-2.49)	(0.22-1.17)
0.02 – 0.06 Hz	1.45	1.33	1.47	0.96
	(0.51-2.72)	(0.54-3.25)	(0.28-4.74)	(0.41-2.37)
0.06 – 0.15 Hz	2.87	2.60	2.25	2.22
	(2.11-4.99)	(0.42-4.62)	(1.37-7.04)	(0.21-4.68)
0.15 – 0.40 Hz	0.64	0.77	0.75	0.73
	(0.50-3.12)	(0.17-3.71)	(0.46-1.33)	(0.17-2.65)
0.40 – 1.60 Hz	2.68	2.47	6.26†	2.33‡
	(1.47-5.95)	(1.22-6.66)	(2.24-21.20)	(0.50-4.44)
Total energy density	7.74	9.01	14.05*	8.03§
0.01– 1.60 Hz	(6.02-16.59)	(4.83-16.48)	(6.01-32.70)	(2.24-11.49)

Data are the energy densities within each of the five frequency domains and the total energy density within the interval from 0.01 Hz to 1.6 Hz obtained with Fourier analysis; group median values and ranges are shown; *P<0.05 and †P<0.01 vs. t=-50; ‡P<0.05 and §P<0.01: change during insulin study vs. change during control study.

compared to the control substance (82.8±85.7 vs. 34.4±42.5%, P<0.05; ANOVA for repeated measures F=7.715, P<0.05). Systemic glucose levels did not change during the iontophoresis of insulin (4.4±0.4 vs. 4.5±0.5 mmol/l, P=0.5) or control substance (4.6±0.5 vs. 4.7±0.6 mmol/l, P=0.4).

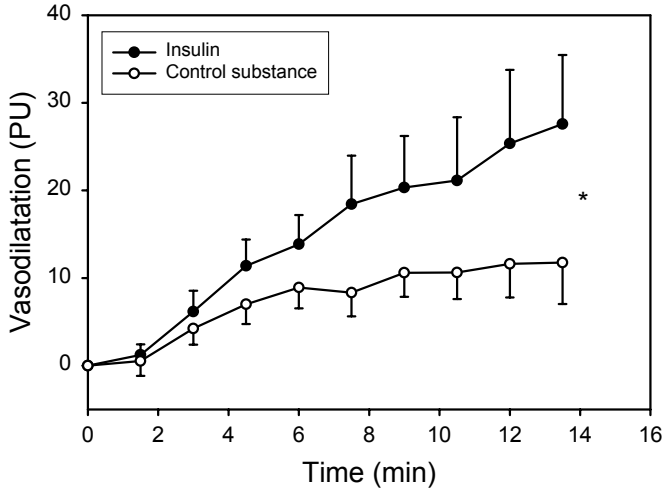

Figure 4. Normalized skin blood flow (PU) during iontophoresis of insulin (closed circles) and a control substance (open circles). *P<0.05 versus control study. The results are expressed as means±SEM.

Discussion

Recent studies[4-8] suggest that insulin-mediated changes in muscle perfusion can modulate insulin-mediated glucose uptake. However, the putative effects of insulin on the microcirculation which may permit such modulation have not been studied. To address this issue, we have examined the effects of systemic physiological hyperinsulinaemia on skin microcirculatory function, the only site available in humans to directly and non-invasively assess microcirculatory dynamics. In addition, the effects of locally administered insulin on skin blood flow were assessed. We report four novel observations. First, hyperinsulinaemia induces recruitment of capillaries in skin. Second, the vasodilatation of skin microcirculation induced by both acetylcholine, an endothelium-dependent vasodilator, and sodium nitroprusside, an endothelium-independent vasodilator, is augmented by hyperinsulinaemia. Third, hyperinsulinaemia influences vasomotion, i.e. oscillations observed in skin microcirculatory blood flow. Specifically, the relative contribution to total skin blood flow of the spectral components thought to result from the heart rate frequency and from endothelial activity was increased by hyperinsulinaemia. Fourth, insulin induces an increase in microcirculatory blood flow, independent of its systemic actions.

Recently, the concept of cross talk between insulin's vascular and metabolic actions has emerged.[2-9] Specifically, it has been proposed that resting muscle is perfused in an intermittent fashion and that, during the time of non-perfusion, anaerobic metabolism prevails, whereas, when muscle is perfused, aerobic metabolism predominates.[8] At any single time point, blood flow and glucose uptake depend on the fraction of tissue that is in the aerobic mode. Insulin, by reducing precapillary arteriolar tone and/or altering arteriolar vasomotion, redirects blood flow from non-nutritive vessels to nutritive capillary beds with a resultant increase in the overall number of perfused capillaries, which at any given moment, would shift a higher percentage of muscle tissue toward aerobic metabolism and glucose uptake.[8]

Our data demonstrate that hyperinsulinaemia is indeed associated with recruitment of capillaries in human skin. This insulin-dependent capillary recruitment is associated with the number of capillaries recruited during post-occlusive reactive hyperaemia without insulin infusion ($r=0.71$; $P<0.05$), a measure of capillary recruitment which has been shown to be related to insulin-mediated whole body glucose uptake[10,11] and to be decreased in insulin-resistant hypertensive subjects.[11] Interestingly, during hyperinsulinaemia, the number of capillaries perfused during post-occlusive reactive hyperaemia was similar to the maximal number of capillaries that can be observed during venous congestion,[16] a method to expose a maximal number of non-perfused capillaries.[16,21] This suggests that hyperinsulinaemia facilitates recruitment of a maximal number of available capillaries.

Since the intermittent character of capillary flow and capillary recruitment seem, in part, controlled by precapillary vasodilatation and vasomotion,[22] it is of interest to assess the effects of insulin on these variables. In the present study, the increase in baseline skin microcirculatory blood flow during hyperinsulinaemia, in parallel with that in total leg blood flow, was significantly different from the decrease in baseline skin microcirculatory blood flow observed during the control study. Therefore,

consistent with most[23,24] but not all[25] studies, hyperinsulinaemia induced vasodilatation at the microcirculatory level in skin. Moreover, hyperinsulinaemia increased the vasodilatation of skin microcirculation induced by both acetylcholine, an endothelium-dependent vasodilator, and by sodium nitroprusside, an endothelium-independent vasodilator, whereas the nonspecific microcirculatory reactivity to the drug vehicles was unaltered. The insulin-induced augmentation of endothelium-dependent vasodilatation in skin microcirculation is in agreement with similar findings at the level of the resistance vessels, primarily in muscle,[26] but the increase in endothelium-independent vasodilatation is not.[26] Nevertheless, the increase in the effects of sodium nitroprusside, a nitric oxide donor, is consistent with recent findings showing that insulin increases the nitric-oxide-stimulated production of cyclic guanosine monophosphate in vascular smooth muscle cells, regardless of whether the nitric oxide was derived from vascular smooth muscle cells inducible nitric oxide synthase or from an exogenous source.[27] Cyclic guanosine monophosphate attenuates the contractile agonist-induced intracellular calcium transient in vascular smooth muscle cells and affects contractile protein function, thereby decreasing vascular smooth muscle cell contraction[27] and favouring vasodilatation. How these changes relate to capillary recruitment, blood flow redistribution and glucose uptake remains to be determined.

Systemic hyperinsulinaemia also influenced the rhythmic fluctuations, so-called vasomotion, of skin microcirculatory blood flow. Compared to basal skin microcirculatory blood flow, systemic hyperinsulinaemia increased the total spectral energy density of the blood flow signal, the relative energy contributions in the interval from 0.01 to 0.02 Hz, in which local endothelial activity is thought to be manifested,[12] and the relative energy contributions in the interval from 0.4 to 1.6 Hz, which is synchronous with the heart rate.[12] The increased relative contribution of the spectral components resulting from the heart rate, a central control mechanism of microcirculatory flow, is consistent with microcirculatory vasodilatation which facilitates the transmission of a more upstream signal to the microcirculation. The increased relative contribution of local endothelial activity to the total peripheral blood flow may imply that insulin modulates microvascular flow locally via an endothelium-dependent mechanism. How these changes in vasomotion relate to capillary recruitment or blood flow redistribution also remains to be determined.

It should be recognized that the intermittent character of capillary flow and capillary flow distribution is determined not only by the precapillary arteriolar network but also by the characteristics of the capillary network itself.[28,29] This may explain why experimental studies demonstrate that insulin-mediated glucose uptake in skeletal muscle can be influenced by changes in capillary perfusion, even if total flow to the muscle remains constant.[5-7]

Since part of the observed effects of systemic hyperinsulinaemia on skin microcirculatory function may be secondary to other systemic effects of insulin,[1,2] we assessed whether insulin was able to influence skin blood flow directly. Whereas iontophoresis of insulin did not induce systemic effects, it did induce a rapid increase in skin microcirculatory blood flow. Nevertheless, it may not be obvious that insulin, applied topically through iontophoresis, penetrates the skin and evokes a blood flow response. The charge of regular insulin is a function of the pH. Above its isoelectric

point (pKa 5.3) regular insulin is negatively charged.[20] The pH of the diluting medium for soluble insulin is approximately 7.4. The actual range of pHs encountered as a substance progresses into the human skin is from pH 5.4 - 7.3.[20,30] Therefore, regular insulin is predominantly negatively charged in skin, and can be delivered by cathodal iontophoresis. A recent study in humans using cathodal iontophoresis demonstrated that insulin iontophoresis does influence skin blood flow,[18] but no definite conclusions could be reached because a control study was not performed. In the present study, the vascular response to iontophoresed insulin was significantly different from that caused by an appropriate control substance, suggesting that insulin was able to penetrate the skin and reach the microcirculation. Whether this vascular response is due to an insulin-receptor-mediated action is unknown. Nevertheless, the 48% increase in skin microcirculatory blood flow is comparable in magnitude to the increase in total limb blood flow observed during systemic hyperinsulinaemia.[9]

Although muscle is considered the main peripheral site of insulin-mediated glucose uptake[31] and insulin-mediated vasodilatation,[32] comparable metabolic[33,34] and vascular effects[23,24,35] of systemic insulin infusion can be demonstrated in skin. In patients with diabetes, it has been demonstrated that, during systemic insulin administration, in parallel with metabolic improvement, there is a redistribution of skin blood flow which favours the nutritive microcirculation.[23,35] However, these studies[23,35] lacked a control group and were, therefore, not able to discriminate between direct effects on skin blood flow of insulin and of improved diabetic control. Skin microvascular vasodilator capacity is also associated with both insulin's metabolic[10,11,36,37] and vascular actions[11] in skeletal muscle. Moreover, influences on skin blood flow have been described during systemic infusion of C-peptide.[38] Therefore, it appears reasonable to investigate skin to learn more about the potential effects of insulin on the microcirculation. Nevertheless, it should be stated that findings concerning skin microcirculation in humans should be extrapolated to muscle with caution.

In summary, our data provide the first direct evidence for insulin-dependent capillary recruitment in human skin. In addition, systemic physiological hyperinsulinaemia induces, in parallel with the increase in total leg blood flow (mainly muscle), an increase in total skin microcirculatory blood flow, and augments nitric oxide-mediated vasodilatation in skin microcirculation. It increases the relative contribution of local endothelial activity to skin microcirculatory flow. Moreover, insulin, locally administered and independent of its systemic actions, induces vasodilatation in skin microcirculation. These findings offer a potential physiological framework for further study of the functional coupling between insulin's metabolic and vascular actions.

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4

Cigarette smoking is associated with an acute impairment of microvascular function in humans

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Abstract

Background Microvascular function has been proposed as a possible mechanism explaining the association of acute smoking with an increased blood pressure and a decreased insulin sensitivity. However, the effects of smoking on microvascular function have not been studied. We have investigated the acute effects of smoking on microvascular function in 12 healthy smokers.

Methods Before and after smoking, we measured heart rate; blood pressure; capillary recruitment during peak reactive hyperaemia; and endothelium-(in)dependent vasodilatation of skin microcirculation with iontophoresis of acetylcholine and sodium nitroprusside combined with laser Doppler fluxmetry. To exclude non-specific changes, a control study with sham smoking was performed. The smoking and sham smoking studies were conducted in a randomised order.

Results Compared to sham smoking, acute smoking caused an increase in heart rate (9.3±4.1 vs. -1.3±3.0 bpm; P<0.001) and systolic blood pressure (6.3±8.8 vs. 0.8±4.4 mmHg; P<0.05); a decrease in absolute and relative capillary recruitment during peak reactive hyperaemia (-4.9±6.9 vs. 0.8±2.1 per mm2, P=0.01, and -13.8±21.4 vs. 1.9±6.9%, P=0.02, respectively); a decrease in the absolute and relative vasodilatation caused by acetylcholine (-62.4±47.7 vs. -30.8±32.6 PU (perfusion units), P=0.04, and -147±163 vs. 32±225%, P=0.07, respectively). The absolute and relative vasodilatation caused by sodium nitroprusside were not affected (-31.6±58.5 vs. -8.4±44.0 PU, P=0.3, and -50.2±219.0 vs. -17.1±139%, P=0.7, respectively).

Conclusions Acute smoking is associated with an impaired capillary recruitment during peak reactive hyperaemia and an impaired microvascular endothelium-dependent vasodilatation. These findings may explain the increased blood pressure and decreased insulin sensitivity that have been observed after acute smoking.

Introduction

One of the major risk factors for cardiovascular disease is cigarette smoking.[1;2] Cigarette smoking is associated with an acute increase in arterial wall stiffness[3;4] and an immediate endothelial dysfunction of the large arteries,[5;6] which are recognized to be important early phenomena in the pathogenesis of atherosclerosis. In addition, cigarette smoking is associated with an acute increase in blood pressure.[7-10] The mechanism behind this relationship is unclear. It has been proposed that the increased blood pressure is caused by an impaired microvascular function, which may increase vascular resistance.[11] In addition, an impaired microvascular function may also reduce insulin action and explain the decrease in insulin sensitivity that has been observed after smoking a cigarette.[7;12] In support of this concept, we have previously shown that microvascular function, measured as capillary recruitment during post-occlusive reactive hyperaemia and acetylcholine-mediated vasodilatation in skin, is related to blood pressure[13]and insulin sensitivity.[14] However, the effects of smoking on these microvascular functions have not been reported.

To determine whether smoking induces microvascular dysfunction, we assessed the acute effects of smoking a cigarette on capillary recruitment during post-occlusive reactive hyperaemia as well as on skin microvascular reactivity to acetylcholine and sodium nitroprusside.

Methods

Subjects

Twelve healthy smokers (average age: 26±6.2 years, range 19-37 years) were included in this study. All subjects were healthy as judged by medical history and non-diabetic according to ADA criteria.[15] The daily intake of cigarettes ranged from 1 to 23 (mean: 12±7). They were normotensive as determined by triplicate office blood pressure measurement and did not use medication. Characteristics of the study subjects are given in Table 1. The study protocol was approved by the local Ethics Committee and conformed with the principles outlined in the Declaration of Helsinki. Informed consent was obtained from each subject.

Study design

The measurements were conducted in a quiet, temperature-controlled room (T=23.4±0.4ºC) at 8.00 AM, after a 10-hour fast, with the subjects in the sitting position. All subjects abstained from caffeine- and alcohol-containing drinks overnight, and had to refrain from smoking for at least 6 h before examination. All microcirculatory measurements were performed with the investigated hand at heart level. Measurements were obtained after allowing 20 min of rest and acclimatization.

Table 1. Characteristics of the subjects

n (male/female)	12 (9/3)
Age (years)	26±6.2
Waist-to-hip-ratio	0.83±0.06
Body mass index (kg/m^2)	22.6±2.9
Fasting plasma glucose (mmol/l)	4.9±0.8
Fasting serum total cholesterol (mmol/l)	4.6±0.8
Fasting HDL-cholesterol (mmol/l)	1.5±0.4

The acute effects of smoking were compared to the effects sham smoking. The study design was randomised, with two sessions. These were performed on separate days within two weeks. After 20 minutes of rest, haemodynamic and microvascular measurements were obtained. The participants were then asked to smoke a cigarette of a filter type (containing ~1.0 mg nicotine and ~15 mg tar) or simulate smoking with a drinking straw with a filter (sham smoking). Subjects were instructed to take a puff of 5 seconds every 15 seconds, and the whole cigarette had to be smoked within 5 minutes. After (sham) smoking, microvascular measurements were repeated. In addition, blood pressure and heart rate were determined before and after smoking and sham smoking.

Haemodynamic measurements

Systolic blood pressure, diastolic blood pressure, and heart rate were determined with an automatic device (Colin Press-Mate BP-8800, Colin, Japan). Measurements were performed before (sham) smoking and from 20-30 min after (sham) smoking. The average of three measurements during each period was calculated. The interval between the three consecutive measurements was 5 min.

Assessment of capillary recruitment

The capillaroscopy studies were conducted before and 30 min after (sham) smoking. Nailfold capillaries in the dorsal skin of the third finger of the left hand were visualized by an epi-illuminated microscope as described previously.[13;14;16] Capillaries were visualized in a standardized manner, making it possible to visualize the same field in the smoking and sham smoking studies. Capillaries were visualized approximately 1.5 mm proximal to the terminal row of capillaries. One visual field of 1 mm^2 was recorded before and after four minutes of arterial occlusion and the images were stored on videotape. The number of capillaries was counted off-line by an experienced investigator (RGIJ) from a freeze-framed reproduction of the videotape and from the running videotape when it was uncertain whether a capillary was present or not. The investigator counting the capillaries was unaware of whether the videotapes were from the smoking or the sham smoking experiment. Capillary density was defined as the number of erythrocyte-perfused capillaries per square millimeter of nailfold skin. Post-occlusive reactive hyperaemia (PRH) after 4 minutes of arterial occlusion with a digital

cuff was used to assess functional recruitment of capillaries.[13;14;16] The number of capillaries in the resting state was counted during a 15-second period, only counting continuously perfused capillaries, as previously described.[17] Directly after release of the cuff, the number of perfused capillaries was counted. Percentage capillary recruitment during PRH was assessed by dividing the increase in capillary density during PRH by the capillary density in the resting state. Intrasubject coefficient of variation was 17.2±12.1% (measured on two occasions in 7 subjects).

Assessment of endothelium-(in)dependent vasodilatation

Endothelium-(in)dependent vasodilatation of the skin microcirculation was evaluated by iontophoresis of acetylcholine and sodium nitroprusside in combination with laser Doppler fluxmetry[13;14] before (sham) smoking and 15 and 35 minutes after (sham) smoking, respectively. A protocol of multiple fixed doses (current intensity X delivery time) was employed resulting in an incremental dose-response curve. Skin temperature was monitored. Acetylcholine (1%; Miochol, Bournonville Pharma, The Netherlands) was delivered using an anodal current; 7 doses (0.1 milliamps (mA) for 20 s) were delivered, with a 60-s interval between each dose. Sodium nitroprusside (0.1%; Nipride, Roche, The Netherlands) was delivered using a cathodal current; 9 doses (0.2 mA for 20 s) were delivered, with a 90-s interval between each dose. Acetylcholine-dependent laser Doppler flux was measured before and after (sham) smoking on the right hand on dorsal skin of the middle phalanx of the third and second finger, respectively. Nitroprusside-dependent laser Doppler flux was measured before and after (sham) smoking on the left hand on the middle phalanx of the fourth and second finger, respectively. Approximately 5 minutes elapsed between the acetylcholine and nitroprusside measurements. During the smoking and sham smoking studies, the same fingers were used for each measurement. Intrasubject coefficients of variation of the percentage increase from baseline to the plateau phase (final two iontophoretic deliveries) was 13.5±7.7% for acetylcholine and 18.7±23.4% for sodium nitroprusside (measured on two occasions in 7 subjects).

Statistical analysis

Data are expressed as mean ± SD, unless stated otherwise. Comparison of haemodynamic variables and microvascular measurements before and after (sham) smoking was performed with ANOVA for repeated measures. To investigate whether the influence of smoking was significantly different from the influence of sham smoking, the interaction between the factors smoking and time was used in ANOVA for repeated measures. Pearson's correlation was used to investigate the association between changes in microvascular function and the number of cigarettes smoked per day. A two-tailed P-value of < 0.05 was considered significant. All analyses were performed using the statistical software package SPSS version 9.0.

Results

Haemodynamic variables

Table 2 shows the haemodynamic variables before and after smoking and sham smoking. Heart rate was significantly increased after smoking (P<0.001); this increase was significantly different from the change in the sham smoking study (9.3±4.1 vs. -1.3±3.0 bpm; P<0.001). Systolic blood pressure was also significantly increased after smoking (P<0.05); this increase was significantly different from the change in the sham smoking study (6.3±8.8 vs. 0.8±4.4 mmHg; P<0.05). Diastolic blood pressure was neither influenced by smoking nor by sham smoking.

Table 2. Metabolic and haemodynamic variables before and after smoking and sham smoking

	Smoking		Sham smoking	
Variable	Before	After	Before	After
Heart rate (bpm)	66.6±7.0	75.9±7.5*†	65.2±7.5	63.9±6.7
Systolic blood pressure (mmHg)	114.7±10.1	120.9±12.4*†	115.6±7.4	114.8±8.0
Diastolic blood pressure (mmHg)	61.8±7.2	64.8±7.2	63.1±6.8	62.7±4.6

Values are expressed as means±SD; bpm = beats per minute
*P<0.05: before vs. after; †P<0.05: change during smoking study vs. change during sham smoking study.

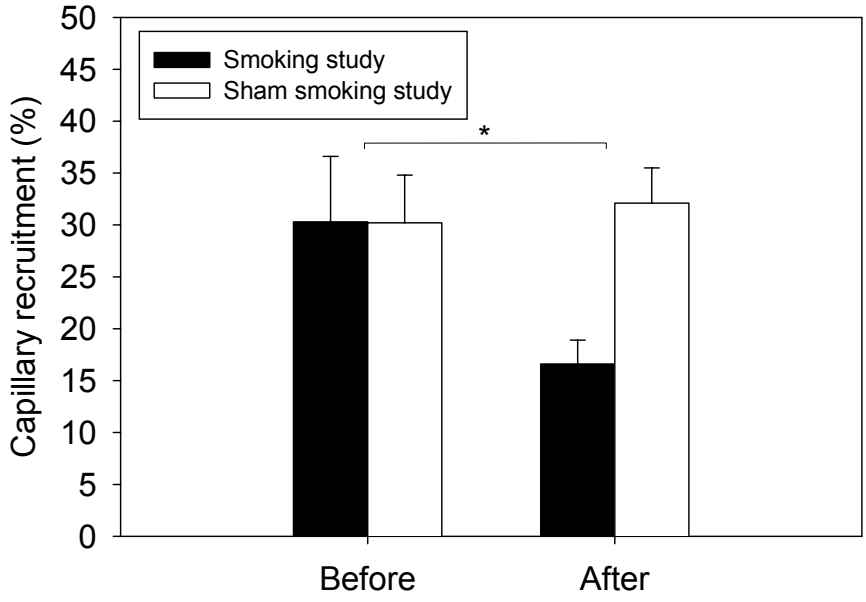

Figure 1. Capillary recruitment during peak reactive hyperaemia before and after smoking and sham smoking. *P<0.05: change during smoking study vs. change during sham smoking study. The results are expressed as means±SEM.

Table 3. Microvascular function before and after smoking and sham smoking

	Smoking		Sham smoking	
Variable	Before	After	Before	After
Capillary recruitment				
Baseline density (per mm^2)	41.1±11.4	41.7±10.4	40.9±8.8	41.0±9.9
Peak density (per mm^2)	52.8±13.8	48.5±12.0*§	53.5±14.2	54.4±15.0
Absolute increase (per mm^2)	11.8±7.7	6.8±3.7*§	12.6±7.6	13.4±6.7
Cap. recruitment (%)	30.3±21.8	16.6±8.1*§	30.2±16.1	32.1±11.7
ACh-mediated vasodilatation				
Skin temperature (ºC)	31.0±0.8	29.5±1.1†	30.8±0.7	30.2±1.8
Baseline perfusion (PU)	31.5±12.6	21.4±11.7*	33.8±13.7	24.4±11.7†
Plateau perfusion (PU)	140.9±55.3	68.2±59.8†	162.2±57.9	121.9±63.7†
Absolute increase (PU)	109.4±50.0	47.0±52.9†§	128.4±55.8	97.6±60.6†
ACh-vasodilatation (%)	368±157	221±203†	419±165	450±275
SNP-mediated vasodilatation				
Skin temperature (ºC)	31.4±1.6	30.0±1.5†§	31.3±1.1	30.8±1.7*
Baseline perfusion (PU)	29.6±7.7	21.5±9.6†§	23.5±5.3	23.9±7.6
Plateau perfusion (PU)	120.2±52.9	80.4±64.1†	88.4±40.8	80.4±42.7
Absolute increase (PU)	90.6±50.2	59.0±56.9†	64.9±39.1	56.5±41.4
SNP-vasodilatation (%)	313±181	263±162	277±156	260±201

Values are expressed as means±SD; PRH indicates post-occlusive reactive hyperaemia; Cap, capillary; ACh, acetylcholine; SNP, sodium nitroprusside

*P<0.05, and †P<0.01: before vs. after; §P<0.05: change during smoking study vs. change during sham smoking study.

Capillary recruitment

Capillaroscopy data are shown in Table 3 and figure 1. Capillary density in the resting state was neither influenced by smoking nor by sham smoking. However, the absolute and relative capillary recruitment were decreased after smoking (P<0.05 vs. baseline). During the sham smoking study, no changes were observed. The observed decrease in the absolute and relative capillary recruitment during the smoking study were significantly different from the changes observed during the sham smoking study (-4.9±6.9 vs. 0.8±2.1 per mm^2, P=0.01, and -13.8±21.4 vs. 1.9±6.9%, P=0.02, respectively). The effect of smoking a cigarette on capillary recruitment was not related to the average number of cigarettes smoked per day (r=0.24, P=0.5).

Endothelium-(in)dependent vasodilatation of skin microcirculation.

Results are shown in Table 3 and figure 2. Smoking significantly diminished the absolute and relative vasodilatation induced by acetylcholine (109.4±50.0 vs. 47.0±52.9 PU, P<0.01, and 368±157 vs. 221±203%, P<0.01, respectively). The significant reduction in the absolute and the relative increase after iontophoresis of acetylcholine during the smoking study were different from the changes observed during the sham smoking study (-62.4±47.7 vs. -30.8±32.6 PU, P=0.04, and -147±163 vs. 32±225%, P=0.07, respectively). The effect of smoking a cigarette on the absolute and the relative

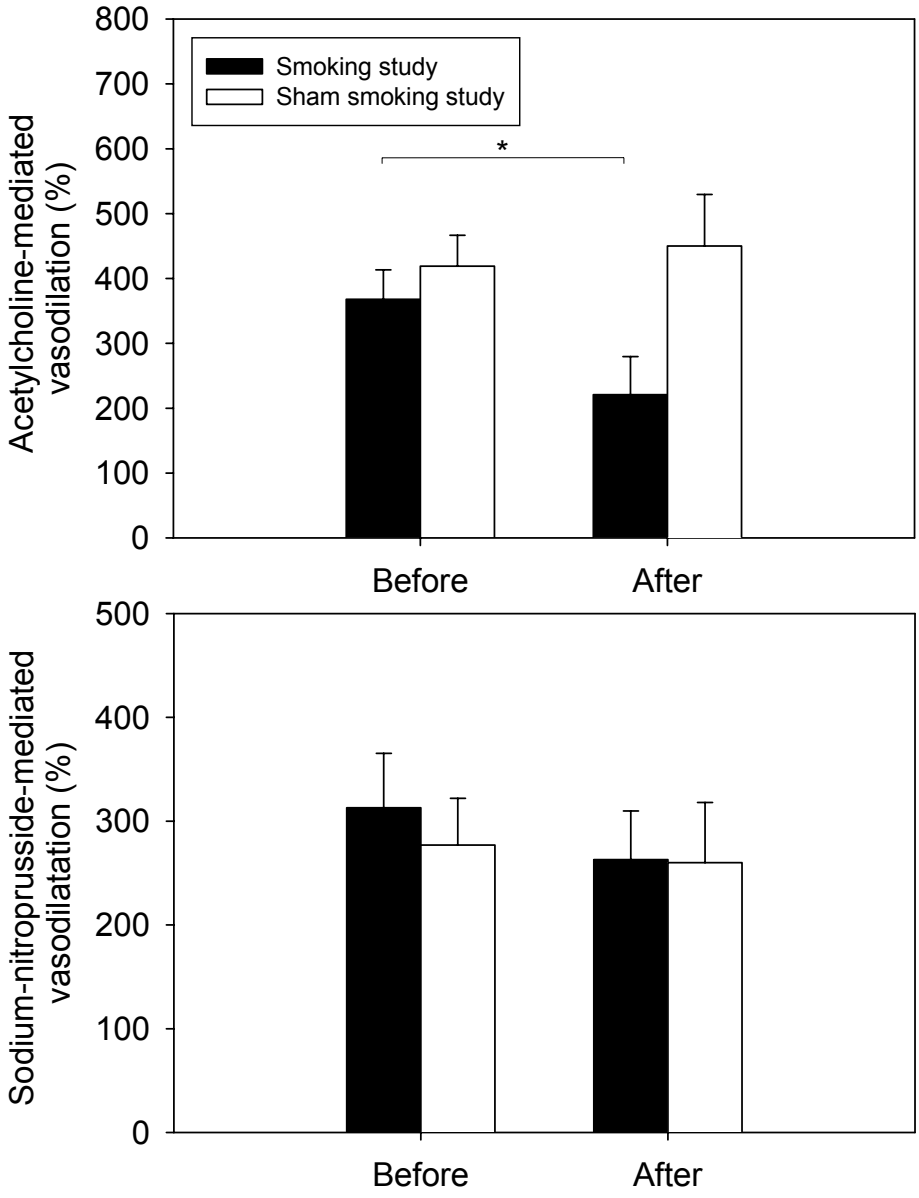

Figure 2. Acetylcholine and sodium nitroprusside mediated vasodilatation before and after smoking and sham smoking.
*P<0.05: before smoking vs. after smoking. The results are expressed as means±SEM.

increase after iontophoresis of acetylcholine was not related to the average number of cigarettes smoked per day (r=-0.01, P=1.0, and r=-0.16, P=0.6).

Smoking did not impair the vasodilatation induced by sodium nitroprusside. The changes in the absolute and the relative increase after iontophoresis of sodium nitroprusside during the smoking study were not different from the changes observed

during the sham smoking study (-31.6±58.5 vs. -8.4±44.0 PU, P=0.3 and -50.2±219.0 vs. -17.1±139%, P=0.7, respectively).

The decrease in skin temperature during the smoking study was larger than the decrease in skin temperature observed during the sham smoking study (-1.4±1.4 vs. -0.6±1.5 ºC, P=0.08, for the baseline measurements preceding the acetylcholine procedure, and -1.4±0.9 vs. -0.6±0.8 ºC, P=0.02, for the baseline measurements preceding the sodium nitroprusside procedure).

Discussion

We have examined the acute effects of smoking on skin microcirculatory function, the only site available in humans to directly and non-invasively assess microcirculatory dynamics. Acute smoking was associated with an impaired recruitment of capillaries and an impaired microvascular endothelium-dependent vasodilatation, whereas the endothelium-independent vasodilation was not influenced. This is in accordance with experimental studies that have demonstrated detrimental effects of cigarette smoke on the microcirculation.[18;19] Our findings are also in accordance with a study by Tur et al., who demonstrated a lower peak and a slower recovery in skin laser Doppler blood flow during peak reactive hyperaemia after smoking in humans.[20] However, capillary recruitment and microvascular endothelium-dependent vasodilatation were not measured, and a control experiment with sham smoking was not performed.

One could argue that the decreased microvascular function after smoking is due to the observed decrease in skin temperature. However, we find this unlikely, because the microvascular dilatation after the iontophoresis of sodium nitroprusside was not affected by smoking. Therefore, the decreased skin temperature may be the consequence, not the cause, of the decreased microvascular function.

The present study does not clarify the mechanism responsible for the smoking induced decrease in microvascular vasodilatation, but a role for nicotine or oxygen free radicals has been suggested. It has been demonstrated that administration of nicotine is associated with an acute impairment of vascular function in experimental studies[21;22] and in human veins.[23] Nicotine may cause sympathetic activation and, in addition, inhibit the activity of nitric oxide synthase,[22] which may explain our finding of an impaired endothelium-dependent vasodilatation. As an alternative, recent clinical[24] and experimental[25] studies suggest a role for oxygen-derived free radicals. Cigarette smoke contains large amounts of free radicals,[26;27] which may injure the endothelium. This hypothesis is supported by studies demonstrating that the antioxidants vitamin C and vitamin E can attenuate the acute impairment of endothelium-dependent vasodilatation in the brachial artery.[6;28] Whether these findings can be extrapolated to the microvascular function remains to be established.

In our study, acute smoking caused an increase in heart rate. The size of this effect is in accordance with other studies reporting the effect of acute smoking on heart rate.[5;6;29-31] The increase in systolic blood pressure after acute smoking is also in accordance with other studies.[7-10] In the present study, we did not measure insulin sensitivity. However, previous studies have demonstrated that acute cigarette smoking is associated with a decrease in insulin sensitivity.[7;12] This decreased insulin sensitivity after smoking may, in part, be caused by the effect of smoking on microvascular function.[11] Experimental[32;33] and human studies[34-36] suggest that a decreased microvascular function contributes to an increase in vascular resistance and antedates an increase in blood pressure. In addition, an impaired microvascular function has been suggested to reduce insulin sensitivity by decreasing the delivery of insulin and glucose.[11;14;37] Our finding of an acute effect of smoking on microvascular function may be relevant in understanding how smoking can increase blood pressure and decrease insulin sensitivity. However, it should be emphasized that other mechanisms, such as sympathetic activation[30] and increased stiffness of large vessels,[3;4] may also be involved in the increased blood pressure after smoking.

We did not measure microvascular function in an age- and sex-matched group of non-smokers. However, skin capillary recruitment, as well as endothelium-dependent and endothelium-independent vasodilation were diminished in the smokers compared with non-smokers that have been investigated in our previous studies. The finding of an impaired skin endothelium-dependent and endothelium-independent vasodilation in smokers is in accordance with a recent study in chronic smokers.[38]

In summary, our data provide the first direct evidence for a smoking induced acute impairment of capillary recruitment in humans. In addition, our data demonstrate that smoking induced an impaired microvascular endothelium-dependent vasodilatation, without an influence on microvascular endothelium-independent vasodilatation. These findings offer a potential explanation for the association of acute smoking with an increased blood pressure and a decreased insulin sensitivity.

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5

TNF-α levels are associated with skin capillary recruitment A potential explanation for the relationship between TNF-α and insulin resistance

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Abstract

Background The mechanism by which tumour necrosis factor α (TNF- α) may cause insulin resistance is not clear. On the basis of experiments in rats, TNF- α a has been suggested to cause defects in capillary function, with a decreased access of insulin and glucose to tissues. To test this hypothesis in humans, we assessed serum TNF- α concentrations, skin capillary recruitment and insulin sensitivity in a group of 37 healthy adults. In addition, we measured these variables in 21 of their prepubertal children.

Methods Serum TNF- α levels were measured by sandwich enzyme immuno-assay and insulin sensitivity was assessed with the hyperinsulinaemic euglycaemic clamp technique. Capillary recruitment during post-occlusive reactive hyperaemia was evaluated by videomicroscopy.

Results In the adults, serum TNF- α levels were associated with both capillary recruitment and insulin sensitivity ($r=-0.40$, $P<0.05$, and $r=-0.33$, $P=0.05$, respectively). In addition, capillary recruitment was associated with insulin sensitivity ($r=0.34$, $P<0.05$). Regression analysis showed that the association between TNF- α and insulin sensitivity (-0.527 mg/kg/min per pmol/l per pg/mL TNF- α [95%-CI: -1.066 to 0.011]; $P=0.05$) decreased by 30% after adjustment for capillary recruitment. In the children, neither capillary recruitment nor insulin sensitivity was significantly associated with TNF- α ($r=+0.33$, $P=0.4$, and $r=-0.24$, $P=0.2$, respectively).

Conclusions In adults, but not in children, serum TNF-α levels are associated with capillary recruitment during post-occlusive hyperaemia, which, in part, can explain the relationship between TNF-α and insulin resistance. Our data suggest that these relationships are initiated during growth from childhood to adulthood.

Introduction

The inflammatory cytokine tumour necrosis factor α (TNF-α), has been reported to play an important role in the insulin resistance of obesity, type 2 diabetes and sepsis.[1,2] Several studies have demonstrated that high levels of TNF-α mRNA and protein are associated with insulin resistance in adult animals and humans.[3-7] In addition, administration of TNF-α to animals can induce insulin resistance,[8,9] and, although neutralisation of TNF-α was not associated with an improved insulin sensitivity in patients with diabetes,[10] neutralisation of TNF-α can improve insulin sensitivity in animals.[3,11]

The mechanism by which TNF-α may cause insulin resistance is not clear. Studies in isolated cells have shown that TNF-α has direct effects on the insulin signalling cascade.[12,13] However, these effects could not be found in isolated skeletal muscle.[14] As an alternative, experiments in rats have suggested that TNF-α causes defects in microvascular function, in particular insulin-induced capillary recruitment, with a decreased access of insulin and glucose to tissues.[15]

An important role for microvascular function as a partial explanation for the demonstrated defects in the ability of insulin to increase glucose uptake, limb blood flow and blood volume in insulin-resistant states has been suggested by several animal and human studies.[16-20] In rats, insulin has been shown to cause capillary recruitment in hind-leg muscles,[16] and changes in insulin-induced capillary recruitment were associated with changes in insulin-mediated glucose uptake.[17] In humans, we have recently demonstrated that insulin infusion induced capillary recruitment in human skin, and this insulin-induced capillary recruitment was associated with the degree of capillary recruitment during post-occlusive hyperaemia.[21] Capillary recruitment after post-occlusive reactive hyperaemia, as well as microvascular endothelium-dependent vasodilation in skin, have been shown to be associated with insulin's metabolic and vascular actions in both normal and hypertensive individuals.[22,23]

Therefore, elevated levels of TNF-α may be associated with impaired microvascular function in humans, which may decrease the delivery of glucose and insulin to skeletal muscle, and play a role in the link between TNF-α and insulin resistance. To test this hypothesis, we assessed serum TNF-α concentrations, microvascular function (i.e. skin capillary recruitment during post-occlusive reactive hyperaemia, and endothelium-(in)dependent vasodilation) and insulin sensitivity in a group of 37 healthy adult individuals. Growth may play an important role in the development of insulin sensitivity and its determinants.[24] Therefore, to investigate whether the associations among TNF-α concentrations, capillary recruitment and insulin resistance are already present in prepubertal children, we measured these variables in 21 of their children.

Methods

Subjects

This study is part of a larger ongoing project in which vascular and metabolic variables were studied in prepubertal children[25] and their parents. They were recruited from the same catchment area and the children had been born at the VU University Medical Center in Amsterdam. Families still living at the same address were contacted by letter and phone. All family members were of Caucasian origin. The current report investigates the relationships among TNF-α, skin capillary recruitment during post-occlusive reactive hyperaemia and insulin sensitivity. After the exclusion of subjects with non–insulin-dependent diabetes mellitus, recent vaccinations and rheumatoid arthritis, 37 healthy adult individuals and 21 children participated in this study. Characteristics of the individuals are summarised in Table 1. The investigation conforms with the principles outlined in the Declaration of Helsinki. The study protocol was approved by the local ethical committee. Informed consent was obtained from each adult individual. For the children, written informed consent was obtained from both parents and verbal informed consent was obtained from the children.

Measurements

The microvascular measurements were conducted in the morning after 30 minutes of acclimatisation in a quiet, temperature-controlled room (T=23.4±0.4ºC), with the individuals in the sitting position and the investigated, non-dominant hand at heart level. All individuals had abstained from caffeine-containing drinks overnight. Nailfold and iontophoresis studies were performed on the same day. The microvascular and metabolic studies (see below) were carried out on separate days approximately one week apart, and performed by two different investigators (RGIJ and JJV, respectively). Both investigators were cross-blinded to the microvascular reactivity and insulin sensitivity results.

Perfused nailfold capillaries in the dorsal skin of the third finger were visualised by a capillary microscope, as described previously.[22,23,26] Two separate visual fields of 1 mm^2 were recorded before and after four minutes of arterial occlusion with a digital cuff, and the images were stored on videotape. Capillaries were visualised approximately 1.5 mm proximal to the terminal row of capillaries in the middle of the nailfold of the third finger. At this spot capillaries are located perpendicular to the skin and appear mostly as dots with only a small part of the arteriolar and venular limb being visible. The number of capillaries at baseline and directly after release of the cuff were counted off-line for respectively 15 and 30 seconds by a single experienced investigator (RGIJ) from a freeze-framed reproduction of the videotape and from the running videotape when it was uncertain whether a capillary was present or not. (The major part of the increase in capillary number occurs within a few seconds.) Capillary density was defined as the number of erythrocyte-perfused capillaries per square millimeter of

Table 1. Characteristics of the parents and the children

	parents	children
n (male/female)	37 (21/16)	21 (11/10)
Age (years)	44.5±4.9	8.6±1.2
Waist (cm)	87.8±12.2	57.9±8.8
Hip (cm)	100.8±7.8	70.3±8.6
Waist-to-hip ratio, men	0.80±0.06	
women	0.75±0.07	
children		0.82±0.04
Height (cm)	175.9±9.1	136.6±8.0
Weight (kg)	78.0±14.8	30.7±7.2
Body mass index (kg/m^2), men	26.0±2.6	
women	23.9±5.3	
children		16.3±2.9
Fat-free mass (kg)	58.3±11.5	22.9±4.1
24-h systolic blood pressure (mmHg)	122.7±15.9	111.4±11.9
24-h diastolic blood pressure (mmHg)	76.0±9.8	65.8±8.3
24-h heart rate (bpm)	61.2±11.2	81.4±8.5
Insulin sensitivity (mg/kg/min per pmol/l) x 100	1.70±1.17	3.6±1.9
Fasting plasma glucose (mmol/l)	4.8±0.7	4.6±0.2
Fasting plasma insulin (pmol/l)	45.0 (37.5-73.0)*	45.0 (35.5-64.5)*
Fasting serum total cholesterol (mmol/l)	5.3±1.0	3.9±0.6
Capillary recruitment during PRH		
Baseline capillary density (per mm^2)	45.1±11.1	39.8±6.6
Peak capillary density (per mm^2)	61.6±16.4†	52.5±11.7†
Capillary recruitment (%)	38±24	33±24
Acetylcholine-mediated vasodilation (%)	337±132	424±270
Sodium nitroprusside-mediated vasodilation (%)	298±198	319±208
TNF-α, pg/mL	1.97±0.74	5.1 (2.7-7.6)*

Data are presented as mean±SD, or as *median (25th-75th percentile),
†*P*<0.001 vs. baseline
PRH indicates post-occlusive reactive hyperaemia

nailfold skin. Post-occlusive reactive hyperaemia after 4 minutes of arterial occlusion with a digital cuff was used to assess functional recruitment of capillaries.[22,23,26] The number of capillaries in the resting state was counted during a 15-second period, only counting continuously perfused capillaries, as previously described.[27] Percentage capillary recruitment during post-occlusive reactive hyperaemia was assessed by dividing the increase in capillary density during post-occlusive reactive hyperaemia by the capillary density in the resting state. Intra-subject coefficient of variation (CV) was 17.2±12.1% (measured on two occasions in 7 individuals).

Endothelium-dependent and -independent vasodilatation of skin microcirculation was evaluated by iontophoresis of acetylcholine and sodium nitroprusside in combination with laser Doppler fluxmetry as previously described in more detail.[22,23] Acetylcholine dissolved in mannitol 3% in water (1% acetylcholine; Miochol, Bournonville Pharma, The Netherlands) was delivered using an anodal current; 7 doses (0.1 milliamps (mA) for 20 s) were delivered, with a 60-s interval between each dose.

Sodium nitroprusside dissolved in water for injection (0.1% sodium nitroprusside; Nipride, Roche, The Netherlands) was delivered using a cathodal current; 9 doses (0.2 mA for 20 s) were delivered, with a 90-s interval between each dose. Acetylcholine-dependent laser Doppler flux was measured on the middle phalanx of the third finger, whereas nitroprusside-dependent laser Doppler flux was measured on the middle phalanx of the fourth finger. Approximately 15 minutes elapsed between these two measurements. The responses to acetylcholine and sodium nitroprusside were calculated as the percentage increase from baseline to the plateau phase (during the final two iontophoretic deliveries). Intrasubject coefficients of variation (CV) of the percentage increase from baseline to the plateau phase (final two iontophoretic deliveries) was 13.5±7.7% for acetylcholine and 18.7±23.4% for sodium nitroprusside (measured on two occasions in 7 subjects).

Sensitivity to insulin-mediated glucose uptake was assessed by the hyperinsulinaemic, euglycaemic clamp technique, as described previously.[22] Briefly, insulin (Velosulin; Novo Nordisk, Bagsvaerd, Denmark) was infused in a primed continuous manner at a rate of 60 mU kg^{-1} $hour^{-1}$ for 2 h. Normoglycaemia was maintained by adjusting the rate of a 20% D-glucose infusion based on plasma glucose measurements performed at 5-min intervals. Whole body glucose uptake (M) was calculated from the glucose infusion rate during the last 60 minutes and expressed per unit of plasma insulin concentration (M/I).[28] Plasma insulin concentrations were measured by radio-immunoassay techniques (Immunoradiometric Assay, Medgenix Diagnostics, Fleurus, Belgium). For convenience the M/I ratio was multiplied by 100.

Anthropometric measurements (which included weight, height, waist circumference and hip circumference) were performed on all participants by one trained investigator (JJV) as described previously.[22] The body mass index was calculated by dividing weight in kilograms by height in meters squared. The waist-to-hip ratio was calculated as a measure of body fat distribution. Fat-free mass was measured using a 4-terminal bioimpedance analyzer (RJL Spectrum Bioelectrical Impedance, BIA 101/S Akern, RJL-System) and equations based on total body water.[29,30]

Human TNF-α was measured by sandwich enzyme immunoassay (Quantikine High Sensitivity, R&D Systems, Oxon, United Kingdom. The intra-assay and inter-assay coefficient of variation for TNF-α were 7.3 and 8.8%, respectively.

Statistical Methods

Variables are presented as mean ± standard deviation (SD), or, in case of a skewed distribution, as median and interquartile range (IQR). Partial correlation analyses were used to investigate the relationships among serum TNF-α, capillary recruitment, insulin sensitivity, and measures of obesity after adjustment for age and sex. Subsequently, a multiple regression analysis was used to analyse whether the association between TNF-α concentrations and insulin sensitivity remained when allowing for capillary

Table 2. Correlation analysis of TNF-α, capillary recruitment, insulin sensitivity and features of obesity in 37 adult individuals after adjustment for age and sex

	TNF-α		Insulin sensitivity	
	r	***P***	**r**	***P***
Insulin sensitivity	-0.33	0.05		
Capillary recruitment	-0.40	0.02	0.34	0.04
Waist-to-hip ratio	0.15	0.4	-0.53	<0.001
Body mass index	0.29	0.09	-0.63	<0.001

recruitment. Multiple regression analysis requires that the measurements be independent. Because our study of 37 adult individuals includes 16 couples, the restriction of independence may not be met and TNF-α levels may correlate positively among spouses. We, therefore, investigated the correlation of TNF-α in the fathers with that in the mothers. Higher TNF-α levels in the father were not related to higher TNF-α levels in the mother (r= –0.31, P=0.2). Therefore, in the subsequent analyses, the TNF-α measurements were treated as independent measurements. Interaction analyses were performed to investigate whether the associations were different between the adults and children. For the linear regression analyses in children, and in the combined population of adults and children, TNF-α levels were log-transformed to achieve a normal distribution. A two-tailed P-value of <0.05 was considered significant. All analyses were performed on a personal computer using the statistical software package SPSS version 9.0 (SPSS, Chicago, IL, USA).

Results

In the adults, the mean serum TNF-α concentration was 2.0±0.7 pg/mL (Table 1). In the children, TNF-α showed a skewed distribution. The median TNF-α concentration was 5.1 pg/mL (25^{th}-75^{th} percentile: 2.7-7.6 pg/mL). During the hyperinsulinaemic, euglycaemic clamp, glucose levels were maintained at 5.0±0.2 mmol/l. Attained serum free insulin concentrations averaged 523±145 pmol/L in the adults and 330±159 pmol/l in the children. The mean rate of glucose uptake, expressed per kg body weight (M-value), was 7.9±0.7 $mg.kg^{-1}.min^{-1}$ in the adults and 10.1±4.2 $mg.kg^{-1}.min^{-1}$ in the children. Table 1 shows the insulin sensitivity expressed as whole body glucose uptake per minute per kg body weight per unit of plasma insulin concentration (mg/kg/min per pmol/l).

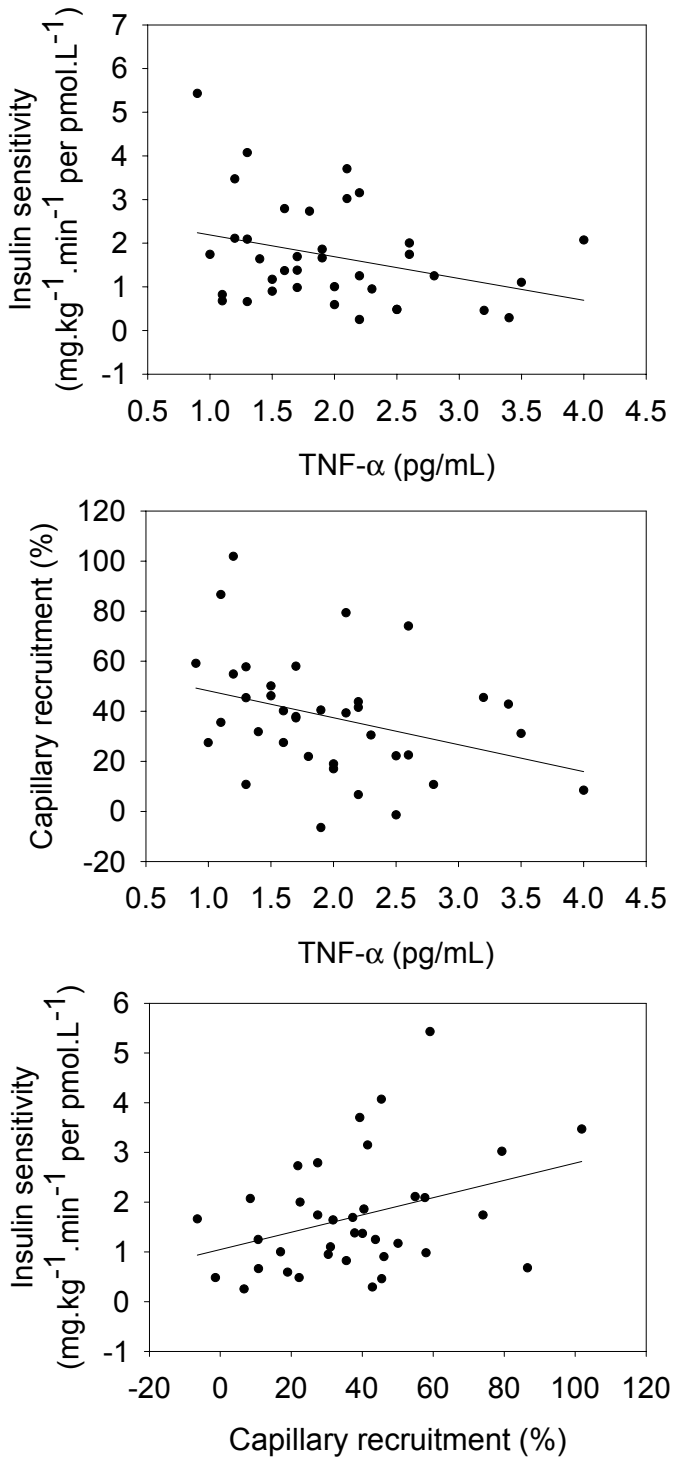

Figure 1. Correlations among TNF-α, insulin sensitivity and capillary recruitment. After adjustment for age and sex, the correlation coefficients were -0.33 (*P*=0.05) for the association between TNF-α and insulin sensitivity, -0.40 (*P*=0.02) for the association between TNF-α and capillary recruitment, and -0.34 (*P*=0.04) for the association between capillary recruitment and insulin sensitivity. Without adjustment for age and sex, the correlation coefficients were -0.32 (*P*=0.06), -0.37 (*P*=0.02) and -0.28 (*P*=0.09), respectively.

Table 3. Regression analysis with insulin sensitivity as dependent variable in 37 adult individuals

Model	B	95%-CI	Standardized Beta	P-value
1.				
TNF-α (per pg/mL)	-0.527	-1.066 to 0.011	-0.33	0.05
2.				
TNF-α (per pg/mL)	-0.367	-0.946 to 0.212	-0.27	0.2
Capillary recruitment (per %)	1.282	-0.564 to 3.128	-0.16	0.2

Both model 1 and 2 are adjusted for age and sex. The R^2 of models 1 and 2 were 0.12 and 0.14, respectively.

Correlation and linear regression analyses

In the adults, the correlation coefficients were adjusted for age and sex. Resting capillary density was not related to TNF-α or insulin sensitivity (r=-0.05, *P*=0.8 and r=-0.06, *P*=0.7, respectively). However, TNF-α concentrations were associated with both capillary recruitment and insulin sensitivity (Table 2 and figure 1). Capillary recruitment was associated with insulin sensitivity (Table 2 and figure 1). TNF-α was not associated with waist-to-hip ratio (Table 2). TNF-α was positively associated with body mass index (*P*=0.09; Table 2). Subsequently, we investigated whether the association between TNF-α and insulin sensitivity could be explained by capillary recruitment (Table 3). Model 1 shows that TNF-α (independent variable), after adjustment for age and sex, was related to insulin sensitivity (dependent variable). In addition, after adjustment for age and sex, an increase in TNF-α of 1 pg/mL was associated with a decrease in capillary recruitment of 13 percent points (95%-CI: -23 to -2; *P*<0.05). This association was similar after adjustment for body mass index (11 percent points per pg/mL TNF-α [95%-CI: -21 to -0.1]; *P*<0.05). Model 2 shows that, after adjustment for capillary recruitment, the regression coefficient of the association between TNF-α and insulin sensitivity decreased by 30% and was no longer statistically significant (Table 3). This suggests that, at least statistically, capillary recruitment may partly explain the relationship between TNF-α and insulin sensitivity. After additional adjustment for body mass index, the association between TNF-α and insulin sensitivity was further reduced (-0.183 mg/kg/min per pmol/l per pg/mL TNF-α [95%-CI: -0.678 to 0.313]; P=0.5).

In the children, TNF-α was not significantly associated with capillary recruitment, insulin sensitivity or measures of obesity (Table 4). If anything, the association of TNF-α with capillary recruitment and body mass index was opposite to that in the parents. Interaction analyses suggested that the associations of TNF-α with capillary recruitment (*P*=0.01) and body mass index (*P*=0.09) were different between adults and children. The association of TNF-α with insulin sensitivity was not clearly different between adults and children (*P*=0.3)

Table 4. Correlation analysis of TNF-α, capillary recruitment, insulin sensitivity and features of obesity in 21 children

	TNF-α		Insulin sensitivity	
	r	*P*	r	*P*
Insulin sensitivity	-0.24	0.4		
Capillary recruitment	0.33	0.2	-0.02	0.9
Waist-to-hip ratio	0.06	0.8	-0.24	0.3
Body mass index	-0.26	0.3	-0.45	0.06

TNF-α was not significantly associated with acetylcholine-mediated vasodilation or nitroprusside-mediated vasodilation in the adults (r=-0.06, *P*=0.7 and r=0.01, *P*=0.9), nor in the children (r=0.07, *P*=0.8 and r=0.26, *P*=0.3, respectively).

Skin temperature was not different between children and adults (30.81±1.0 vs. 31.15±0.9, p=0.2). Similar conclusions were reached when statistical analyses were performed using different indices of insulin sensitivity (M-value, M/I-value or M/I-value per kilogram fat-free mass). The same held true for the absolute versus the percentage increase of capillary density. The associations were not significantly different between men and women, nor between boys and girls (data not shown).

Discussion

The central novel finding of the present study is the inverse association between TNF-α and capillary recruitment in adults. Importantly, our results confirm the inverse relationship of TNF-α with insulin sensitivity[6,31,32] as well as the positive relationship of insulin sensitivity with capillary recruitment.[22,23] Subsequent regression analyses were consistent with the hypothesis that the relationship between TNF-α and insulin sensitivity in adults might be explained, at least in part, by capillary recruitment.

To our knowledge, this is the first study to report on the associations of TNF-α with microvascular function, insulin sensitivity and measures of obesity in children. These associations were less clear or in the opposite direction to those in their parents. The association between TNF-α and insulin sensitivity was not clearly different between adults and children. However, the association between TNF-α and capillary recruitment in children was in the opposite direction and significantly different from that in adults, which suggests that, in contrast to adults, microvascular mechanisms do not play a role in the TNF-α-insulin sensitivity relationship in children. In addition, the association between TNF-α and body mass index in adults was clearly different from that in children. Taken together, our cross-sectional findings in two different age groups suggest, therefore, that the relationships among TNF-α, vascular function, insulin sensitivity and obesity are initiated during growth from childhood to adulthood.

Interestingly, the initiation of relationships of certain variables with haemodynamic phenomena during growth has also been observed in investigations of the fetal origins

hypothesis. For example, the association of birth weight with blood pressure and insulin resistance is weak or absent in children and much stronger in adults.[33-38] In addition, puberty is an important contributor to the development of type 2 diabetes and cardiovascular risk,[24] whereas Elhadd et al. have recently shown that puberty modulates microvascular endothelial function and antioxidant mechanisms in childhood diabetes.[39] Our finding that the relationships among TNF-α, vascular function, insulin sensitivity and obesity are initiated during growth from childhood to adulthood fits with this concept.

The mechanism explaining the association between TNF-α and insulin resistance is not clear. Although studies in isolated cells have shown that TNF-α has direct effects on the insulin signalling cascade,[12,13] it has also been suggested that TNF-α causes defects in capillary function, with a decreased access of nutrients to tissues. In the present study, capillary recruitment was related to both TNF-α and insulin sensitivity. Moreover, it statistically explained part of the association between TNF-α and insulin sensitivity. These cross-sectional findings in humans are consistent with the experimental data of Youd et al. in rats,[15] who demonstrated that infusion of TNF-α impaired the insulin-induced capillary recruitment and glucose uptake in skeletal muscle.[15] Taken together, these data suggest that the association between TNF-α and insulin resistance is in part explained by vascular mechanisms and in part by direct effects of TNF-α on the insulin signalling cascade in muscle cells.

Levels of TNF-α as measured in the circulation are low and it is not known whether circulating TNF-α levels are biologically active. Nevertheless, several studies have shown an inverse relationship of plasma levels of TNF-α with insulin sensitivity,[6,31,32] a concept further supported by studies demonstrating associations between TNF-α mRNA and insulin resistance,[40,41] and by animal studies demonstrating that administration and/or neutralisation of TNF-α have direct effects on insulin resistance.[3,8,9,11] These data suggest that circulating TNF-α-levels provide information that is useful to study the link between TNF-α and insulin resistance. Circulating TNF-α may either be a marker of the amount of TNF-α that is produced locally or act synergistically with locally produced TNF-α.

TNF-α infusion is associated with an impaired endothelium-dependent vasodilation in large vessels in animals[42] and resistance vessels in humans.[43] A reduced endothelium-dependent vasodilation at the precapillary level may be the cause of changes in capillary recruitment.[27] Our findings of an association of TNF-α levels with capillary recruitment, but not with microvascular endothelium-dependent vasodilation, do not support this mechanism, at least not with respect to the TNF-α-capillary recruitment relationship. Therefore, we suggest that there is a direct association between TNF-α and the functional or structural characteristics of the capillary network, as assessed by capillary recruitment during post-occlusive reactive hyperaemia.

Although acute infusion of TNF-α, while impairing endothelium-dependent vasodilation, increases total forearm blood flow,[43] this does not exclude a role for TNF-

α in impairing capillary recruitment. Several studies have shown that it is capillary flow, and not total limb blood flow, that is important in the determination of insulin sensitivity.[16,17,20,44]

It has been suggested that adipose tissue is a significant source of serum TNF-α. In vitro release of TNF-α by adipocytes has been reported and circulating concentrations of TNF-α have been found to be associated with body mass index,[45] a finding compatible with our data. Although our finding that TNF-α was not related to waist-to-hip ratio is in contrast to findings in healthy elderly individuals and a study in obese women,[32,45] it is in accordance with a study by Nilsson et al, who demonstrated in elderly men that TNF-α concentrations were associated with body mass index, but not with waist-to-hip ratio.[6] This may be related to the relatively low amount of visceral fat in our middle-aged individuals. A relatively low amount of visceral compared to peripheral fat mass may result in a more pronounced association of TNF-α with body mass index than with waist-to-hip ratio. Additional adjustment for body mass index further reduced the association between TNF-α and insulin sensitivity. This may indicate that, besides capillary recruitment, obesity-related mechanisms play a role in the TNF-α- insulin resistance relationship, but we cannot exclude that this statistical analysis resulted in overadjustment, i.e. underestimated the remaining association between TNF-α and insulin sensitivity.

It should be emphasized that any interpretation of our results must take into account the cross-sectional design of our study. However, intervention studies in animals with administration and/or neutralisation of TNF-α also suggest a direct link between TNF-α, insulin resistance[3,8,9,11] and insulin-mediated capillary recruitment.[15]

In conclusion, we have demonstrated that serum TNF-α is associated with capillary recruitment during post-occlusive hyperaemia in adults. In addition, this capillary recruitment can partly explain the previously described relationship between TNF-α and insulin resistance. Our findings thus provide support for a vascular component of TNF-α-induced insulin resistance. These associations, however, were not present in the prepubertal children of these adults. Our findings, therefore, suggest that the relationships among TNF-α, vascular function and insulin sensitivity are initiated during growth from childhood to adulthood.

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6

Individuals at an increased coronary heart disease risk are characterized by an impaired microvascular function in skin

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Abstract

Background To investigate whether microvascular function in skin is a valid model for the study of the relationships between cardiovascular risk factors and microvascular function, we investigated skin microvascular function in individuals with an increased coronary heart disease risk.

Methods Forty-six healthy, white individuals aged 30-70 years were studied. Coronary heart disease risk was assessed with the use of the coronary heart disease risk score according to the Framingham Heart Study, which is based on the risk factors age, blood pressure, cigarette smoking, total cholesterol, HDL cholesterol and diabetes. Endothelium-dependent and -independent vasodilation in skin were evaluated with laser Doppler after iontophoresis of acetylcholine and sodium nitroprusside. Videomicroscopy was used to measure recruitment of skin capillaries after arterial occlusion.

Results The coronary heart disease risk score (i.e., the 10-year probability of coronary heart disease) varied form 1 to 37%. Microvascular function decreased with increasing quartiles of coronary heart disease risk (for acetylcholine-mediated vasodilation: 687, 585, 420 and 326%, P=0.002; for nitroprusside-mediated vasodilation: 776, 582, 513 and 366%, P=0.02; for capillary recruitment: 49.9, 44.6, 27.2 and 26.7%, P=0.001). These trends were similar in men and women (P for interaction >0.2), and independent of body mass index.

Conclusions An increased coronary heart disease risk is associated with an impaired endothelium-dependent vasodilatation and capillary recruitment in skin, suggesting that microvascular function in skin is a valid model for the study of the relationships between cardiovascular risk factors and microvascular function.

Introduction

Coronary heart disease is the leading cause of morbidity and mortality among adults in Europe and North-America.[1] The relative contribution of microvascular and macrovascular processes to the risk of coronary heart disease is unknown, but its elucidation is important from etiological, preventive, and therapeutic perspectives.[2,3] Coronary microvascular disease may explain the occurrence of myocardial ischaemia without overt coronary artery blockage,[4-8] as well as heart failure[9,10] and mortality[10] after myocardial infarction. However, most studies of microvascular dysfunction have been conducted in small numbers of highly selected symptomatic patients.[4-8,10] This is partly because methods to assess the coronary microcirculation are invasive and applicable only in experimental settings.[2-8]

The skin microcirculation offers an opportunity to noninvasively explore the relation of systemic microvascular dysfunction to (risk factors for) coronary heart disease.[11] In support of this concept, we and others have previously demonstrated that impaired microvascular responses in skin are associated with elevated blood pressure[12-17] and insulin resistance.[12,14,15,17] The question thus arises whether skin microvascular function is associated with coronary heart disease and its risk factors in general. The coronary heart disease risk score derived from the Framingham Heart Study can be used to estimate coronary heart disease risk in middle-aged white populations.[18-22] This risk score can be calculated for men and women from risk factors for coronary heart disease that can easily be obtained, i.e., age, blood pressure, cigarette smoking, total cholesterol, HDL cholesterol and diabetes.[23]

To investigate whether microvascular function in skin is a valid model for the study of the relationships between cardiovascular risk factors and microvascular function, we investigated whether the coronary heart disease risk score according to the Framingham Study is associated with microvascular function in skin in a randomly selected population of middle-aged individuals. In addition, we examined the association of microvascular function with the individual risk factors that constitute the coronary heart disease risk score.

Methods

Subjects

Forty-six white individuals (28 men) aged 30-70 years participated in this study (Table 1). They were recruited by local advertisements and word of mouth. Five individuals had type 2 diabetes and nine were smokers. None had a history of cardiovascular disease. The diabetic participants were on average 47.4±11.7 years old, and their average HbA1c was 6.2±1.4%. Among the diabetic participants, 1 had

Table 1. Characteristics of the study population

	Mean ± SD	(range)
N (men/women)	46 (28/18)	
Age, years	49.2 ± 12.2	(30 – 70)
Body mass index, kg/m²	26.6 ± 3.9	(19.0 – 37.8)
Systolic blood pressure, mmHg	135.2 ± 13.9	(112 – 161)
Diastolic blood pressure, mmHg	84.1 ± 9.6	(68 – 112)
Serum cholesterol, mmol/l	5.3 ± 1.0	(3.4 – 8.0)
HDL cholesterol, mmol/l	1.3 ± 0.4	(0.71 – 2.27)
LDL cholesterol, mmol/l	3.4 ± 1.0	(1.4 – 5.6)
Smoking, yes/no	9/37	
Diabetes, yes/no	5/41	
ACh-mediated vasodilatation, %	502 ± 253	(131 – 1102)
SNP-mediated vasodilatation, %	555 ± 317	(105 – 1384)
Capillary recruitment, %	37 ± 18	(-3 – 82)
CHD risk score, %	11.0 ± 9.9	(1 – 37)

ACh indicates acetylcholine; SNP, sodium nitroprusside; CHD risk score, Coronary Heart Disease risk score derived from the Framingham Heart Study

microalbuminuria, 1 was taking aspirin, and 2 were taking ACE-inhibitors. None of the non-diabetic participants was treated with anti-hypertensive medication. We did not include individuals younger than 30 or older than 70 years because the coronary heart disease risk score has not been defined for these age categories.[18] The study protocol was approved by the local Ethics Committee, and informed consent was obtained from each subject. The investigation conforms with the principles outlined in the Declaration of Helsinki.

Measurements

Microvascular function in skin was measured after 30 minutes of acclimatisation in a quiet, temperature-controlled room, with the subjects in sitting position and the investigated non-dominant hand at heart level. The subjects were asked to refrain from caffeine, alcohol containing drinks and meals for 4 h preceding the test. Nailfold and iontophoresis studies were performed on the same day and subjects were studied at the same time of the day. Skin temperature was monitored continuously during the measurements.

Endothelium-dependent and -independent vasodilatation in skin was evaluated by iontophoresis of acetylcholine and sodium nitroprusside in combination with laser Doppler fluxmetry as previously described in more detail.[14,17,24] Laser Doppler fluxmetry measures microvascular perfusion, the product of red blood cell velocity and concentration.[25] A protocol of multiple fixed doses (current intensity × delivery time) was employed resulting in an incremental dose-response curve. Acetylcholine (1%;

Miochol, Bournonville Pharma, The Netherlands) was delivered using an anodal current; 7 doses (0.1 milliamps (mA) for 20 s) were delivered, with a 60-s interval between each dose. Sodium nitroprusside (0.1%; Nipride, Roche, The Netherlands) was delivered using a cathodal current; 9 doses (0.2 mA for 20 s) were delivered, with a 90-s interval between each dose. Acetylcholine-dependent laser Doppler flux was measured on the middle phalanx of the third finger, whereas nitroprusside-dependent laser Doppler flux was measured on the same spot at the opposite hand. Approximately 15 minutes elapsed between these two measurements. The day-to-day coefficient of variation of the percentage increase from baseline to the plateau phase (final two iontophoretic deliveries) was 9.8±5.6% for acetylcholine and 8.3±5.4% for sodium nitroprusside, as determined in 9 subjects on two occasions.

Nailfold capillaries in the dorsal skin of the third finger were visualised by a capillary microscope linked to a television camera, a video recorder and a monitor as previously described in more detail.[13,14,17,24] Two separate visual fields of 1 mm² were recorded before and after 4 minutes of arterial occlusion with a digital cuff, and the images were stored on videotape. The number of capillaries was counted at baseline and directly after release of the cuff from a freeze-framed reproduction of the videotape. If the presence of a capillary was uncertain the counting was done by a running videotape. Capillary density is defined as the number of erythrocyte-perfused capillaries per square millimetre of nailfold skin. Percentage capillary recruitment was assessed by dividing the increase in capillary density after 4 minutes of arterial occlusion by the baseline capillary density. The day-to-day coefficient of variation was 8.3±4.9%, as determined in 9 subjects on two occasions.

The systolic and diastolic blood pressure was measured with ambulatory monitoring (Spacelabs 90207, Redmond, Washington, USA). This was used to obtain 24 h recordings of blood pressure and heart rate. The measurements were done at the non-dominant arm and the monitors were programmed to take blood pressure and heart rate readings every 15 minutes from 07.00-22.00, and every 20 minutes from 22.00-07.00. Mean 24 h blood pressure was used for the coronary heart disease risk score calculations.

Total, LDL and HDL cholesterol levels were measured according to standard laboratory procedures. Diabetes was defined by fasting plasma glucose concentrations according to the ADA criteria.[26] Anthropometric measurements (which included weight, height, waist circumference and hip circumference) were performed as described previously.[14] The body mass index was calculated by dividing weight in kilograms by height in meters squared. The waist-to-hip ratio was calculated as a measure of body fat distribution. Waist circumference and hip circumference were not available in 4 individuals.

The coronary heart disease risk score predicts coronary heart disease for men and women, and is based on the risk factors age, total cholesterol (or LDL cholesterol), HDL cholesterol, blood pressure, diabetes and smoking. The coronary heart disease

score sheets based on a prediction algorithm were used to calculate the coronary heart disease risk score.[18] For example, in men, Equation 1: L=0.04826xage (in years) -0.65945 (if total cholesterol (in mg/dL) <160) +0.0 (if total cholesterol 160 to 199) +0.17692 (if total cholesterol 200 to 239) +0.50539 (if total cholesterol 240 to 279) +0.65713 (if total cholesterol >=280) +0.49744 (if HDL cholesterol (in mg/dL) <35) +0.24310 (if HDL cholesterol 35 to 44) +0.0 (if HDL cholesterol 45 to 49) -0.05107 (if HDL cholesterol 50 to 59) -0.48660 (if HDL cholesterol >=60) -0.00226 (if blood pressure [BP] optimal) +0.0 (if BP normal) +0.28320 (if BP high normal) +0.52168 (if BP stage I hypertension) +0.61859 (if BP stage II hypertension) +0.42839 (if diabetes present) +0.0 (if diabetes not present) +0.52337 (if smoker) +0.0 (if not smoker). The function is evaluated at the values of the means for each variable in the equation. Call it G, where Equation 1: G =0.04826x48.5926 -0.65945x0.07433 +0.17692x0.38851 +0.50539 x0.16673 +0.65713x0.05826 +0.49744x0.19285 +0.24310x0.35476 -0.05107x0.19646 -0.48660x0.10727 -0.00226x0.20048 +0.28320x0.20048 +0.52168x0.22820 +0.61859 x0.13057 +0.42839x0.05223 +0.52337x0.40458 =3.0975. This value of G is subtracted from function L to produce function A (Equation 2), which is then exponentiated, to produce B (Equation 3). The latter represents the relative odds for coronary heart disease. The survival value s(t) is exponentiated by B and subtracted from 1.0 to calculate the 10-year probability of coronary heart disease (Equation 4). Equation 2: A=L-G (where G=3.0975). Equation 3: $B=e^{A}$. Equation 4: Probability=1-[s(t)]B [where s(t) 10 years=0.90015]. With the use of similar equations, coronary heart disease risk can be calculated for women.[18]

Statistical Methods

Data are expressed as mean ± SD, unless stated otherwise. MANOVA was used to investigate the association of coronary heart disease risk score quartiles with microvascular function, before and after adjustment for body mass index or waist-to-hip ratio. An interaction analysis was performed to investigate whether these associations were different between men and women. In an additional analysis, we used linear regression analysis to investigate the association of microvascular function with the individual risk factors that constitute the coronary heart disease risk score. Although it is not known whether the variation in microvascular function is cause or consequence or both of blood pressure, serum lipids and diabetes, we chose to use microvascular function as the dependent variable. The adjusted R^2 was used to determine the proportion of variation in the microvascular function explained by the coronary heart disease risk factors. A two-tailed P-value of < 0.05 was considered significant. All analyses were performed on a personal computer using the statistical software package SPSS version 9.0 (SPSS, Chicago, Illinois, USA).

Results

The coronary heart disease risk score (i.e., the 10-year probability of coronary heart disease) varied form 1 to 37%. Figure 1 shows that an increased risk for coronary heart disease was associated with a lower acetylcholine- and nitroprusside-mediated vasodilation, and with lower capillary recruitment. These trends were similar in men and women (*P* for interaction >0.2), and significant after adjustment for body mass index (*P*=0.02, *P*=0.1 and *P*=0.006, respectively). After adjustment for waist-to-hip ratio, these trends were of borderline significance (*P*=0.1, *P*=0.1 and *P*=0.07, respectively).

Table 2 shows the association of the individual risk factors that constitute the coronary heart disease risk score with the microvascular measurements. These associations were adjusted for age and sex, except when age or sex was the variable under investigation. Although not all associations were statistically significant, it can be seen that adverse risk factors were related to a lower acetylcholine-mediated vasodilation and capillary recruitment. Sodium nitroprusside-mediated vasodilation only appeared to be associated with the risk factors age and smoking.

With only systolic and diastolic blood pressure entered into the regression model, they accounted for 56%, 3% and 72% of the interindividual variation in acetylcholine-mediated vasodilation, sodium nitroprusside-mediated vasodilation and capillary recruitment, respectively. With all coronary heart disease risk factors entered into a regression model simultaneously, the explained variation increased to 74%, 45% and 74%, respectively.

When the analyses were performed with the coronary heart disease risk score based on LDL cholesterol instead of total cholesterol, the results were similar (data not shown).

Table 2. Percent point change in acetylcholine- and sodium nitroprusside-mediated vasodilation, and in capillary recruitment per unit change in individual risk factors that constitute the coronary heart disease risk score (with 95% confidence interval)

	ACh-mediated vasodilation (%)	SNP-mediated vasodilation (%)	Capillary recruitment (%)
Gender (men vs women)	-103 (-252 to 45)	-9 (-177 to 160)	-10 (-20 to 1)
Age (10 y)	-70 (-130 to -11)*	-150 (-218 to -82)**	-5 (-9 to -0.01)*
Total cholesterol (mmol/l)	-25 (-104 to 55)	-0.01 (-91 to 90)	-3 (-8 to 3)
HDL cholesterol (mmol/l)	177 (-25 to 379)	-138 (-372 to 96)	18 (4 to 31)*
Systolic BP (10 mmHg)	-128 (-171 to -85)**	7 (-60 to 73)	-11 (-13 to -8)**
Diastolic BP (10 mmHg)	-149 (-213 to -84)*	8 (-82 to 98)	-13 (-17 to -10)**
Smoking (yes vs no)	-293 (-471 to -115)**	-261 (-473 to -49)*	-7 (-21 to 7)
Diabetes (yes vs no)	-205 (-447 to 37)	-15 (-299 to 269)	-5 (-22 to 13)

*p<0.05, **p<0.01, adjusted for age and sex, except when age or sex was the variable under investigation.
ACh indicates acetylcholine; SNP, sodium nitroprusside.

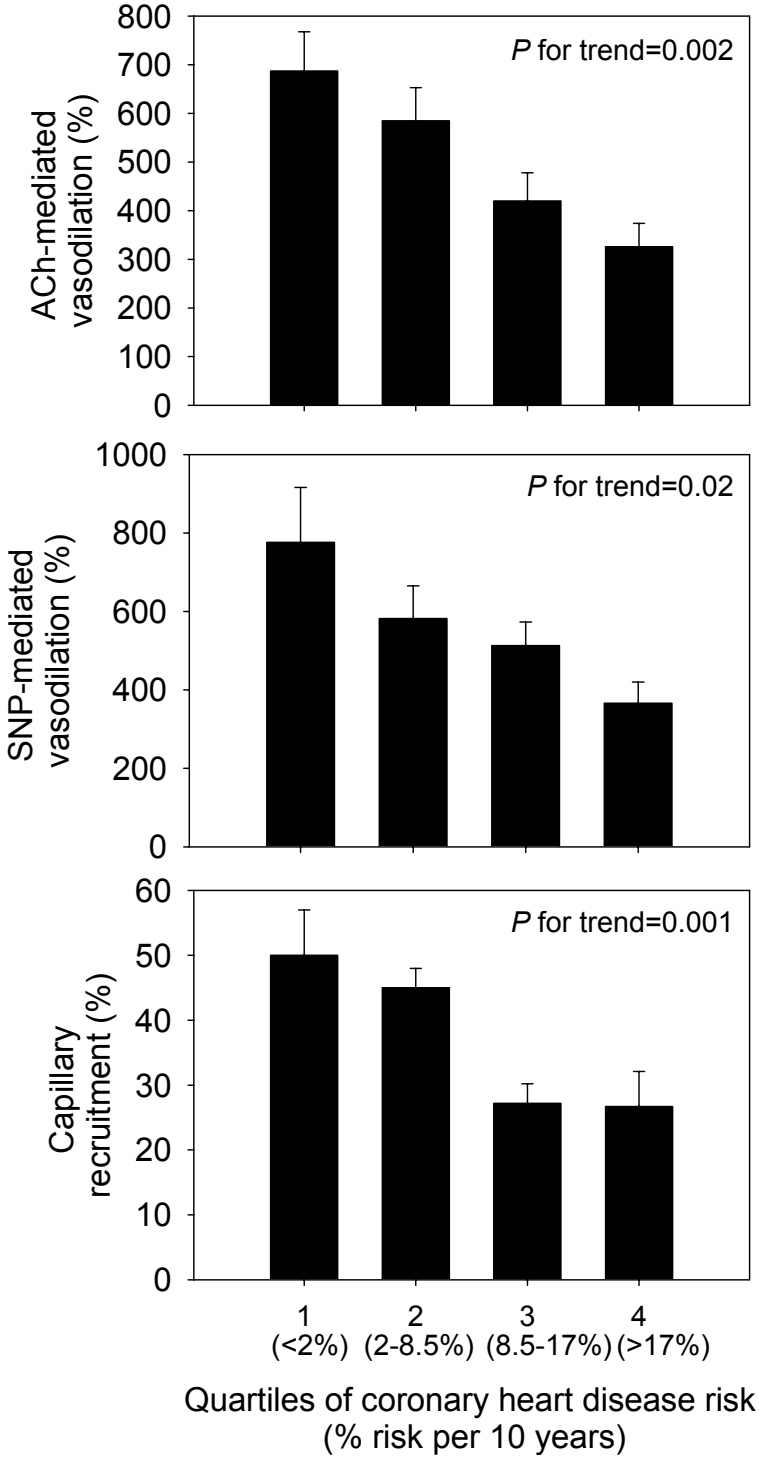

Figure 1. Univariate associations of the coronary heart disease risk score with acetylcholine- and sodium nitroprusside-mediated vasodilation, and with capillary recruitment.

Discussion

Coronary microvascular disease may explain the occurrence of myocardial ischaemia without overt coronary artery blockage,[4-8] as well as heart failure[9,10] and mortality[10] after myocardial infarction. However, methods to assess the coronary microcirculation are invasive and applicable only in experimental settings.[2-8] Therefore, the development of non-invasive methods to measure microvascular function is important. The interesting new finding of the present study was that an increased coronary heart disease risk was associated with a lower endothelium-dependent vasodilatation and capillary recruitment in skin. These findings were similar in men and women and independent of age. Our findings suggest that microvascular function in skin may be a valid model for the study of the relationships between cardiovascular risk factors and microvascular function.

We demonstrated that individuals at an increased risk for coronary heart disease are characterized by an impaired microvascular function in skin. However, our data do not provide direct evidence of an association between coronary microvascular disease and risk of coronary heart disease, as there was no assessment of coronary microcirculation. Nevertheless, it should be appreciated that coronary microvascular dysfunction appears to be part of a systemic microvascular process.[27-29] Several studies have suggested that microcirculation in skin resembles the microcirculation in other tissues. Although skin microvascular resistance does not make a major contribution to the total peripheral vascular resistance, an association between skin microvascular function and blood pressure can be demonstrated.[12,14-17] In addition, in individuals with hypertension, microvascular defects can be demonstrated in heart,[29] skeletal muscle[30] and skin.[13,31] Similarly, although muscle is the main peripheral site of insulin-mediated glucose uptake, an association of diabetes and/or insulin resistance with microvascular function has been reported in heart,[29] skeletal muscle[32,33] and skin.[12,14,15,17] Moreover, metabolic and vascular effects of insulin could be demonstrated in skin.[34,35] Taken together these studies suggest that microvascular function in skin resembles microvascular function in other tissues in many ways.

In our study, microvascular function was measured as endothelium-(in)dependent vasodilatation and capillary recruitment. The association of these microvascular measurements with coronary heart disease risk is in accordance with recent findings of an association between coronary heart disease risk and the laser Doppler signal during peak reactive hyperaemia in skin.[36] These studies provide support for the use of microvascular function in skin as a model for the study of the relationships between cardiovascular risk factors and microvascular function. Additional support comes from studies demonstrating that impaired responses of the skin microcirculation may be reversed after cholesterol-lowering therapy.[37-39]

In the univariate analyses, an increased risk for coronary heart disease was not only associated with a lower acetylcholine-mediated vasodilation, but also with a lower

nitroprusside-mediated vasodilation. This may be in accordance with the results from Schachinger et al., who demonstrated that an impaired endothelial and endothelium-independent coronary vasoreactivity were associated with a significantly higher incidence of cardiovascular events.[40] However, in our study, the association of an increased coronary heart disease risk with a lower nitroprusside-mediated vasodilation was no longer significant after adjustment for body mass index.

Other findings were not affected by adjustment for body mass index. However, after adjustment for waist-to-hip ratio, the association between coronary heart disease risk and microvascular function was diminished. Waist-to-hip ratio is an independent risk factor for coronary heart disease[41] and abdominal fat may secrete substances,[42] such as free fatty acids and cytokines, which may influence microvascular function and coronary heart disease risk. Free fatty acids have been shown to impair endothelium-dependent vasodilation at the level of the resistance vessels[43] and proinflammatory cytokines have been proposed to link central obesity to vascular dysfunction.[44]

The associations between microvascular function on the one hand and the individual cardiovascular risk factors on the other show a pattern that is consistent with the association between microvascular function and the total coronary heart disease risk score. Although not all associations were statistically significant, male gender, age, cholesterol, low HDL cholesterol, systolic and diastolic blood pressure, smoking and diabetes were related to a diminished endothelium-dependent vasodilation and capillary recruitment. A diminished endothelium-independent vasodilation was only associated with age and smoking. These findings reinforce the idea that measurements of microvascular function in skin can be relevant to understanding the pathogenesis of coronary heart disease.

Many studies have demonstrated associations between blood pressure and microvascular function.[12-17] In the present study, blood pressure explained 56% and 3% of the variation in endothelium-dependent and -independent vasodilation, respectively. After adding the other coronary heart disease risk factors to the model, the explained variation increased to 74% and 45%, respectively. These increases in the explained variation in endothelium-dependent and -independent vasodilation suggest that the other risk factors may be of additive relevance for these measures of microvascular function. The explained variation in capillary recruitment, however, was only slightly increased after adding the other risk factors.

A limitation of the present study is the relatively small sample size. However, the characteristics were comparable to the characteristics of the participants of the Framingham Heart Study[18] and European population studies in middle-aged individuals.[19] Another limitation is that it is not possible to distinguish between cause and effect due to the cross-sectional nature of the study. Impaired microvascular function may be the result of higher coronary heart disease risk factors.[23,45,46] However, the opposite may also be true. For example, an impaired microvascular function may result in an increase in peripheral resistance and a decrease the delivery of insulin and

glucose to the tissues, thereby causing an increase in blood pressure and insulin resistance.[47-49] Regardless of whether or not microvascular function is cause or effect, our finding of an impaired skin microvascular function in individuals at an increased risk of coronary heart disease suggests that microvascular function in skin may be a valid model for the study of the relationships between cardiovascular risk factors and microvascular function. Nevertheless, further large scale studies in this area are needed.

In conclusion, our data provide the first evidence for an association of an increased coronary heart disease risk with impaired endothelium-dependent vasodilation and capillary recruitment in skin. These findings suggest that microvascular function in skin provides information about the vascular system relevant to coronary heart disease. The longitudinal investigation of a large population is required to investigate the association of microvascular function in skin with coronary heart disease morbidity and mortality.

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7

Introductory remarks on twin studies as a means of investigating the fetal origins hypothesis

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In this chapter, we will briefly discuss that the association of birth weight with blood pressure and other cardiovascular risk factors is relevant for cardiovascular disease. This association may be confounded or explained by several variables, such as socio-economic status and genetic make-up. We will postulate that twin studies offer a unique opportunity to investigate the influence of such variables. However, we will emphasize that twin studies cannot be used to identify maternal factors. We will also summarise previous studies investigating the fetal origins hypothesis in twins, and discuss several methodological issues. Finally, we will briefly review the twin population and cardiovascular risk factors analysed in the present thesis.

1. Association between birth weight and cardiovascular disease

1.1 Epidemiological studies

As described in Chapter 1, evidence has accumulated that low birth weight is associated with an increased risk of cardiovascular disease.[1-5] This association may be mediated by the association of low birth weight with several established risk factors for cardiovascular disease, such as blood pressure,[6] insulin resistance,[7-12] diabetes,[13] plasma lipids[8,14-16] and fibrinogen.[17-19]

1.2 Is the association of birth weight with blood pressure relevant?

The association between birth weight and subsequent blood pressure levels has been considered to provide some of the strongest, and most consistent, support for the fetal origins hypothesis. A review of 80 studies in more than 444,000 singletons demonstrated that, on average, a 1 kg higher birth weight is associated with a 2 mmHg lower blood pressure.[6] In clinical practice this may seem a small difference, but these are relevant differences between the mean values of populations.[20] For example, lowering mean systolic blood pressure in a population by 2 mmHg corresponds to a 8% reduction in the risk of stroke.[21] Huxley et al. have recently concluded that the size of the association between birth weight and blood pressure may be overestimated due to publication bias and be of little relevance,[22] but this conclusion was based largely on findings in very large population studies, in which birth weight data and/or blood pressure levels were self-reported, probably causing attenuation of the associations in these studies. Interestingly, in two of their three largest studies weighting the meta-analysis, adjusted odds ratios for (self-reported) hypertension in men of less than 5.5 lb at birth were nevertheless significant at 1.26 (95% confidence interval 1.11–1.44).[23] For women in the two Nurses' Health Studies,[24] the adjusted odds ratio was 1.39 (1.29–1.50) and 1.43 (1.31–1.56). The discrepancy between the weak association of birth weight with self-reported blood pressure and the strong association with self-reported hypertension may be due to the fact that self-reported diagnosis of hypertension in these studies was more reliable than self-reported actual blood pressure levels. In addition, it should be appreciated that, in epidemiological studies, associations of birth weight with

determinants of blood pressure are likely to be stronger than with blood pressure itself due to compensatory mechanisms that tightly regulate blood pressure. For example, the associations between birth weight and measurements of vascular function are more pronounced than the associations between birth weight and blood pressure.[25-28]

1.3 Confounding of the association between birth weight and cardiovascular risk

The fetal origins hypothesis suggests that the association between birth weight and cardiovascular risk is due to a programmed response to intrauterine malnutrition. According to this hypothesis, improvements in intrauterine nutrition may prevent disease in later life. This would have major implications for public health. However, an alternative hypothesis states that the association between birth weight and cardiovascular disease may be due to confounding by an adverse environment that is related to small size at birth and later cardiovascular disease. Socioeconomic status in particular may have an important impact on lifestyle choices that are related to both low birth weight[29] and cardiovascular risk (factors).[30-32] For example, reduced size at birth may simply be a marker for poor maternal socioeconomic circumstances that predict relative deprivation of the offspring in adult life, which leads in turn to an increased risk of cardiovascular disease. Although a few studies have shown that adjustment for lifestyle factors, such as smoking, employment, alcohol consumption and exercise, had little effect on the association between birth weight and cardiovascular disease, [2-4] influences of other (unknown) factors cannot be excluded in these studies.

Another alternative explanation for the association between birth weight and cardiovascular disease is that the association arises from pleiotropic genetic factors. Both birth weight and cardiovascular disease are influenced by genetic factors. If there are genetic factors which influence both birth weight and adult cardiovascular disease, these factors may be responsible for the association.[33-35] In other words, a genotype responsible for cardiovascular disease in later life may cause retarded fetal growth in utero. In this case, nutrition-induced changes in fetal growth may not prevent the development of cardiovascular disease.

2. Elimination of confounding factors

2.1 Experimental approach

The epidemiological studies linking low birth weight with cardiovascular risk do not provide evidence for a causal relationship, and further, mechanistic tests of the fetal origins hypothesis are required. Only in experimental settings can we investigate whether the association of one variable with another is causal. An ideal experimental setting creates circumstances across which only one factor affecting the outcome of interest varies. In humans, such an experimental setting is usually achieved in a randomized trial. However, a randomized trial in humans with interventions to influence birth weight is difficult to perform for several reasons. First, it is not known what intervention(s) should be used to increase birth weight. Second, it should be

remembered that, although any intervention in a fetus may have large benefits for disease in later life, unexpected dramatic adverse effects may also develop. Third, the results of these trials will be definitive only after sufficient clinical cardiovascular diseases have developed in the study group, which means a study duration of at least 50 years.

Usually, the term experiment is restricted to situations in which circumstances are manipulated by the investigator. However, intrapair comparisons in twins (and to a lesser extent in siblings) provide a unique opportunity to mimic a scientific experiment. The influence of one factor (in this case birth weight) on the outcome of interest (cardiovascular risk factors) can be investigated independent of many other factors (such as socio-economic and genetic factors), because the influence of these factors is eliminated within pairs. Therefore, twins can be considered as an "experiment of nature".

2.1.1 Comparisons between sibling pairs

Confounding by socio-economic factors can be diminished by comparing siblings, as for all children within a family who have been born from the same mother and father, socio-economic factors are approximately the same. If socio-economic factors do not play a role in the association between birth weight and cardiovascular risk factors, one would expect that the sibling with the lowest birth weight will also have the highest level of the cardiovascular risk factor compared to the sibling with the highest birth weight. In addition, differences in birth weight should be inversely associated with differences in the risk factor of interest. If, however, socio-economic factors do play a role, then these associations would be diminished within the paired sibling analyses as compared to unpaired analyses. To our knowledge, only a few studies have used the comparison of siblings to investigate the fetal origins hypothesis. It has been demonstrated that, at 7 years of age, heights and weights of infants born with a low birth weight remained approximately 0.5 SD less than those of siblings born with a normal birth weight.[36] In addition, Matte et al. have shown that, within same sex sibling pairs, differences in birth weight were directly associated with differences in IQ in boys (predicted IQ difference per 1 kg increase in birth weight = 5.0, 95% confidence interval 2.8 to 7.1) but not in girls (1.0, -0.9 to 3.0).[37] These studies thus suggest that the association of these variables with birth weight are independent of parental socio-economic factors.

2.1.2 Comparison between twin pairs

This comparison between siblings can be taken one step further by investigating twin pairs.

Similar to singleton siblings, the members of a twin pair are raised in the same family and, therefore, the same socio-economic class. In addition, twin pairs provide a unique tool to investigate the influence of genetic factors. In monozygotic twins, who are

genetically identical or nearly identical,[38] the influence of genetic factors on the differences within a twin pair is excluded. In dizygotic twins, who share on average half of their genes, the influence of genetic factors is reduced, but not excluded. If genetic factors do not play a role in the association between birth weight and cardiovascular risk factors, one would expect that, both for dizygotic and for monozygotic twins, the twin with the lowest birth weight from each pair will also have the highest level of the cardiovascular risk factor compared to the cotwin with the highest birth weight. In addition, inverse associations between intrapair differences in birth weight and intrapair differences in the risk factor should exist both in dizygotic and in monozygotic twins. If, however, genetic factors do play a role, these associations would exist only within dizygotic twins, and not within monozygotic twins. In this case, within dizygotic twins, unfavourable genetic factors will cause growth retardation and cardiovascular disease in one twin, but not in the cotwin who does not have the unfavourable genotype. In monozygotic twins, both twin members have the same genotype, so they both have the same unfavourable or favourable genetic factors.

For a mathematical approach to the use of intrapair analyses in twins, see the appendix of this chapter. It seems safe to say that if the intrapair association between two variables in DZ twins is larger than in MZ twins, this always implies that pleiotropic genes mediate the association between them.

3. Can within-pair analyses in twins be used to identify maternal factors?

Dwyer and colleagues suggest a new approach to twin studies,[39] which they claim allows us to gain insights into the particular importance of maternal factors. They suggest that if the regression coefficient seen in unpaired analyses is greatly reduced in within-pair analyses in pooled data of dizygotic and monozygotic twins, then maternal factors (eg, nutrition or socio-economic status) might be largely responsible for the noted association in unpaired analyses, because within-pair analysis has stratified for the mother.[39] This reasoning is not correct.

Although within-pair analyses in twins stratify for all maternal factors, they also stratify for all paternal factors. Furthermore, within-pair analyses in twins to a large extent also stratify for fetal genetic factors. Dizygotic twins share on average 50% of their genes and monozygotic twins are genetically identical. Therefore, if the regression coefficient is greatly reduced in within-pair analyses in twins, then maternal, paternal, or fetal genetic factors might account for the noted association in unpaired analyses. We conclude that the comparison of within-pair analyses with unpaired analyses cannot be used to identify maternal factors.

4. Previous studies investigating the fetal origins hypothesis in twins

When we started our investigations in twins, three studies had used within-pair analyses in twins to investigate the fetal origins hypothesis.[39-41]

Poulsen et al.[40] had investigated elderly twin pairs discordant for non-insulin dependent diabetes. They found that, in both dizygotic and monozygotic twin pairs, the twin members with diabetes had a reduced birth weight compared to their non-diabetic cotwins (monozygotic twin pairs: mean ± SEM 2634±135 vs 2829±131 g, P<0.02; dizygotic twin pairs: 2509±135 vs 2854±168 g, P<0.02).[40] However, in this analysis, zygosity was determined by a questionnaire, and only 14 twin pairs of each zygosity were included.

The association between birth weight and blood pressure had been investigated by Dwyer et al.[39] and Poulter et al.[41] Dwyer et al. investigated dizygotic and monozygotic twins at age 8, and found that blood pressure changed by -7.0 mm Hg (95% confidence interval -10.1 to -3.9) for each 1 kg increase in birth weight in the overall sample of twins. This association was little altered in within-pair analyses (-5.3, -13.8 to +3.2) and was similar for both monozygotic (-6.5, -22.5 to +9.4) and dizygotic (-4.9, -15.8 to +6.0) pairs. Poulter et al. compared intrapair differences in blood pressure in four strata of intrapair differences in birth weight (0, 1-500 g, 501-1000g and greater than 1000 g). A graded inverse relation between strata of within-pair differences in birth weight and differences in adult blood pressure was apparent, with an adjusted blood pressure difference of 8.7 mmHg across the four strata of birth weight (test for trend: *P*=0.05). When differences in blood pressure were stratified for zygosity similar but non-significant trends were apparent. Both Dwyer et al. and Poulter et al. suggested that the association between birth weight and blood pressure is independent of genetic factors. However, in our view both studies are open to another interpretation. In the study of Dwyer et al. only 16 monozygotic twin pairs were included,[39] and the confidence interval of the association between birth weight and blood pressure within monozygotic twins was very wide, ranging from -22.5 to +9.4 mmHg. From this confidence interval, it is difficult to conclude that birth weight was negatively associated with blood pressure within monozygotic twin pairs. Furthermore, it should be noted that blood pressure was measured only three times in each individual and zygosity was determined by a questionnaire, and thus misclassification of zygosity could have complicated the comparison between dizygotic and monozygotic twins. The results of Poulter et al.[41] are also open to another interpretation. As a first intrapair analysis, blood pressure levels between cotwins with the highest and the lowest birth weight from each pair can be compared in all dizygotic and all monozygotic twin pairs, which can be calculated from the data presented in their paper. After adjustment for confounding factors, the 203 dizygotic, but not the 140 monozygotic, twins with the highest birth weight had a systolic blood pressure that was significantly lower compared to their cotwins with the lowest birth weight (difference in blood pressure: -5.37 mm Hg, *P*<0.05 and -0.85 mm Hg, *P*=0.8, respectively). This suggests that the relationship between birth weight and blood pressure within twin pairs is much stronger in dizygotic than in monozygotic twin pairs, which is supportive of genetic influences.

Taken together, the limited number of twin studies investigating the fetal origins hypothesis have several weaknesses:

- small sample size,[39,40]
- zygosity was determined by a questionnaire,[39,40] which will result in a misclassification of zygosity in approximately 7% of the twins[42] and, consequently, complicate the comparison between monozygotic and dizygotic twins,
- use of self-reported birth weight in elderly subjects,[40,41] which is likely to attenuate the association between birth weight and adult outcome,
- blood pressure, which is notoriously variable, was measured twice [39] or three[41] times, which is also likely to attenuate the association between birth weight and blood pressure in comparison with studies using more extensive measurements of blood pressure,
- incomplete interpretation of the data,[41]
- finally, besides blood pressure[41] [39] and diabetes,[40] no other cardiovascular risk factors have been investigated.

5. Methodological considerations

5.1 Birth weight as a measure of intrauterine growth

For methodological considerations concerning birth weight as a measure of intrauterine growth see Chapter 15 (section 1.1).

5.2 Differences in birth weight in twins as a model for differences in birth weight in singletons

It could be argued that differences in birth weight in twins are a poor model for differences in birth weight in singletons. For example, intrauterine growth in twins is different from that in singletons.[43] However, the association between birth weight and blood pressure in the overall sample of our twin cohort (-1.9 mmHg per kg increase of birth weight) was remarkably similar to the well-established association in singletons (approximately -2 mmHg per kg increase of birth weight).[6] The same holds true for the size of the association of birth weight with serum lipids[44] and later height in the overall sample of twins, which were similar to the size of the associations of birth weight with serum lipids[8,14,14,14-16,45,45-49] and height[8,50-63] in singletons. In addition, differences in birth weight within twin pairs have been associated with differences in many variables that have been related to birth weight in singletons, such as blood pressure, [39,64] diabetes,[40,65] serum lipids,[44] fibrinogen,[66] myocardial infarction[67] and height.[68,69] Although intrauterine growth in twins may be different from that in singletons, the associations between birth weight and cardiovascular risk in twins suggest that birth weight in twins is relevant for the development of cardiovascular disease, and that

differences in birth weight in twins can be used as a model for differences in birth weight in singletons.

5.3 Comparison between dizygotic and monozygotic twins

It could further be argued that the association between intrapair differences in dizygotic twins cannot be compared to intrapair differences in monozygotic twins to study the influence of genetic factors, as around two thirds of monozygotic twins are monochorionic (i.e. share a placenta), whereas all dizygotic twins are dichorionic (i.e. have separate placentas). Therefore, besides genetic factors, intrauterine factors may also differ between dizygotic and monozygotic twins and may be the cause of any observed differences in the intrapair association of birth weight. However, we consider it unlikely that the differences in the intrapair associations in dizygotic versus monozygotic twins are due to intrauterine differences. First, the overall associations of birth weight with all investigated variables were similar in dizygotic and monozygotic twins. Second, although a study in 6 twin pairs with twin-twin transfusion syndrome suggested that this syndrome may influence pulse wave velocity in the conduit arteries, chorionicity did not influence blood pressure.[70] In addition, a large prospective twin study (418 twin pairs) demonstrated that chorionicity did not influence the intrapair association between birth weight and blood pressure.[71] Furthermore, it should be noted that intrapair differences in birth weight in monozygotic twins have been related to within-pair differences in HDL cholesterol,[44] insulin sensitivity,[72] diabetes[40,65] and height,[68,69] demonstrating that the twin study design in general is quite capable of showing that intrauterine factors can influence adult outcome.

6. Twin studies described in the present thesis

6.1 Study population

We have studied the association of birth weight with several cardiovascular risk factors in a group of adolescent dizygotic and monozygotic twin pairs. This study is part of a larger project carried out at the Department of Biological Psychology in which cardiovascular risk factors were studied in 160 adolescent twin pairs and their parents.[73-80] Addresses of twins living in Amsterdam and neighbouring cities were obtained from City Council population registries. Twins still living with their biological parents were contacted by letter. From the families initially willing to participate several had to be excluded because not all four family members could find the time to participate or could speak Dutch sufficiently. After including sufficient monozygotic twins only dizygotic twins were included, in order to create approximately equal groups of monozygotic and dizygotic twins. Overall, between 30 and 40% of the families complied.[73] Zygosity was initially determined by typing the following polymorphisms: ABO, MNS, P, Rhesus, Lutheran, Kell, Duffy, Kidd, Gm, Am and Km. In a later stage of the project same-sex twin pairs were also typed by DNA polymorphisms.[81] A questionnaire was used to gather information on various factors including the use of medication and smoking

behaviour. The maternal questionnaire included questions regarding birth weight and gestational age of their children. This questionnaire was sent to the mothers a few weeks ahead of their visit to our department, allowing them to obtain birth data from birth certificates. Opposite-sex dizygotic twin pairs were excluded because of the effects of sex differences within a pair on both birth weight and cardiovascular risk factors. Subjects using oral contraceptives were also excluded for these analyses, and none of the subjects used any other medication that may affect cardiovascular risk factors. Thus, 53 dizygotic and 61 monozygotic twin pairs were eligible for analysis.

6.2 Cardiovascular risk factors measured in the twins

The measurements are described in more detail in the methods section of the following chapters. Briefly, blood pressure was measured 6 times at rest and during two mental stress tasks (Chapter 8). Indicators of sympathetic and parasympathetic activity (i.e. cardiac pre-ejection period and respiratory sinus arrhythmia) were assessed with electrocardiography and impedance cardiography at rest and during two mental stress tasks (Chapter 9). In a subgroup of these twins, insulin sensitivity was calculated from fasting insulin and glucose levels during follow-up (Chapter 10). In all twins, total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride levels, apolipoprotein A1 (the structural apolipoprotein linked to HDL), apolipoprotein B (the structural apolipoprotein linked to LDL) and lipoprotein(a) levels were measured in a fasting blood sample (Chapter 11). In this blood sample, fibrinogen was also measured (Chapter 13). Cholesterol metabolism was measured by markers of cholesterol synthesis (i.e. lathosterol; a precursor of cholesterol) and absorption (campesterol and β-sitosterol; plant sterols) (Chapter 12). Height (Chapter 14) and weight were measured in a standardized way.

6.3 Power considerations

We have thus investigated intrauterine and genetic influences on the association between birth weight and cardiovascular risk factors in a well-characterized cohort of twins with relatively sophisticated measurements of the variables of interest.

As described in Chapter 1, previous studies in singletons have shown that birth weight is consistently associated with several cardiovascular risk factors. Previous studies among dizygotic twin pairs (see section 4 of this chapter) have shown effects of birth weight on blood pressure and diabetes that are very strong. The small study by Dwyer et al. demonstrated that, within dizygotic twin pairs, a difference in birth weight of 1 kilogram was associated with a difference in blood pressure of -4.9 mmHg. In addition, Poulter et al. demonstrated that within dizygotic twin pairs, twins with the highest birth weight from each pair had a systolic blood pressure that was 5.4 mmHg lower compared to their cotwins with the lowest birth weight. Similarly, Poulsen et al.[40] found that a small difference in birth weight within dizygotic twin pairs (195 g) was related to diabetes. These remarkably strong effects in intrapair comparisons in

dizygotic twins are probably due to the elimination of various confounding characteristics, such as gestational age, maternal factors (e.g. height, weight gain, smoking and blood pressure during pregnancy), social class, birth order (in relation to other siblings) and sex.

It should be realised that the fetal origins hypothesis is very clear in stating that the association between birth weight and cardiovascular risk factors is entirely due to intrauterine nutritional factors. The alternative hypothesis states that these associations are due to a genetic factor that is related to both birth weight and cardiovascular risk. These conflicting hypotheses will be tested in this thesis. In our study, the power to detect a difference in systolic blood pressure of 3 mmHg (SD=8 mmHg) in the paired comparison[82] of the 53 dizygotic twin pairs with the lowest birth weight and their cotwins with the highest birth weight is 80% at α=0.05. If the association between birth weight and blood pressure is independent of genetic factors (as stated in the fetal origins hypothesis), a similar difference in blood pressure would be expected within the monozygotic twins. The power to detect this difference within 61 monozygotic twin pairs is 84% at α=0.05. Similarly, the power (α=0.05) to detect a within-pair difference in LDL cholesterol of 0.25 mmol/L and in HDL cholesterol of 0.08 mmol/L in the dizygotic twins is 75 and 70%, respectively. In the monozygotic twins, the corresponding power is 80 and 75%, respectively. If the association between birth weight and cardiovascular risk factors is completely due to genetic factors, it could be expected that these differences in cardiovascular risk factors are present within dizygotic twins, but completely absent within the monozygotic twins. The power to detect intrapair correlations between differences in birth weight and differences in cardiovascular risk factors of 0.35 at α=0.05 is 74% within the dizygotic twin pairs, and 81% within monozygotic twin pairs. If the association between birth weight and cardiovascular risk factors is due to genetic factors, it could be expected that the association between birth weight and cardiovascular risk factors is present within dizygotic twins, but completely abolished within the monozygotic twins. Interaction analyses to detect a difference of 0.45 in the correlation between dizygotic and monozygotic twins has a power of 71% at α=0.05, and 81% at α=0.1. This is the difference in the association between dizygotic and monozygotic twins that can reasonably be expected if genetic factors play a crucial role in the association between birth weight and cardiovascular risk. The analyses on fasting glucose and insulin levels are performed in a small subgroup. The power to detect intrapair correlations between differences in birth weight and differences in glucose and insulin levels of 0.35 at α=0.05 is 52% within the combined sample of dizygotic and monozygotic twin pairs, and 27% for the monozygotic and dizygotic twin pairs separately.

The power of a study is the probability that a study of a given size would detect as statistically significant a real effect of a given magnitude.[82] Therefore, it should be emphasized that the absence of statistically significant results in the analyses in our sample, as well as in other samples, may very well be due to type II errors, which can be

thought of as 'false negative findings'. In other words, we cannot exclude the possibility that an effect exists if it is not found in the analyses. In the interpretation of the results, it is therefore important to take into account the confidence intervals; a large confidence interval is an indicator of low power.[83] Finally, it should be realized that effects of a small magnitude are more difficult to detect. It is possible that the association between birth weight and a cardiovascular risk factor is only *in part* explained by genetic factors. Of course, the power of our study to detect these small effects is lower than the power to detect very large effects.

7. Summary

Twin pairs offer a unique opportunity to investigate the genetic and intrauterine environmental influences on the association of birth weight with cardiovascular risk factors. If genetic factors do not play a role in the aetiology of this association and the association is due to correlated environmental factors, one would expect that, both for dizygotic and for monozygotic twins, negative associations between intrapair differences in birth weight and intrapair differences in the risk factor exist. If, however, genetic factors do play a role, these associations would generally be larger in dizygotic twins than in monozygotic twins. In this case genetic factors are implied, and the fetal origins hypothesis is, at least in part, rejected. It should be emphasized that the comparison of within-pair analyses with unpaired analyses cannot be used to identify maternal factors. So far, only a limited number of twin studies have investigated the fetal origins hypothesis, and these studies have several weaknesses, such as a relatively small sample size and the use of inadequate measurements. Importantly, current data suggest that differences in birth weight in twins can be used as a model for differences in birth weight in singletons, and that intrapair differences in dizygotic twins can be compared to intrapair differences in monozygotic twins to study the influence of genetic factors. We, therefore, have studied the association of birth weight with several cardiovascular risk factors in a group of adolescent dizygotic and monozygotic twin pairs.

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Appendix

Mathematical approach

The expectation of the regression of two difference scores e.g. D(X) is trait X in one twin minus trait X in the cotwin and D(Y) is defined in a similar manner for trait Y) is:

Regression [D(Y), D(X)] = cov [D(Y),D(X)] / var D(Y)

for MZ twins: b(MZ) = 2cov (Ey,Ex) / 2var (Ey) = cov (Ey,Ex) / var (Ey)

for DZ twins: b(DZ) = [2 cov (Ex,Ey) + cov (Gx, Gy)] / [2var (Ey) + var (Gy)] ,

where cov (Ex,Ey) is that part of the covariance between Y and X that is environmentally mediated; var (Ey) stands for the environmental variance of Y; cov (Gx,Gy) represents the covariance between Y and X that is mediated by genetic factors; var (Gy) is the genetic variance of Y.

Now assume that var (Ey) is one (we define the heritability of trait Y relative to an environmental variance of one), then the regressions for MZ and DZ pairs become:

b(MZ) = cov (Ey,Ex)

b(DZ) = [2 cov (Ex,Ey) + cov (Gx, Gy)] / [2 + var (Gy)]

(note that if the genetic variance of Y is zero, then the expectations for the regression coefficients in MZ and DZ pairs is the same, i.e. cov (Ey,Ex). However, in this situation no one would test if the association between X and Y was genetically mediated).

Example

Assume a heritability of 50% for trait Y, i.e. var G = 1.
Then: b(DZ) = 2/3 cov (Ex,Ey) + 1/3 cov (Gx, Gy)

- If the covariance of Ex and Ey is the same as the covariance of Gx and Gy then the regression coefficients of MZ and DZ would be equal, but there would still be genetic mediation of the association between X and Y.
- If Y is a heritable trait, but the covariance of Gx and Gy is zero, then the regression in DZ twins is smaller than in MZ twins.
- If the regression in DZ twins is larger than in MZ twins, this implies genetic mediation of the association between X and Y.

To put this a bit more general (regardless of the heritability of Y, as long as it is not zero):

- There always is a combination of values for the covariance of Ex and Ey and the covariance of Gx and Gy that leads to the same regression coefficients in MZ and DZ twins, while still is genetic mediation of the association between X and Y.
- If Y is a heritable trait, but the covariance of Gx and Gy is zero, then the regression in DZ twins is always smaller than in MZ twins.
- It seems safe to say that if the regression in DZ twins is larger than in MZ twins, this always implies that pleiotropic genes mediate the association between X and Y.

8

Evidence for genetic factors explaining the birth weight-blood pressure relationship Analysis in twins

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Abstract

Background Epidemiological studies have consistently shown an inverse association between birth weight and systolic blood pressure in later life after adjustment for current size. To examine whether this association is explained by intrauterine or genetic factors, we investigated birth weight and blood pressure data in 61 dizygotic and 53 monozygotic adolescent twin pairs.

Methods Birth weight was obtained from the mothers. Blood pressure measurements were performed six times at rest and during mental stress.

Results The dizygotic, but not the monozygotic, twins with the lowest birth weight from each pair had a systolic blood pressure measured at rest and during the reaction time experiment that was higher compared to their cotwins with the highest birth weight (dizygotic twins: blood pressure at rest, 119.4±9.7 mm Hg vs. 117.3±8.5 mm Hg [*P*=0.07] and during a reaction time task, 126.2±10.8 vs. 123.6±9.5 [*P*=0.09]; monozygotic twins: blood pressure at rest 117.4±6.4 vs. 118.4±9.0 [*P*=0.4] and during a reaction time task 122.9±8.4 vs. 124.2±10.8 [*P*=0.2]). The differences in blood pressure between the cotwins with the lowest and the cotwins with the highest birth weight were different in dizygotic compared to monozygotic twin pairs (for blood pressure at rest, *P*=0.05; for blood pressure during reaction time, *P*=0.03). After adjustment for differences in current weight, intrapair differences in birth weight were negatively and significantly associated with differences in systolic blood pressure at rest and during the reaction time task in dizygotic twins (regression coefficient: -5.7 mm Hg/kg [95% confidence interval: [-10.4 to -1.0] and -6.3 [-12.7 to 0], respectively), but not in monozygotic twins (-0.1 [-5.4 to 5.2] and +3.5 [-1.8 to 8.8], respectively). Interaction analysis indicated that the associations were different between dizygotic twins and monozygotic twins (*P*=0.1 and *P*<0.05, respectively).

Conclusions These data suggest that genetic factors may play an important role in the association between birth weight and blood pressure.

Introduction

Epidemiological studies have consistently shown an inverse association between birth weight and systolic blood pressure from childhood to adulthood.[1-5] One leading theory postulates that intrauterine programming in response to fetal malnutrition induces permanent changes in the structure and function of organs, which cause raised blood pressure.[6] However, human exposure to famine in utero did not result in a significantly higher blood pressure.[7,8] Alternatively, it has been proposed that genetic factors influencing both birth weight and blood pressure could explain the relationships between these two factors.[9] In other words, the genotype responsible for raised blood pressure may itself cause retarded fetal growth in utero.

Twin studies offer a unique opportunity to distinguish between intrauterine and genetic influences.[10] Specifically, differences within dizygotic twin pairs are a function of both genetic and nongenetic factors, whereas differences within monozygotic (identical) pairs are almost completely caused by nongenetic factors.[10] If genetic factors do not play a role in the association between birth weight and blood pressure, it could be expected that both for dizygotic and for monozygotic twins the twin with the lowest birth weight from each pair will also have the highest blood pressure compared to the cotwin with the highest birth weight. In addition, negative associations between intrapair differences in birth weight and intrapair differences in blood pressure should exist both in dizygotic and in monozygotic twins. If, however, genetic factors do play a role, these associations would hold true only for dizygotic twins, not for monozygotic twins. In two previous twin studies, it has been suggested that the association between birth weight and blood pressure is independent from genetic factors.[11,12] However, these studies[11,12] could not specifically examine differences between dizygotic and monozygotic twins, because the results of the intrapair analyses of the differences in birth weight with differences in blood pressure in both dizygotic and monozygotic twins were not statistically significant in either study. To re-examine this issue, we analyzed birth weight and blood pressure data in a large group of adolescent twin pairs still living with their parents. Blood pressure was measured at rest and during mental stress, which is an important early predictor for the development of essential hypertension.[13,14]

Methods

Subjects

This study is part of a larger project in which cardiovascular risk factors were studied in 160 adolescent twin pairs and their parents.[15-17] Addresses of twins living in Amsterdam and neighboring cities were obtained from City Council population registries. Twins still living with their biological parents were contacted by letter. From the families initially willing to participate several had to be excluded because not all four family

members could find the time to participate or could speak Dutch sufficiently. After including sufficient monozygotic twins only dizygotic twins were included, in order to create approximately equal groups of monozygotic and dizygotic twins. Overall, between 30 and 40% of the families complied.[15] Zygosity was determined as described in detail previously.[15] A questionnaire was used to gather information on various factors including the use of medication and smoking behavior. The maternal questionnaire included questions regarding birth weight and gestational age of their children. This questionnaire was sent to the mothers a few weeks ahead of their visit to our department, allowing them to obtain birth data from birth certificates. Opposite-sex dizygotic twin pairs were excluded because of the effects of sex differences within a pair on both birth weight and blood pressure. Subjects using oral contraceptives were excluded for these analyses. None of the subjects used any other medication that may affect blood pressure. Thus, 53 dizygotic and 61 monozygotic twin pairs were eligible for analysis.

Measurements

Height and weight were measured in a standardized way. After acclimatization blood pressure was measured six times at rest and during reaction time (RT) and mental arithmetic (MA) tasks as described in detail previously.[15] During RT, subjects had to press a 'yes'-button when a high tone and a 'no'-button when a low tone was heard over the earphones. During MA, subjects had to add up three numbers that were presented in succession on a television screen. After 5 seconds an answer to the addition problem appeared on the screen. Subjects were asked to press the 'yes'-button when a correct answer and the 'no'-button when a wrong answer appeared on a screen. The means of the 6 measurements at rest and during both stress tasks were calculated. All blood pressure measurements were performed with an oscillometric technique (Dinamap 845XT, Critikon Inc).

Statistical Methods

In the total group, linear regression analysis was used to investigate the influence of birth weight on blood pressure after adjustment for sex and after additional adjustment for current weight.[1-5] Associations of current weight with birth weight and blood pressure were investigated with correlation analysis after adjustment for sex. An interaction analysis was performed to investigate whether zygosity, current weight or current body-mass index influenced the associations between birth weight and blood pressure by introducing a product term of these variables and birth weight into the regression model. The paired *t* test was used to compare twins with the lowest birth weight from each pair with their cotwins with the highest birth weight. For this analysis, 2 dizygotic and 2 monozygotic twin pairs had to be excluded because the birth weight of the twins within a pair was equal. The differences in dizygotic twin pairs and in monozygotic twin pairs were compared using the independent samples *t* test. Linear

Table 1. Association between birth weight and blood pressure in twins

Variable	Beta (95%-CI)*	*P*
Adjusted for sex:		
Systolic blood pressure		
at rest	-0.5 (-2.6 to 1.5)	0.6
during reaction time task	-2.1 (-4.5 to 0.3)	0.09
during mental arithmetic task	-2.8 (-5.6 to 0)	0.05
Adjusted for sex and current weight:		
Systolic blood pressure		
at rest	-1.9 (-3.9 to 0.0)	0.05
during reaction time task	-3.6 (-6.0 to -1.2)	<0.01
during mental arithmetic task	-4.6 (-7.4 to -1.8)	<0.01

*Beta (95%-CI) in mm Hg/kg.
CI indicates confidence interval

regression analysis was used to analyze whether intrapair differences in birth weight influenced intrapair differences in blood pressure before and after adjustment for differences in current weight in dizygotic and monozygotic twins (including the 4 twin pairs in which the birth weight of the twins within a pair was equal). In order to create a wide range in intrapair differences with both positive and negative values, intrapair differences in birth weight were calculated by randomly subtracting the cotwin with the lowest birth weight from the cotwin with the highest birth weight or vice versa. After ensuring that the regression lines passed through the origin in both dizygotic and monozygotic twins (i.e. the intercept was not significantly different from 0), interaction analysis was performed to investigate whether zygosity influenced the associations between intrapair differences in birth weight and differences in blood pressure, A two-tailed P-value < 0.05 was considered significant. All analyses were performed on a personal computer using the statistical software package SPSS version 7.5 (SPSS Inc).

Results

In the total group of twins, negative associations between birth weight and blood pressure were found after adjustment for sex, although the association with blood pressure at rest and during RT was not significant (Table 1, upper panel). After adjustment for sex, current weight was associated with birth weight ($r= 0.26$, $P<0.001$) and blood pressure measured at rest, during RT and during MA ($r= 0.32$; $r=0.23$; and $r= 0.23$, respectively, $P<0.001$) more strongly than was body-mass index (data not shown). After additional adjustment for current weight, the negative associations of birth weight with systolic blood pressure were strengthened (Table 1, lower panel). Interaction analysis indicated that these associations were not significantly modified by zygosity, current weight or current body-mass index (data not shown).

Table 2. Clinical characteristics of the cotwins with the lowest and the highest birth weight in dizygotic and monozygotic twin pairs

	Dizygotic Twin Pairs			Monozygotic Twin Pairs		
Variable	Cotwins with the lowest birth weight	Cotwins with the highest birth weight	*P*	Cotwins with the lowest birth weight	Cotwins with the highest birth weight	*P*
Birth weight, g	2246±493	2626±558	<0.001	2336±528	2636±485	<0.001
GA, weeks	36±8.4	36±8.4	-	37±2.8	37±2.8	-
n (male/female)	59(32/27)	59(32/27)	-	51(30/21)	51(30/21)	-
Age, years	17.0±1.7	17.0±1.7	-	16.0±1.8	16.0±1.8	-
BMI, kg/m^2	20.0±1.9	20.3±2.2	0.5	19.5±2.2	19.7±2.2	0.2
Current weight, kg	59.9±7.8	61.8±10.1	0.09	57.7±9.6	58.9±9.3	0.03
Smoking, n	7	9	-	4	4	-
SBP, mm Hg						
at rest	119.4±9.7	117.3±8.5	0.07	117.4±6.4	118.2±9.0	0.4
during RT	126.2±10.8	123.6±9.5	0.09	122.9±8.4	124.2±10.8	0.2
during MA	129.2±12.6	128.1±12.6	0.6	128.1±9.2	128.2±10.9	0.9

Mean±SD.

GA indicates gestational age; BMI, body mass index; SBP, systolic blood pressure; RT, reaction time task; MA mental arithmetic task.

Comparison between cotwins with the lowest and cotwins with the highest birth weight

Birth weight and gestational age were similar in dizygotic and monozygotic twins (Table 2). The differences in birth weight between the cotwins with the lowest birth weight and those with the highest birth weight from each pair were similar for dizygotic and monozygotic twin pairs (380 g and 300 g, respectively; *P* for the difference, 0.2; Table 2). Both dizygotic and monozygotic twins with the lowest birth weight from each pair were lighter than their cotwins with the highest birth weight, whereas BMI was similar. The dizygotic twins with the lowest birth weight had a systolic blood pressure measured at rest and during RT that was higher than that of their cotwins with the highest birth weight. However, the monozygotic twins with the lowest birth weight had a systolic blood pressure that was similar to that of their cotwins with the highest birth weight (Table 2). The differences in blood pressure between the cotwins with the lowest and the cotwins with the highest birth weight were different in dizygotic compared to monozygotic twin pairs (for blood pressure at rest, P=0.05; for blood pressure during RT, P=0.03).

Associations between intrapair differences

To further characterize the relation between birth weight and blood pressure, we determined the associations between intrapair differences in birth weight and differences in blood pressure. Table 3 shows that intrapair differences in birth weight were negatively associated with differences in systolic blood pressure at rest and during

Table 3. Associations between intrapair differences in birth weight and differences in systolic blood pressure in dizygotic and monozygotic twin pairs

	Dizygotic Twin Pairs		**Monozygotic Twin Pairs**	
Variable	Beta (95%-CI)*	*P*	Beta (95%-CI)*	*P*
Unadjusted:				
Systolic BP, mm Hg				
at rest	-3.7 (-8.1 to 0.8)	0.10	+0.4 (-4.7 to 5.6)	0.9
during RT	-5.2 (-11.0 to 0.7)	0.08	+3.7 (-1.4 to 8.8)	0.2
during MA	-0.6 (-8.3 to 7.0)	0.9	+1.6 (-4.2 to 7.3)	0.6
Adjusted for differences in current weight:				
Systolic BP, mm Hg				
at rest	-5.7 (-10.4 to -1.0)	0.02	-0.1 (-5.4 to 5.2)	0.9
during RT	-6.3 (-12.7 to 0)	0.05	+3.5 (-1.8 to 8.8)	0.2
during MA	-2.6 (-11.0 to 5.7)	0.5	+1.0 (-4.9 to 7.0)	0.7

*Beta (95%-CI) in mm Hg/kg.
CI indicates confidence interval; BP, blood pressure; RT, reaction time task; MA, mental arithmetic task.

RT in dizygotic twins, but not in monozygotic twins. After adjustment for differences in current weight, intrapair differences in birth weight were significantly and negatively associated with differences in blood pressure at rest and during RT (Table 3, lower panel). For example, a positive difference in birth weight of 1 kg within pairs was associated with a negative difference in systolic blood pressure at rest of 5.7 mm Hg in dizygotic twin pairs and a negative difference of 0.1 mm Hg in monozygotic twin pairs. Interaction analysis indicated that the associations were significantly different between dizygotic twins and monozygotic twins for systolic blood pressure during RT ($P<0.05$), and the associations tended to be significantly different for systolic blood pressure at rest ($P=0.1$).

If subjects with a gestational age shorter than 37 weeks (21 dizygotic and 24 monozygotic twin pairs) were excluded the results were similar. Adjustment for gestational age or (differences in) smoking did not change the results. For diastolic blood pressure comparable results were obtained as for systolic blood pressure, but the differences between dizygotic and monozygotic twins were not significant (data not shown).

Discussion

In accordance with previous studies in singletons,[1-5] we found negative associations between birth weight and blood pressure after adjustment for current weight in twins. In dizygotic twin pairs, the twins with the lowest birth weight from each pair tended to have a higher blood pressure compared to their cotwins with the highest birth weight. In addition, significant negative associations between intrapair differences in birth weight

and intrapair differences in systolic blood pressure measured at rest and during RT were observed after adjustment for differences in current weight. To eliminate the influence of genetic factors on these associations, we also studied monozygotic twin pairs. Despite a similar difference in birth weight as in dizygotic twins, the monozygotic twins with the lowest birth weight had blood pressures similar to those of their cotwins with the highest birth weight. In addition, in the monozygotic twins, there was no negative association between intrapair differences in birth weight and differences in systolic blood pressure. When dizygotic and monozygotic twins were compared, the differences in blood pressure between the twins with the lowest birth weight and their cotwins with the highest birth weight were significantly different for blood pressure measured at rest and during RT. These data provide the first evidence that genetic factors influence the association between the variance in birth weight and that in blood pressure.

Because the intrapair analyses could not exclude a negative association between birth weight and blood pressure in monozygotic twins, the possibility that intrauterine factors also influence the relationship between birth weight and blood pressure cannot be ruled out. However, the comparison of dizygotic twins with monozygotic twins demonstrates that elimination of genetic factors abolishes the strong association between birth weight and blood pressure. Therefore, our results suggest that genetic factors may play an important role in the birth weight-blood pressure relationship, but cannot exclude additional intrauterine influences.

Our results seem contradictory to the conclusions from two previous twin studies.[11,12] However, these studies[11,12] could not specifically examine differences between dizygotic and monozygotic twins, because the results of the intrapair analyses of the differences in birth weight with differences in blood pressure in both dizygotic and monozygotic twins were not statistically significant in either study. In the study of Dwyer et al. only 16 monozygotic twins were included[11] and the results of Poulter et al.[12] are also open to another interpretation. Poulter et al. compared intrapair differences in blood pressure in four strata of intrapair differences in birth weight (0, 1-500 g, 501-1000g and greater than 1000 g) and concluded that the relationship between birth weight and blood pressure is probably independent of genetic factors. However, a closer look at their data shows that the opposite may be true. As a first intrapair analysis, blood pressure levels between cotwins with the highest and the lowest birth weight from each pair should be compared in all dizygotic and monozygotic twins, which can be calculated from the data presented in their paper. After adjustment for confounding factors, the 203 dizygotic, but not the 140 monozygotic, twins with the highest birth weight had a systolic blood pressure that was significantly lower compared to their cotwins with the lowest birth weight (difference in blood pressure: -5.37 mm Hg, $P<0.05$ and -0.85 mm Hg, $P=0.8$, respectively). This suggests that the relationship between birth weight and blood pressure within twin pairs differs between dizygotic and monozygotic twins, which is in accordance with our results.

Around two thirds of monozygotic twins are monochorionic (i.e. share a placenta), whereas all dizygotic twins are dichorionic (i.e. have separate placentas). Therefore, it could be argued that, besides genetic factors, intrauterine factors may also differ between dizygotic and monozygotic twins and may be the cause of the difference in the intrapair association between birth weight and blood pressure. We do not have data on chorionicity in our group of monozygotic twins, but a recent study in monozygotic twins demonstrated that, in both monochorionic and dichorionic monozygotic twins, the twins with the lowest birth weight from each pair had a blood pressure that was lower than their cotwins with the highest birth weight.[18] Although focused on another subject and based on a relatively small number of twins, these data demonstrate that differences in the intra-uterine environment between dizygotic and monozygotic twins are not a likely explanation for the differences in the intrapair associations between birth weight and blood pressure.

In animal studies, it has been demonstrated that maternal undernutrition during pregnancy retards fetal growth and elevates blood pressure.[19] This may, however, reflect the selective survival of fetuses genetically susceptible to hypertension, with a possible role for insulin resistance.[20] Interestingly, in human studies of maternal undernutrition,[7,8] birth weight in the offspring was lowered, but blood pressure in later life was not elevated, which is consistent with an important role for genetic factors.

The size of the association between intrapair differences in birth weight and differences in blood pressure in dizygotic twins is larger than the size of the association between birth weight and blood pressure observed in previous studies of singletons.[1-5] This is probably due to the elimination of various confounding characteristics, such as gestational age, maternal factors (e.g. height, weight gain, smoking and blood pressure during pregnancy), social class, birth order (in relation to other siblings), ethnic origin and sex.

To our knowledge, this is the first study to examine the association between birth weight and blood pressure measured during mental stress. It has been demonstrated that an enhanced cardiovascular response to stress is an early predictor for the development of essential hypertension.[13,14] We found that the association of birth weight with blood pressure during both stress tasks was higher than with blood pressure measured at rest. This emphasizes the increased risk of the development of future hypertension in subjects born with a low birth weight and suggests that the mechanism responsible for the enhanced response to stress plays a role in the association between low birth weight and hypertension. The associations between intrapair differences in birth weight and differences in blood pressure were in the same direction for blood pressure at rest, during RT and during MA. However, the differences between dizygotic and monozygotic twins were less clear cut for blood pressure measured during MA than at rest and during RT. Although it has been demonstrated that heritability of blood pressure increases during both MA and RT compared to blood pressure at rest,[15,21] our

findings suggest that genetic factors are less important in the association of birth weight with blood pressure during MA than with blood pressure at rest and during RT.

In our group of twins, the negative associations between birth weight and blood pressure were strengthened after adjustment for current weight, which showed a stronger association with both birth weight and blood pressure than did body-mass index. Furthermore, the negative associations between intrapair differences in birth weight and differences in blood pressure in dizygotic twin pairs were strengthened after the adjustment for differences in current weight. This is in accordance with previous studies that show that adjustment for current size (i.e. weight in young subjects and body-mass index in adults) increases the strength of the association of birth weight with blood pressure.[3,4, 22-24] Adjusting for current size has often been justified on the grounds that birth weight is positively related to later size, and also that current weight is positively related to blood pressure, and if not adjusted for could obscure a negative relation between birth weight and blood pressure.[25] However, Lucas et al. suggested that this interpretation is incorrect and proposed that it is the change in size from birth to later life rather than size at birth itself that is implicated.[25] In our study, the associations were strengthened after adjustment for current size. This suggests that both size at birth and change in size from birth to later life are associated with higher blood pressure in later life.

In our study, no significant interaction of either current body-mass index or weight on the relationship between birth weight and blood pressure could be observed, suggesting that the strength of the association between birth weight and blood pressure was not larger in subjects with a high than in subjects with a low current body-mass index or weight. This is consistent with the results from some studies,[26,27] but is in contrast to the findings of others.[5,23,24]

It has been suggested that improvement of fetal nutrition and thereby, intrauterine growth may prevent the development of cardiovascular disease.[6] However, if the relationship between low birth weight and raised blood pressure is caused by genetic factors, improvement of fetal nutrition may not prevent the development of raised blood pressure. Low birth weight may only serve as a marker of increased risk of raised blood pressure.

In summary, we found a tendency towards higher blood pressure levels in the twins with the lowest birth weight from each pair compared to their cotwins with the highest birth weight and negative associations between intrapair differences in birth weight and differences in blood pressure in dizygotic twins, but not in monozygotic twins. This difference in the birth weight-blood pressure relationship between dizygotic and monozygotic twin pairs suggests that genetic factors may play an important role in the association between birth weight and blood pressure.

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9

Low birth weight is associated with increased sympathetic activity Dependence on genetic factors

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Abstract

Background Low birth weight may be associated with high blood pressure in later life through genetic factors, an association that may be explained by alterations in sympathetic and parasympathetic activity. We examined the association of birth weight with cardiac pre-ejection period and respiratory sinus arrhythmia (indicators of cardiac sympathetic and parasympathetic activity, respectively), and with blood pressure in 53 dizygotic and 61 monozygotic adolescent twin pairs.

Methods Birth weight of the twins was obtained from the mothers. Pre-ejection period and respiratory sinus arrhythmia were measured with electrocardiography and impedance cardiography at rest, during a reaction time task and during a mental arithmetic task.

Results In the overall sample, lower birth weight was significantly associated with shorter pre-ejection period at rest, during the reaction time task and during the mental arithmetic task ($P=0.0001$, $P<0.0001$, and $P=0.0001$, respectively), and with larger pre-ejection period reactivity to the stress tasks ($P=0.02$ and $P=0.06$, respectively). In within-pair analyses, differences in birth weight were associated with differences in pre-ejection period at rest and during both stress tasks in dizygotic twin pairs ($P=0.01$, $P=0.06$, and $P=0.2$, respectively), but not in monozygotic twin pairs ($P=0.9$, $P=1.0$, and $P=0.5$, respectively). Shorter pre-ejection period explained approximately 63-84% of the birth weight-blood pressure relation.

Conclusions Low birth weight is associated with increased sympathetic activity, and this explains a large part of the association between birth weight and blood pressure. In addition, our findings suggest that the association between birth weight and sympathetic activity depends on genetic factors.

Introduction

Low weight at birth is associated with raised blood pressure in later life.[1] This association has been attributed to a programmed response to intrauterine malnutrition,[2] but the alternative view is that genetic factors influencing both birth weight and blood pressure are responsible.[3]

Studies in dizygotic and monozygotic twin pairs offer a unique opportunity to investigate the influence of intrauterine and genetic factors. Specifically, differences within dizygotic twin pairs are a function of both genetic and nongenetic factors, whereas differences within monozygotic (genetically identical) pairs can only be caused by nongenetic factors. In our cohort of adolescent twin pairs, within-pair differences in birth weight were associated with differences in blood pressure in dizygotic twin pairs, but not in monozygotic twin pairs.[4] These data are consistent with several other twin studies,[5-7] and demonstrated that genetic factors play an important role in the association between birth weight and blood pressure in later life.[4]

The mechanisms that underlie the association between birth weight and blood pressure are largely unknown. Changes in autonomic nervous system activity are involved in the development of high blood pressure.[8] However, it is not known whether birth weight is associated with the activity of the sympathetic and parasympathetic nervous systems, nor whether any such associations can explain the birth weight-blood pressure relationship.

To examine the association of birth weight with indicators of cardiac autonomic nervous activity and blood pressure, and the possible influence of genetic factors, we investigated birth weight, the cardiac pre-ejection period and respiratory sinus arrhythmia at rest and during mental stress in dizygotic and monozygotic twin pairs. Cardiac pre-ejection period and respiratory sinus arrhythmia are indicative of sympathetic and parasympathetic nervous system control of the heart, respectively.[9-11]

Methods

Subjects

This study is part of a larger project in which cardiovascular risk factors have been studied in 160 adolescent twin pairs and their parents.[4;12;13] A maternal questionnaire included questions regarding birth weight and gestational age of their children.[4] Opposite-sex dizygotic twin pairs were excluded because of the effects of sex differences within a pair. Individuals using oral contraceptives were excluded. None of the participants used any other medication that may affect measurements of cardiac autonomic nervous system or blood pressure. Thus, 53 dizygotic and 61 monozygotic twin pairs were eligible for analysis. The study was approved by an institutional review committee and the subjects gave informed consent.

Experimental protocol

After acclimatization, measurements were performed at rest and during reaction time and mental arithmetic tasks as described in detail previously.[12] During the reaction time task, participants had to press a 'yes'-button when a high tone and a 'no'-button when a low tone was heard. During the mental arithmetic task, participants had to add up numbers that were presented on a television screen.

Measurements

The pre-ejection period is the time interval between the onset of ventricular depolarization and the opening of the semilunar valves. The pre-ejection period is an index of cardiac contractility that indicates beta-adrenergic inotropic drive to the left ventricle:[9;10] the shorter the pre-ejection period, the stronger the sympathetic control of heart rate. The pre-ejection period was measured as described previously.[14;15] Pre-ejection period was defined as the time in ms from the onset of the Q-wave on the ECG to the B-point (the opening of the aortic valves) in the impedance cardiogram. Pre-ejection period reactivity was calculated by subtracting the pre-ejection period during the stress tasks from the pre-ejection period at rest. A larger pre-ejection period reactivity then is an indicator of an increased sympathetic activity.[9;10]

Respiratory sinus arrhythmia refers to the cyclic variations in heart rate that are related to respiration. These variations are largely due to respiratory modulation of the outflow of the vagal nerve: the larger the respiratory sinus arrhythmia, the stronger the vagal control of heart rate.[11] Respiratory sinus arrhythmia was measured as described previously.[16;17] Respiratory sinus arrhythmia reactivity was calculated by subtracting the respiratory sinus arrhythmia during the stress tasks from the respiratory sinus arrhythmia at rest.

Blood pressure measurements from an arm cuff around the non-dominant arm were performed automatically with an oscillometric technique.[4]

Statistical Methods

Data are expressed as mean ± SD, unless stated otherwise. The paired Student's t-test was used to compare measurements before and during mental stress. In the overall sample, linear regression analysis was used to investigate the influence of birth weight on heart rate, respiratory sinus arrhythmia and pre-ejection period, and to analyse within-pair associations. Interaction analysis was performed to investigate whether the associations were modified by zygosity, current body mass index or current weight. Linear regression analysis was also used to investigate whether the association between birth weight and blood pressure[4] remained when allowing for the pre-ejection period. A two-tailed P-value < 0.05 was considered significant. All analyses were performed using the statistical software package SPSS version 9.0 (SPSS Inc).

Table 1. Clinical characteristics of the twins

	Dizygotic twins	Monozygotic twins
n (men)	106 (60)	122 (68)
Age, years	17.0±1.7	16.0±1.8
Birth weight, g	2438±547	2488±519
Gestational age, weeks	37±3	36±8
Body mass index, kg/m^2	20.2±2.0	19.6±2.2
Systolic blood pressure, mmHg	118.3±9.0	117.8±7.8
Diastolic blood pressure, mmHg	67.3±6.5	66.3±5.9
Total cholesterol, mmol/L	4.1±0.7	4.3±0.8
Smoking, n (men)	14 (10)	10 (8)
Heart rate, bpm		
At rest	67.4±11.5	68.7±10.4
During reaction time task	72.8±13.5*	74.0±11.7*
During mental arithmetic task	75.9±15.7*	78.1±13.2*
Reactivity to reaction time task	5.4±4.3	5.4±4.2
Reactivity to mental arithmetic task	8.5±7.9	9.5±6.7
Pre-ejection period, ms		
At rest	118.8±14.3	114.8±15.3
During reaction time task	115.5±17.2*	110.5±20.2*
During mental arithmetic task	111.8±19.3*	105.4±19.5*
Reactivity to reaction time task	3.3±9.1	4.4±10.4
Reactivity to mental arithmetic task	7.0±13.8	9.5±10.8
Respiratory sinus arrhythmia, ms		
At rest	103.3±55.5	116.1±59.2
During reaction time task	89.4±51.5*	94.1±48.8*
During mental arithmetic task	79.4±51.3*	83.7±49.4*
Reactivity to reaction time task	13.5±28.0	21.0±28.8
Reactivity to mental arithmetic task	23.7±32.8	31.9±35.7

*P<0.001 vs. at rest

Results

The characteristics of the twins are shown in Table 1. Heart rate was significantly increased and pre-ejection period and respiratory sinus arrhythmia were significantly decreased during mental stress.

Association of birth weight with pre-ejection period and respiratory sinus arrhythmia in the overall sample (Table 2)

Birth weight was not significantly related to heart rate measurements. Birth weight was positively associated with the absolute pre-ejection period and negatively with the pre-ejection period reactivity to mental stress. In other words, low birth weight was associated with a shorter pre-ejection period and a higher reactivity to mental stress,

Table 2. Associations of birth weight with heart rate, pre-ejection period and respiratory sinus arrhythmia in twins

	Beta (95%-CI)*	*P*
Heart rate		
At rest	-0.4 (-3.1 to 2.4)	0.8
During reaction time task	-1.2 (-4.4 to 1.9)	0.4
During mental arithmetic task	-2.0 (-5.7 to 1.5)	0.2
Reactivity to reaction time task	-0.9 (-1.95 to 0.2)	0.1
Reactivity to mental arithmetic task	-1.7 (-3.6 to 0.1)	0.06
Pre-ejection period, ms		
At rest	6.8 (3.1 to 10.6)	0.0001
During reaction time task	9.7 (5.0 to 14.5)	<0.0001
During mental arithmetic task	9.8 (4.8 to 14.7)	0.0001
Reactivity to reaction time task	-2.9 (-5.4 to -0.4)	0.02
Reactivity to mental arithmetic task	-3.0 (-6.1 to 0.2)	0.06
Respiratory sinus arrhythmia, ms		
At rest	2.3 (12.7 to 17.3)	0.8
During reaction time task	4.7 (-8.3 to 17.6)	0.5
During mental arithmetic task	4.6 (-8.2 to 17.4)	0.5
Reactivity to reaction time task	-3.3 (-10.7 to 4.0)	0.4
Reactivity to mental arithmetic task	-2.6 (-11.5 to 6.3)	0.6

*Beta (95%-CI) per kg birth weight after adjustment for age, sex and current body mass index

Table 3. Associations of within-pair differences in birth weight (independent variable) with within-pair differences in pre-ejection period and respiratory sinus arrhythmia (dependent variables) in twins

	Dizygotic Twin Pairs		**Monozygotic Twin Pairs**	
	Beta (95%-CI)*	*P*	Beta (95%-CI)*	*P*
Pre-ejection period, ms				
At rest	12.8 (3.0 to 22.7)	0.01	-0.7 (-9.0 to 7.5)	0.9
During reaction time task	13.1 (1.8 to 24.4)	0.02	-0.2 (-9.7 to 9.5)	1.0
During mental arithmetic task	9.7 (-3.6 to 23.0)	0.2	-3.9 (-14.8 to 7.0)	0.5
Reactivity to reaction time task	-0.2 (-6.4 to 5.9)	0.9	-0.6 (-7.6 to 6.5)	0.9
Reactivity to mental arithmetic task	1.3 (-8.1 to 10.7)	0.5	3.1 (-4.9 to 11.2)	0.4
Respiratory sinus arrhythmia, ms				
At rest	8.2 (-37.9 to 54.2)	0.7	24.1 (-25.8 to 74.0)	0.3
During reaction time task	21.5 (-17.3 to 60.3)	0.3	27.2 (-5.4 to 59.8)	0.1
During mental arithmetic task	18.2 (-18.3 to 54.6)	0.3	22.4 (-11.3 to 56.1)	0.2
Reactivity to reaction time task	-18.1 (-41.1 to 4.9)	0.1	-5.5 (-34.7 to 23.8)	0.7
Reactivity to mental arithmetic task	-10.1 (-35.4 to 15.1)	0.4	1.8 (-30.4 to 34.1)	0.9

*Beta (95%-CI) per kg birth weight after adjustment for differences in current BMI.

both indicative of an increased sympathetic activity. These associations were not significantly modified by zygosity, current weight or current body mass index (P >0.6). Specifically, the association between birth weight and the pre-ejection period was similar in dizygotic and monozygotic twins (at rest: 6.2 [95%-confidence interval: -0.9 to 11.4], P=0.02 vs. 8.4 [2.8-13.9], P=0.004; during the reaction time test: 8.0 [1.7-14.3], P=0.03 vs. 12.5 [5.1-19.7], P=0.001; during the mental arithmetic test: 8.4 [1.4-15.4], P=0.02 vs. 12.5 [5.5-19.6], P=0.001).

Birth weight was not associated with respiratory sinus arrhythmia.

Within-pair association of birth weight with pre-ejection period and respiratory sinus arrhythmia (Table 3)

Within-pair differences in birth weight were positively associated with differences in the pre-ejection period at rest and during the reaction time task in dizygotic twins, but not in monozygotic twins. For example, in dizygotic twins, a difference in birth weight of 1 kg within pairs was associated with a pre-ejection period at rest that was 12.8 ms *shorter* (indicative of increased cardiac sympathetic activity) in the twin with the lowest birth weight compared to the cotwin with the highest birth weight after adjustment for differences in current body mass index. The associations of birth weight with the pre-ejection period at rest, during the reaction time task and during the mental arithmetic task were different between dizygotic twins and monozygotic twins (P=0.04, P=0.07, and P=0.1, respectively). Within-pair differences in birth weight were not associated with the pre-ejection period reactivity. Within-pair differences in birth weight were also not significantly associated with differences in respiratory sinus arrhythmia (Table 2) or heart rate (data not shown).

When subjects with a gestational age shorter than 37 weeks (21 dizygotic and 24 monozygotic twin pairs) were excluded the results were similar. When the analyses were performed without adjustment for current body mass index or after adjustment for current weight instead of current body mass index, the results were also similar (data not shown).

Association of pre-ejection period and respiratory sinus arrhythmia with blood pressure (Tables 4 and 5)

The pre-ejection period as well as the pre-ejection period reactivity were associated with systolic blood pressure, indicating that increased sympathetic activity was associated with higher blood pressure. In addition, respiratory sinus arrhythmia was negatively associated with systolic blood pressure (Table 4), indicating that decreased parasympathetic activity was associated with higher blood pressure.

Within-pair differences in the pre-ejection period were negatively associated with differences in systolic blood pressure (Table 5), indicating that increased sympathetic activity was associated with higher blood pressure within twin pairs. Within-pair differences in respiratory sinus arrhythmia were negatively associated with differences

Table 4. Associations of pre-ejection period and respiratory sinus arrhythmia with systolic blood pressure in the overall sample of twins

	Beta (95%-CI)*	*P*
Pre-ejection period		
At rest	-0.18 (-0.24 to -0.11)	<0.00001
During reaction time task	-0.16 (-0.21 to -0.11)	<0.00001
During mental arithmetic task	-0.15 (-0.20 to -0.10)	<0.00001
Reactivity to reaction time task	0.20 (0.10 to 0.31)	0.0001
Reactivity to mental arithmetic task	0.12 (0.04 to 0.20)	0.004
Respiratory sinus arrhythmia, ms		
At rest	-0.02 (-0.04 to -0.002)	0.03
During reaction time task	-0.04 (-0.06 to -0.02)	0.0001
During mental arithmetic task	-0.05 (-0.07 to -0.03)	<0.00001
Reactivity to reaction time task	0.03 (-0.002 to 0.07)	0.06
Reactivity to mental arithmetic task	0.05 (0.02 to 0.08)	0.002

*Beta (95%-CI) mm Hg per ms after adjustment for age, sex and current BMI

in systolic blood pressure, but only the association of respiratory sinus arrhythmia during the mental arithmetic task with blood pressure in dizygotic twins was statistically significant (Table 5).

Subsequently, we examined whether birth weight effects on sympathetic activity could explain the association between birth weight and blood pressure. After adjustment for pre-ejection period at rest, during the reaction time task or during the mental arithmetic task, the regression coefficient of the previously[4] described association between birth weight and blood pressure in the overall sample of twins (slope: -1.9 [95%-conficence interval: -3.9 to 0.0]; *P*=0.05) decreased by 63, 84 and 74%, respectively (slope after adjustment: -0.7 [-2.7 to 1.2], *P*=0.5; -0.3 [-2.2 to 1.5], *P*=0.7; and -0.5 [-2.4 to 1.4], *P*=0.6, respectively). This is illustrated in the figure.

Adjustment for pre-ejection period reactivity or respiratory sinus arrhythmia (or reactivity) did not influence the association between birth weight and blood pressure (data not shown). The results were similar when blood pressure during stress was used instead of blood pressure at rest (data not shown). All results were similar when adjusted for smoking and gestational age (data not shown).

Discussion

We report three novel findings. Firstly, in our overall sample, low birth weight was strongly associated with a shorter pre-ejection period and a larger pre-ejection period decrease in response to stress, which are indicative of increased sympathetic nervous system activity. Secondly, a shorter pre-ejection period was related to higher blood pressure, and statistically explained a large part of the association between birth weight

Table 5. Associations of within-pair differences in pre-ejection period and respiratory sinus arrhythmia (independent variables) with within-pair differences in systolic blood pressure (dependent variable) in twins

	Dizygotic Twin Pairs		**Monozygotic Twin Pairs**	
	Beta (95%-CI)*	*P*	Beta (95%-CI)*	*P*
Pre-ejection period, ms				
At rest	-0.18 (-0.30 to -0.06)	0.0003	-0.23 (-0.38 to -0.07)	0.005
During reaction time task	-0.18 (-0.28 to -0.08)	0.001	-0.15 (-0.28 to -0.01)	0.03
During mental arithmetic task	-0.15 (-0.24 to -0.06)	0.002	-0.16 (-0.27 to -0.04)	0.01
Reactivity to reaction time task	0.14 (-0.07 to 0.35)	0.2	-0.03 (-0.23 to 0.16)	0.7
Reactivity to mental arithmetic task	0.08 (-0.06 to 0.22)	0.3	0.05 (-0.12 to 0.22)	0.6
Respiratory sinus arrhythmia, ms				
At rest	-0.01 (-0.04 to 0.02)	0.4	-0.004 (-0.03 to 0.02)	0.8
During reaction time task	-0.03 (-0.06 to 0.003)	0.08	-0.02 (-0.06 to 0.02)	0.3
During mental arithmetic task	-0.04 (-0.08 to -0.01)	0.02	-0.03 (-0.07 to 0.01)	0.1
Reactivity to reaction time task	0.04 (-0.02 to 0.09)	0.2	-0.01 (-0.05 to 0.04)	0.8
Reactivity to mental arithmetic task	0.04 (-0.01 to 0.09)	0.1	0.02 (-0.02 to 0.06)	0.2

*Beta (95%-CI) mmHg per ms after adjustment for differences in current BMI.

and blood pressure. Thirdly, within-pair differences in birth weight were associated with differences in the pre-ejection period in dizygotic twins, but not in monozygotic twins, which demonstrates that the birth weight – sympathetic activity relationship depends on genetic factors.

The association between low birth weight and increased sympathetic activity in the overall sample is consistent with several experimental studies.[18;19] In addition, low birth weight is associated with high resting heart rate in adult life in middle-aged individuals.[20]

A shorter pre-ejection period was strongly associated with higher systolic blood pressure in these twin subjects. This is consistent with studies in singletons that investigated the association of blood pressure with sympathetic activity, as indicated by muscle sympathetic nerve activity,[21] plasma levels of norepinephrine[22] or spectral analysis of heart rate.[23]

The association between birth weight and blood pressure in this twin sample was remarkably similar to the well-established association in singletons (approximately -2 mm Hg per kg increase of birth weight[1]). This association diminished greatly (i.e. 63-84%) after adjustment for the cardiac pre-ejection period. These findings are consistent with the hypothesis that the association between birth weight and blood pressure can be explained, at least partially, by sympathetic nervous system activity.

The within-pair analyses suggest that the association between birth weight and sympathetic activity is dependent on genetic factors. This finding adds to our previous observation of a genetic explanation for the birth weight-blood pressure relationship in twins.[4] If the relationship between low birth weight and sympathetic activity depends on genetic factors, improvement of fetal nutrition may not prevent the development of increased sympathetic nervous system activity.

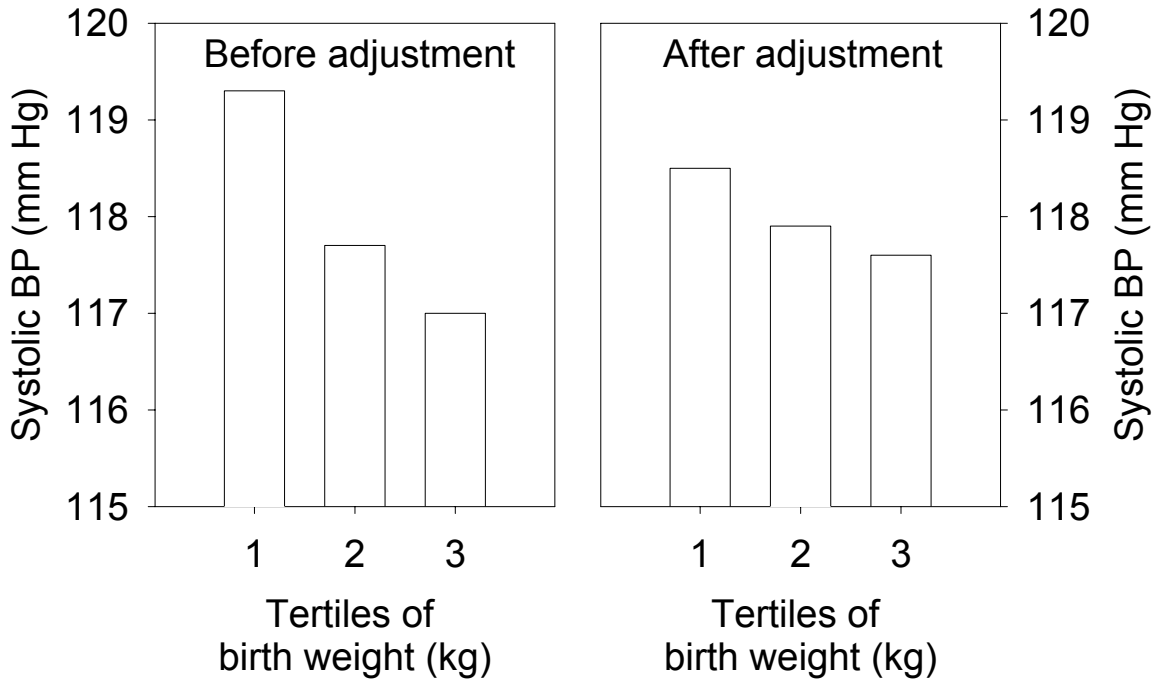

Figure. Illustration of the influence of adjustment for cardiac sympathetic activity on the birth weight-blood pressure relationship. Systolic blood pressure level is shown according to tertiles of birth weight before and after adjustment for the pre-ejection period during the reaction time task. Division points for tertiles: 2.25 and 2.75 kg.

Our findings are relevant for understanding the development of hypertension and cardiovascular disease. A recent meta-analysis demonstrated that, on average, a 1 kg higher birth weight is associated with a 2 mmHg lower blood pressure.[1] In clinical practice this may seem a small difference, but these are relevant differences between the mean values of populations. For example, lowering mean systolic blood pressure in a population by 2 mmHg corresponds to a 8% reduction in the risk of stroke.[24] Huxley et al. have recently concluded that the size of the association between birth weight and blood pressure may be overestimated due to publication bias,[25] but this conclusion was largely based on findings in very large population studies, in which birth weight data and/or blood pressure levels were self-reported, causing attenuation of the associations in these studies. In addition, our findings may have important implications that extend beyond blood pressure. An increased sympathetic activity may be important for the development of insulin resistance, atherosclerosis and cardiac hypertrophy.[26-28]

Differences in birth weight in twins can be used as a model for differences in birth weight in singletons, as a number of studies have demonstrated that birth weight in twins is associated with many variables that have been related to birth weight in singletons.[4-7;13;29-31]

It could be argued that, besides genetic factors, intrauterine factors in monozygotic twins may also be different from those in dizygotic twins. An important intrauterine difference between monozygotic and dizygotic twins is the placentation. Around two thirds of monozygotic twins are monochorionic (i.e. share a placenta), whereas all dizygotic twins are dichorionic (i.e. have separate placentas). We do not have data on chorionicity in our group of monozygotic twins, but we consider it unlikely that differences in chorionicity between dizygotic and monozygotic twins can fully explain the difference in the association of birth weight with indices of sympathetic activation and blood pressure. First, the overall association between birth weight and indices of sympathetic activation was similar in dizygotic and monozygotic twins. Second, others have shown that chorionicity did not influence the intrapair association between birth weight and blood pressure.[7;32] Furthermore, it should be noted that intrapair differences in birth weight in monozygotic twins have been related to intrapair differences in HDL cholesterol,[13] insulin sensitivity,[30] diabetes[33] and height,[34]demonstrating that the twin study design in general is quite capable of showing that intrauterine factors can influence adult outcome.

In summary, we have shown that low birth weight is associated with increased sympathetic activity, and that a large part of the association between birth weight and blood pressure is explained by this increase. In addition, the within-pair analyses suggest that the association between low birth weight and increased sympathetic activity depends on genetic factors.

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10

The association of low birth weight with insulin resistance is, at least in part, independent of genetic factors

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Abstract

Background Epidemiological studies have consistently shown an association of low birth weight with insulin resistance in later life after adjustment for current size. To examine whether the association between low birth weight and insulin resistance is explained by intrauterine or genetic factors, we investigated birth weight and insulin resistance in 17 dizygotic and 17 monozygotic young adult twin pairs.

Methods Birth weight was obtained from the mothers. Insulin resistance was calculated from fasting insulin and glucose levels using the homeostasis model assessment formula.

Results Intrapair differences in birth weight were negatively associated with differences in insulin resistance in both dizygotic twins and monozygotic twins (-3.0 units per kilogram birth weight [95%-confidence interval -8.7 to 2.7], P=0.3, and -13.2 [-25.0 to -1.3], P<0.05). Interaction analyses demonstrated that the association in dizygotic twins was not significantly different from the association in monozygotic twins. Interaction analyses further demonstrated that the associations were not significantly modified by current body mass index, weight or sex. The results were similar after adjustment for (differences in) body mass index.

Conclusions These data suggest that the association between low birth weight and insulin resistance is, at least in part, due to intrauterine factors.

Introduction

Epidemiological studies have consistently shown an inverse association between low birth weight and the development of type 2 diabetes.[1] This association may be mediated by alterations in insulin sensitivity, insulin secretion, or a combination of both.[1] One leading theory postulates that the association between birth weight and diabetes is due to intrauterine programming in response to fetal malnutrition that induces permanent changes in structure and function of organs, which may cause diabetes in later life.[2] According to this hypothesis, improvement of intrauterine nutrition may decrease the risk of diabetes in later life. The alternative hypothesis, however, suggests that the association between low birth weight and diabetes is due to genetic factors.[3] If the same genetic factor influences both birth weight and diabetes, this could at least partly account for the association of low birth weight with diabetes. In this case, improvement of nutrition may not decrease the risk of diabetes in later life.

Twin pairs offer a unique opportunity to investigate the effects of intrauterine nutrition and genetic influences.[4] Specifically, studying differences within monozygotic (genetically identical) twin pairs allows almost complete elimination of the influence of genotype on the association between size at birth and disease in later life.[4] It has been demonstrated that monozygotic diabetic twins have a lower birth weight compared with their non-diabetic co-twins.[5;6] This excludes the possibility that the association between birth weight and diabetes is explained entirely by genetic factors. The association between birth weight and diabetes may, in part, be mediated by insulin resistance, but the association between birth weight and insulin resistance has not been investigated in twins. If intrapair differences in birth weight are associated with intrapair differences in insulin resistance in monozygotic twins, this suggests that the association between these variables is due to intrauterine influences and independent of genetic factors.

We have therefore examined the association between birth weight and indicators of insulin resistance in a cohort of dizygotic and monozygotic twin pairs.

Methods

Subjects

Individuals were recruited from the Netherlands Twin Register as part of a large ongoing project in twin families.[7-9] The sample consisted of two cohorts (young adults, mean age 26 years and older adults, mean age 50 years). In the young cohort, reliable data on birth weight and gestational age were obtained from the mother, when, on average, the twins were aged 16.[10;11] A maternal questionnaire that included questions regarding birth weight and gestational age of their children was sent to the mothers a few weeks ahead of their visit to our department, allowing them to obtain birth data from birth certificates.[10;11] Zygosity was determined as described in detail previously.[12]

Table 1. Clinical characteristics of the twins

	Dizygotic twins	Monozygotic twins
n (men)	34 (14)	34 (20)
Age, years	26.3±2.4	25.9±2.8
Birth weight, kg	2.6±0.6	2.4±0.5
Body mass index, kg/m^2	23.6±5.6	22.1±2.4
Smoking, n	3	7
Fasting insulin, pmol/L*	51.5 (40.0-79.8)	41.0 (32.8-57.8)
Fasting glucose, mmol/L	5.1±0.6	4.9±0.5
Insulin sensitivity, HOMA*	11.0 (8.9-19.1)	9.5 (6.7-12.6)

Data are presented as mean±SD, or as *median (25th-75th percentile)

Subjects who did not fast from 23.00 hours the night before were excluded. The opposite-sex dizygotic twin pairs were excluded because of the effects of sex differences within a pair on both birth weight and glucose metabolism. Thus, 68 individuals (17 dizygotic and 17 monozygotic twin pairs) were eligible for analysis. The characteristics of the dizygotic and monozygotic twins are shown in Table 1. The study was approved by the Vrije Universiteit Ethics Committee, and all subjects gave written informed consent prior to their participation.

Measurements

Height and weight were measured in a standardized way. Plasma insulin concentrations were measured by RIA techniques (Immunoradiometric Assay, Medgenics Diagnostics, Fleurus, Belgium). The intra-assay and inter-assay coefficient of variation were 5% and 6%, respectively. Glucose in plasma was determined on a Hitachi 747 analyser (Roche) with gluco-quant glucose/HK reagent (Roche diagnostics, Mannheim, Germany). Insulin resistance was calculated using the homeostasis model assessment (HOMA) formula: insulin resistance (dimensionless) = fasting insulin (pmol/L) *fasting glucose (mmol/L) /22.4.[13] A questionnaire was used to gather information on various factors including the use of medication and smoking behaviour.

Statistical Methods

Data are expressed as mean ± SD, unless stated otherwise. Fasting insulin levels and insulin resistance were log-transformed to achieve normality before analysis. In the overall sample, linear regression analysis was used to investigate the influence of birth weight on fasting insulin, fasting glucose and insulin resistance after adjustment for age, sex and after additional adjustment for current body mass index. Linear regression analysis was used to analyze whether intrapair differences in birth weight influenced intrapair differences in fasting insulin, fasting glucose and insulin resistance before and after adjustment for differences in current body mass index in dizygotic and monozygotic twins. Intrapair differences in birth weight were calculated by randomly subtracting the birth weight of the cotwin with the lowest birth weight from that of the

Table 2. Associations of birth weight with insulin, glucose and insulin resistance (HOMA) in twins

	Beta (95%-CI)[a]	*P*
Adjusted for age and sex:		
Insulin (log pmol/L)	-0.02 (-0.26 to 0.22)	0.9
Glucose (mmol/L)	-0.13 (-0.36 to 0.11)	0.3
Insulin sensitivity, log HOMA	-0.02 (-0.13 to 0.09)	0.5
Adjusted for age, sex and BMI:		
Insulin (log pmol/L)	-0.07 (-0.30 to 0.16)	0.6
Glucose (mmol/L)	-0.12 (-0.36 to 0.12)	0.3
Insulin sensitivity, log HOMA	-0.04 (-0.15 to 0.07)	0.5

[a]Beta (95%-CI) per kg birth weight. CI indicates confidence interval; BMI, body mass index

cotwin with the highest birth weight or vice versa.[10;11;14] An interaction analysis was performed to investigate whether zygosity and sex influenced the associations between intrapair differences in birth weight and differences in fasting insulin, fasting glucose and insulin resistance by introducing a product term of these variables and the differences in birth weight into the regression model. A two-tailed P-value < 0.05 was considered significant. All analyses were performed on a personal computer using the statistical software package SPSS version 9.0 (SPSS Inc).

Results

In the overall sample, after adjustment for age and sex, birth weight was not associated with fasting insulin, fasting glucose and insulin resistance (Table 2, upper panel). After additional adjustment for current body mass index, the associations of birth weight with fasting insulin, fasting glucose and insulin resistance were similar (Table 2, lower panel).

Intrapair association of differences in birth weight with differences in fasting insulin, fasting glucose and insulin resistance

Intrapair analyses in twins allow the elimination of various confounding characteristics, such as gestational age, maternal factors (e.g. height, weight gain, smoking and blood pressure during pregnancy), social class, birth order (in relation to other siblings) and sex. In addition, intrapair analyses in monoygotic twins also allow the elimination of genetic influences. Intrapair differences in birth weight were negatively associated with differences in fasting insulin and insulin resistance in both dizygotic twins and monozygotic twins (Table 3, upper panel). These associations were similar after adjustment for differences in current body mass index (Table 3, lower panel). For example, in monozygotic twins, a difference in birth weight of 1 kg within pairs was

Table 3. Associations between intrapair differences in birth weight and differences in insulin, glucose and insulin sensitivity (HOMA) in dizygotic and monozygotic twin pairs

	Dizygotic Twin Pairs		Monozygotic Twin Pairs	
	Beta (95%-CI)[a]	*P*	Beta (95%-CI)[a]	*P*
Unadjusted				
Insulin (pmol/L)	-11.9 (-34.4 to 10.7)	0.3	-46.7 (-95.2 to 1.8)	0.06
Glucose (mmol/L)	0.02 (-0.43 to 0.48)	0.9	-0.02 (-0.56 to 0.51)	0.9
Insulin sensitivity, HOMA	-3.0 (-8.7 to 2.7)	0.3	-13.2 (-25.0 to -1.3)	<0.05
Adjusted for differences in BMI				
Insulin (pmol/L)	-12.7 (-36.0 to 10.6)	0.3	-50.2 (-100.2 to -0.2)	<0.05
Glucose (mmol/L)	-0.06 (-0.40 to 0.27)	0.7	-0.02 (-0.58 to 0.5)	0.9
Insulin sensitivity, HOMA	-3.5 (-9.0 to 2.1)	0.2	-12.5 (-23.6 to 0.3)	0.05

[a]Beta (95%-CI) per kg birth weight. CI indicates confidence interval; BMI, body mass index.

associated with an insulin resistance that was 12.5 units higher in the twin with the lowest birth weight compared to the cotwin with the highest birth weight after adjustment for differences in current body mass index. Although these associations were not statistically significant in dizygotic twins, interaction analyses demonstrated that the associations in dizygotic twins were not significantly different from the associations in monozygotic twins (P for all >0.1), indicating that the difference between the association between birth weight and insulin resistance in monozygotic and that in dizygotic twins is likely due to chance. Differences in birth weight were not associated with differences in plasma glucose.

The results were similar after adjustment for (differences in) current weight instead of body mass index (data not shown). In addition, interaction analyses demonstrated that the associations were not significantly modified by either current body mass index or weight. Interaction analysis also indicated that the associations of (intrapair differences) in birth weight with (differences in) fasting insulin, fasting glucose and insulin resistance were similar in men and women. (*P* for all >0.1).

Discussion

The finding of a significant association between intrapair differences in birth weight and intrapair differences in fasting insulin and insulin resistance in monozygotic twins excludes the possibility that the association between low birth weight and insulin resistance is entirely due to a common genetic factor. Therefore, the association between these two variables is, at least in part, due to intrauterine factors.

Our findings add to the findings of two previous twin studies that demonstrated that monozygotic diabetic twins have a lower birth weight compared with their non-diabetic co-twins.[5;6] Our findings are in accordance with a recent study that showed that, within monozygotic twin pairs, differences in birth weight were related to differences in insulin

resistance, as measured with the euglycaemic hyperinsulinaemic clamp technique.[15] Taken together, the previous[5;6] and current data in monozygotic twins suggest that the relationship of low birth weight with insulin resistance and diabetes is, at least in part, caused by intrauterine factors. Therefore, improvement of fetal growth may have beneficial effects on glucose metabolism.

The results of the twin studies investigating the association of birth weight with insulin resistance and diabetes are in contrast with the published studies investigating birth weight and blood pressure within twins, which all have demonstrated that differences in birth weight within monozygotic twins were not significantly related to differences in systolic blood pressure in later life.[10;16-19] This suggests a differential influence of intrauterine and genetic factors on the association between birth weight and glucose metabolism on the one hand and the association between birth weight and blood pressure on the other. Although the results from the Dutch famine birth cohort may have been influenced by selection bias, analyses in this cohort have nevertheless demonstrated a similar pattern, as intrauterine exposure to famine was related to changes in glucose metabolism,[20] but not to changes in blood pressure.[21] In conclusion, the results from twin studies as well as the results from the Dutch famine study suggest that intrauterine nutrition may explain the association between size at birth and glucose metabolism, whereas genetic factors may explain the link between size at birth and blood pressure.

Our finding of an association between birth weight and insulin resistance suggests a role for insulin resistance in the birth weight-diabetes relationship. Several studies in singletons have shown that birth weight is associated with indicators of insulin resistance.[1] In contrast, it is not clear whether low birth weight is also associated with a defect in insulin secretion. Some studies have demonstrated that insulin secretion is reduced in low-birth-weight subjects,[22;23] but others have not.[24-28]

Birth weight was associated with insulin resistance in the intrapair analyses, but not in the unpaired analyses in the overall sample. This is probably due to the elimination of various confounding characteristics in the intrapair analyses, such as gestational age, maternal factors (e.g. height, weight gain, smoking and blood pressure during pregnancy), social class, birth order (in relation to other siblings) and sex. A much stronger association in intrapair analyses has also been found in several other studies investigating the association between birth weight and adult outcome in twins.[5;10;11;29]

Our data do not support an important role for current size (i.e. body mass index or weight) in the associations between birth weight and insulin resistance. No significant interaction of birth weight with either current body mass index or weight could be observed, suggesting that the strength of the association of birth weight with insulin resistance was similar in individuals with a high and in individuals with a low current body mass index or weight. In addition, the (intrapair) associations were similar before and after adjustment for measures of current size.

In summary, we have shown that, within monozygotic twin pairs, lower birth weight is associated with indicators of insulin resistance. This eliminates the possibility that the association between birth weight and insulin resistance is entirely due to a common genetic factor. These data support the concept that improvement of intrauterine nutrition may have beneficial effects on insulin sensitivity in adult life.

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11

Evidence for genetic factors explaining the association between birth weight and LDL cholesterol, and possible intrauterine factors influencing the association between birth weight and HDL cholesterol
Analysis in twins

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Abstract

Backgound Several recent studies have demonstrated an association between low weight at birth and an atherogenic lipid profile in later life.

Methods To examine the influences of nongenetic intrauterine and genetic factors, we investigated 61 dizygotic and 53 monozygotic adolescent twin pairs. Birth weight was obtained from the mothers.

Results Regression analysis demonstrated that low birth weight was associated with high levels of total cholesterol, LDL cholesterol and apolipoprotein B (-0.17 mmol/L per kg; P=0.07, -0.18 mmol/L per kg; P=0.04 and -0.07 g/L per kg; P=0.02, respectively), and with low levels of HDL cholesterol (+0.04 mmol/L per kg; P=0.1), after adjustment for age, sex and body mass index. Intrapair differences in birth weight were significantly associated with differences in total cholesterol, LDL cholesterol and apolipoprotein B in dizygotic twins after adjustment for differences in current body mass index (-0.49 mmol/L per kg; P=0.02, -0.51 mmol/L per kg; P=0.01 and -0.10 g/L per kg; P=0.04, respectively), demonstrating that the larger the difference in birth weight, the higher these risk factors in the twin with the lowest birth weight compared to the cotwin with the highest birth weight. In monozygotic twins, however, the associations between intrapair differences in birth weight and differences in total cholesterol, LDL cholesterol and apolipoprotein B were in the opposite direction (+0.32 mmol/L per kg; P=0.03, +0.23 mmol/L per kg; P=0.08 and +0.06 g/L per kg; P=0.04, respectively). The association between intrapair differences in birth weight and differences in HDL cholesterol was not significant in dizygotic twins (+0.04 mmol/L per kg; P=0.6) and of borderline significance in monozygotic twins (+0.11 mmol/L per kg; P=0.05).

Conclusions These data suggest that genetic factors account for the association of low birth weight with high levels of total cholesterol, LDL cholesterol and apolipoprotein B, whereas nongenetic intrauterine factors possibly play a role in the association between low birth weight and low levels of HDL cholesterol.

Introduction

Studies from different areas have shown that indices of fetal growth, such as birth weight, are inversely associated with cardiovascular morbidity and mortality in men and women.[1-3] Although the mechanisms are not known, it has been suggested that abnormalities in the metabolism of serum lipids may, in part, explain these associations.[4] Several recent studies have demonstrated that total cholesterol,[4-6] low density lipoprotein (LDL) cholesterol[4;5] and apolipoprotein B,[4;7-9] known risk factors for cardiovascular disease, are inversely related to size at birth. In addition, there is some evidence that small size at birth is associated with decreased levels of high density lipoprotein (HDL) cholesterol[10;11] and apolipoprotein A1.[12] These associations have been attributed to a programmed response to intrauterine malnutrition that induces permanent changes in the structure and function of organs, which cause an atherogenic lipid profile in adult life.[13] This theory is supported by a study demonstrating that exposure to the Dutch famine in utero influenced lipid levels in later life.[14] However, exposure to famine in utero during the Leningrad siege was not associated with effects on lipid levels.[15] The alternative view is that genetic factors influencing both birth weight and lipid profile could explain the relationships between these two factors.[16] Genetic factors play an important role in the determination of serum lipids[17] and, to a lesser extent, in the determination of birth weight.[18] It could be proposed that the genotype responsible for an atherogenic lipid profile may itself cause retarded fetal growth in utero.

Twin studies offer a unique opportunity to distinguish between nongenetic intrauterine and genetic influences.[19] Specifically, differences within dizygotic twin pairs are a function of both genetic and nongenetic factors, whereas differences within monozygotic (identical) pairs are almost completely caused by nongenetic factors.[19] If genetic factors do not play a role in the association between birth weight and cardiovascular risk factors, it could be expected that, both for dizygotic and for monozygotic twins, the twin with the lowest birth weight from each pair will also have the highest levels of cardiovascular risk factors compared to the cotwin with the highest birth weight. In addition, it could be expected that the larger the difference in birth weight, the higher these levels of cardiovascular risk factors in the twin with the lowest birth weight compared to the cotwin with the highest birth weight. If, however, genetic factors do play a role, these associations would hold true only for dizygotic twins, not for monozygotic twins. In a group of adolescent twin pairs still living with their parents, we have previously shown that genetic factors play an important role in the association between low birth weight and high blood pressure.[20] We here investigated whether the relationship between birth weight and an atherogenic lipid profile is influenced by nongenetic intrauterine or by genetic factors.

Methods

Subjects

This study is part of a larger project in which cardiovascular risk factors were studied in 160 adolescent twin pairs and their parents.[17;20-22] Addresses of twins living in Amsterdam and neighboring cities were obtained from City Council population registries. Twins still living with their biological parents were contacted by letter. Overall, between 30 and 40% of the families complied.[21] Zygosity was determined as described in detail previously.[21] A questionnaire was used to gather information on various factors including the use of medication and smoking behavior. The maternal questionnaire included questions regarding birth weight and gestational age of their children. This questionnaire was sent to the mothers a few weeks ahead of their visit to our department, allowing them to obtain birth data from birth certificates. Opposite-sex dizygotic twin pairs were excluded because of the effects of sex differences within a pair on both birth weight and serum lipid profile. Subjects using oral contraceptives were also excluded for these analyses. None of the subjects used any other medication that may affect serum lipid profile. Thus, 53 dizygotic and 61 monozygotic twin pairs were eligible for analysis.

Measurements

Height and weight were measured in a standardized way. After acclimatization EDTA blood was obtained between 8:30 and 10:30 AM by venipuncture after overnight fasting. Plasma was separated from cells after centrifugation for 10 minutes at 3000 rpm. Part of the plasma was kept at 4°C for lipid determinations within the next 5 days. The remainder was frozen at -20°C for later use. Total cholesterol and triglyceride levels were determined using enzymatic methods (Boehringer Mannheim, FRG, CHOD-PAP kit number 236691 and GPO-PAP kit number 701904). HDL cholesterol was measured after precipitation of VLDL, IDL, and LDL.[23] LDL cholesterol was calculated by the formula of Friedewald,[24] which is valid if triglyceride concentrations do not exceed 4.52 mmol/L.[25] There were no subjects with triglycerides above 4.07 mmol/L. Apolipoprotein A1 (the structural apolipoprotein linked to HDL) and apolipoprotein B (the structural apolipoprotein linked to LDL) were quantified by radial immunodiffusion.[26;27] Lipoprotein(a) levels were measured with a “bi-site” sandwich ELISA as described previously.[22]

Statistical Methods

In the total group, linear regression analysis was used to investigate the influence of birth weight on serum lipids after adjustment for age and sex, and after additional adjustment for body mass index (BMI). An interaction analysis was performed to investigate whether zygosity or current BMI influenced the associations between birth weight and serum lipid profile. As in previous twin studies examining the association

between birth weight and adult health, intrapair analyses were performed to investigate the influence of intrauterine and genetic factors.[20;28-33] As a first intrapair analyses, we compared twins with the lowest birth weight from each pair with their cotwins with the highest birth weight. Because the variability of the within pair differences rather than between pair variation is of interest, the paired *t* test was used.[34] For this analysis, 2 dizygotic and 2 monozygotic twin pairs had to be excluded because the birth weight of the twins within a pair was equal. The differences in dizygotic twin pairs and in monozygotic twin pairs were compared using the independent samples *t* test. As a first analysis the comparison of serum lipids between twins with the lowest and the highest birth weight is very simple and illustrative. However, twin pairs that differ 1 gram in birth weight are not differentiated from twin pairs who differ many hundreds of grams in birth weight. As a further and better method of analysis, linear regression analysis was used to analyze whether intrapair differences in birth weight influenced intrapair differences in serum lipids before and after adjustment for differences in current BMI in dizygotic and monozygotic twins (including the 4 twin pairs in which the birth weight of the twins within a pair was equal). In order to maintain the normal distribution of intrapair differences, intrapair differences in birth weight were calculated by randomly subtracting the cotwin with the lowest birth weight from the cotwin with the highest birth weight or vice versa.[35] Interaction analysis was performed to investigate whether zygosity influenced the associations between intrapair differences in birth weight and differences in serum lipids and lipoproteins. Serum concentrations of triglycerides had a skewed distribution and were transformed using natural logarithms. For presentation in Table 2, they were transformed back into the original units by taking the antilogs. A two-tailed P-value < 0.05 was considered significant. All analyses were performed on a personal computer using the statistical software package SPSS version 9.0 (SPSS Inc).

Results

In the total group of twins, low birth weight was associated with high serum levels of total cholesterol, LDL cholesterol, triglycerides and apolipoprotein B after adjustment for age and sex (Table 1, upper panel). In addition, low birth weight was associated with low HDL cholesterol and apolipoprotein A1 levels. However, only the association of birth weight with apolipoprotein B and apolipoprotein A1 was statistically significant. After additional adjustment for current BMI, the associations with birth weight were similar (Table 1, lower panel). Interaction analysis indicated that the associations between birth weight and serum lipids were not significantly modified by zygosity (data not shown). The associations of birth weight with total cholesterol, LDL cholesterol and apolipoprotein B were stronger in subjects with a high current BMI (*P* for interaction <0.01).

Table 1. Associations between birth weight and serum lipids and lipoproteins in twins

	Beta (95%-CI)[a]	*P*
Adjusted for age and sex:		
Total cholesterol (mmol/L)	-0.16 (-0.35 to 0.02)	0.08
LDL cholesterol (mmol/L)	-0.17 (-0.35 to 0.01)	0.06
HDL cholesterol (mmol/L)	+0.04 (-0.02 to 0.10)	0.2
Triglycerides (mmol/L)[b]	-0.08 (-0.16 to -0.02)	0.08
Apolipoprotein A1 (g/L)	+0.05 (0.01 to 0.09)	0.03
Apolipoprotein B (g/L)	-0.07 (-0.11 to -0.03)	0.002
Lp (a) (g/L)	+2.4 (-0.8 to 6.4)	0.2
Adjusted for age, sex and body mass index:		
Total cholesterol (mmol/L)	-0.17 (-0.35 to 0.01)	0.07
LDL cholesterol (mmol/L)	-0.18 (-0.36 to -0.01)	0.04
HDL cholesterol (mmol/L)	+0.04 (-0.02 to 0.10)	0.1
Triglycerides (mmol/L)[b]	-0.08 (-0.17 to 0.01)	0.08
Apolipoprotein A1 (g/L)	+0.05 (0.01 to 0.09)	0.02
Apolipoprotein B (g/L)	-0.07 (-0.11 to -0.03)	0.002
Lp (a) (g/L)	+0.03 (-0.01 to 0.06)	0.2

[a]Beta (95%-CI) per kg birth weight. [b]log-transformed. CI indicates confidence interval.

Comparison between cotwins with the lowest and cotwins with the highest birth weight

Birth weight and gestational age were similar in dizygotic and monozygotic twins (Table 2). The differences in birth weight between the cotwins with the lowest birth weight and those with the highest birth weight from each pair were similar for dizygotic and monozygotic twin pairs (380 g and 300 g, respectively; *P* for the difference, 0.2; Table 2). Although none of the differences in serum lipids between the cotwins with the lowest and the highest birth weight were statistically significant, several interesting trends could be observed. The dizygotic twins with the lowest birth weight had total cholesterol, LDL cholesterol and apolipoprotein B concentrations that were higher than those of their cotwins with the highest birth weight. However, the monozygotic twins with the lowest birth weight had total cholesterol, LDL cholesterol and apolipoprotein B concentrations that were lower than those of their cotwins with the highest birth weight (Table 2). The differences in total cholesterol, LDL cholesterol and apolipoprotein B between the cotwins with the lowest and the cotwins with the highest birth weight were different in dizygotic compared to monozygotic twin pairs (for cholesterol, *P*=0.03; for LDL cholesterol, *P*=0.03; for apolipoprotein B, *P*=0.1).

Both dizygotic and monozygotic twins with the lowest birth weight had HDL cholesterol and apolipoprotein A1 levels that were lower than those of their cotwins with the highest birth weight, whereas levels of triglycerides and Lp (a) were similar.

Table 2. Clinical characteristics of the cotwins with the lowest and the highest birth weight in dizygotic and monozygotic twin pairs

	Dizygotic Twin Pairs			Monozygotic Twin Pairs		
	Cotwins with the lowest birth weight	Cotwins with the highest birth weight	*P*	Cotwins with the lowest birth weight	Cotwins with the highest birth weight	*P*
Birth weight, g	2246±493	2626±558	<0.001	2336±528	2636±485	<0.001
GA, weeks	36±8.4	36±8.4	-	37±2.8	37±2.8	-
n (male/female)	59(32/27)	59(32/27)	-	51(30/21)	51(30/21)	-
Age, years	17.0±1.7	17.0±1.7	-	16.0±1.8	16.0±1.8	-
BMI, kg/m^2	20.0±1.9	20.3±2.2	0.5	19.5±2.2	19.7±2.2	0.2
Smoking, n	7	9	-	4	4	-
Total chol (mmol/L)	4.15±0.71	3.99±0.62	0.1	4.23±0.80	4.32±0.79	0.1
LDL chol (mmol/L)	2.58±0.68	2.40±0.57	0.07	2.63±0.75	2.69±0.75	0.2
HDL chol (mmol/L)	1.25±0.24	1.27±0.25	0.5	1.29±0.25	1.33±0.24	0.1
Triglycerides(mmol/L)	0.63±1.54	0.64±1.45	0.7	0.63±1.38	0.63±1.40	0.9
Apolipoprotein A1(g/L)	1.34±0.15	1.38±0.17	0.06	1.35±0.16	1.38±0.17	0.08
Apolipoprotein B (g/L)	0.78±0.15	0.75±0.18	0.2	0.77±0.16	0.79±0.16	0.2
Lp (a) (g/L)	0.10±0.10	0.10±0.10	0.9	0.15±0.15	0.15±0.15	0.9

Mean±SD. GA indicates gestational age; BMI, body mass index; chol, cholesterol.

Associations between intrapair differences in birth weight and serum lipid profile

To further characterize the relation between birth weight and serum lipid profile, we determined the associations between intrapair differences in birth weight and differences in serum lipids and lipoproteins. Table 3 shows that, in dizygotic twins, intrapair differences in birth weight were associated with differences in total cholesterol, LDL cholesterol and apolipoprotein B. The larger the difference in birth weight, the higher these risk factors in the twin with the lowest birth weight compared to the cotwin with the highest birth weight. In monozygotic twins, intrapair differences in birth weight were also significantly associated with differences in total cholesterol, LDL cholesterol and apolipoprotein B. However, the direction of the effect was opposite to that in the dizygotic twins: the larger the difference in birth weight, the lower these risk factors in the twin with the lowest birth weight compared to the cotwin with the highest birth weight. For example, in dizygotic twins, a difference in birth weight of 1 kg within pairs was associated with an LDL cholesterol that was 0.51 mmol/L higher in the twin with the lowest birth weight compared to the cotwin with the highest birth weight after adjustment for differences in BMI. In contrast, in monozygotic twins, a difference in birth weight of 1 kg within pairs was associated with an LDL cholesterol that was 0.23 mmol/L lower in the twin with the lowest birth weight compared to the cotwin with the highest birth weight. Interaction analysis indicated that the associations were significantly different between dizygotic twins and monozygotic twins (P<0.01 for total cholesterol, LDL

cholesterol and apolipoprotein B). The results were similar before and after adjustment for differences in BMI.

Before and after adjustment for differences in BMI, the association between intrapair differences in birth weight and differences in HDL cholesterol was not significant in dizygotic twins and of borderline significance in monozygotic twins (Table 3). Intrapair differences in birth weight were not associated with differences in triglycerides, apolipoprotein A1 and Lp (a) in either dizygotic or monozygotic twins. Interaction analysis indicated that the associations between intrapair differences in birth weight and differences in HDL cholesterol, triglycerides, apolipoprotein A1 and Lp (a) were not significantly different between dizygotic twins and monozygotic twins (P >0.3).

If subjects with a gestational age shorter than 37 weeks (21 dizygotic and 24 monozygotic twin pairs) were excluded the results were similar. Adjustment for gestational age or (differences in) smoking did not change the results (data not shown). If the associations were adjusted for (differences in) current weight instead of current BMI, the results were similar.

Table 3. Associations between intrapair differences in birth weight and differences in serum lipids and lipoproteins in dizygotic and monozygotic twin pairs

	Dizygotic Twin Pairs		**Monozygotic Twin Pairs**	
	Beta (95%-CI)[a]	*P*	Beta (95%-CI)[a]	*P*
Unadjusted:				
Total cholesterol (mmol/L)	-0.47 (-0.86 to -0.09)	0.02	+0.31 (0.02 to 0.61)	0.04
LDL cholesterol (mmol/L)	-0.50 (-0.87 to -0.13)	0.01	+0.23 (-0.03 to 0.48)	0.08
HDL cholesterol (mmol/L)	+0.01 (-0.12 to 0.14)	0.9	+0.11 (-0.01 to 0.23)	0.08
Triglycerides (mmol/L)	+0.03 (-0.13 to 0.18)	0.7	-0.04 (-0.19 to 0.10)	0.5
Apolipoprotein A1 (g/L)	+0.06 (-0.04 to 0.15)	0.2	+0.04 (-0.04 to 0.13)	0.5
Apolipoprotein B (g/L)	-0.10 (-0.18 to -0.01)	0.08	+0.06 (0.01 to 0.12)	0.03
Lp (a) (g/L)	+0.04 (-0.03 to 0.10)	0.3	-0.01 (-0.05 to 0.02)	0.5
Adjusted for differences in BMI:				
Total cholesterol (mmol/L)	-0.49 (-0.89 to -0.08)	0.02	+0.32 (0.03 to 0.62)	0.03
LDL cholesterol (mmol/L)	-0.51 (-0.90 to -0.13)	0.01	+0.23 (-0.03 to 0.49)	0.08
HDL cholesterol (mmol/L)	+0.04 (-0.10 to 0.16)	0.6	+0.11 (-0.00 to 0.23)	0.05
Triglycerides (mmol/L)	-0.02 (-0.17 to 0.14)	0.8	-0.05 (-0.19 to 0.10)	0.6
Apolipoprotein A1 (g/L)	+0.07 (-0.03 to 0.16)	0.17	+0.04 (-0.04 to 0.13)	0.3
Apolipoprotein B (g/L)	-0.10 (-0.19 to -0.01)	0.04	+0.06 (0.03 to 0.12)	0.04
Lp (a) (g/L)	+0.03 (-0.03 to 0.10)	0.3	-0.01 (-0.05 to 0.02)	0.4

[a]Beta (95%-CI) per kg birth weight. CI indicates confidence interval; BMI, body mass index

Discussion

In accordance with previous studies in singletons,[4-10] we found that low birth weight was associated with high serum concentrations of total cholesterol, LDL cholesterol and apolipoprotein B in twins. In dizygotic twin pairs, the twins with the lowest birth weight from each pair tended to have higher levels of total cholesterol, LDL cholesterol and apolipoprotein B compared to their cotwins with the highest birth weight. In addition, the associations between intrapair differences in birth weight and intrapair differences in levels of total cholesterol, LDL cholesterol and apolipoprotein B demonstrated that the larger the difference in birth weight, the higher these risk factors in the smaller baby compared to the larger baby. To eliminate the influence of genetic factors on these associations, we also studied monozygotic twin pairs. Despite a similar difference in birth weight as in dizygotic twins, the monozygotic twins with the lowest birth weight tended to have lower, not higher, levels of total cholesterol, LDL cholesterol and apolipoprotein B than their cotwins with the highest birth weight. In addition, the associations between intrapair differences in birth weight and differences in levels of total cholesterol, LDL cholesterol and apolipoprotein B demonstrated that the larger the difference in birth weight, the lower these risk factors in the twin with the lowest birth weight compared to the cotwin with the highest birth weight. The differences in the intrapair associations between dizygotic and monozygotic twins provide the first evidence that genetic factors importantly influence the association between the variance in birth weight and that in levels of total cholesterol, LDL cholesterol and apolipoprotein B.

The association between low birth weight and low levels of HDL cholesterol is in accordance with some,[10;11] but not all,[4] studies in singletons. In both dizygotic and monozygotic twin pairs, the twins with the lowest birth weight from each pair tended to have lower levels of HDL cholesterol compared to their cotwins with the highest birth weight. In addition, in both dizygotic and monozygotic twins, intrapair differences in birth weight tended to be associated with intrapair differences in levels of HDL cholesterol. These data suggest that the association between the variance in birth weight and that in levels of HDL cholesterol may be independent of genetic factors.

The association between low birth weight and low levels of apolipoprotein A1 is consistent with one study in singletons,[12] but in contrast to other studies.[4;10] Although levels of apolipoprotein A1 tended to be lower in dizygotic and monozygotic twins with the lowest birth weight from each pair compared to their cotwins with the highest birthweight, intrapair differences in birth weight were not associated with intrapair differences in apolipoprotein A1. This makes it difficult to interpret the relative contribution of nongenetic intrauterine versus genetic factors to the association between birth weight and apolipoprotein A1.

Although exposure to famine in utero during the Leningrad siege was not associated with lipid levels,[15] exposure to the Dutch famine in utero influenced lipid levels in later

life.[14] This may, however, reflect the selection of fetuses genetically susceptible to an increased cardiovascular risk.[36] During the famine, the number of conceptions was about 50% lower than the pre-famine level and perinatal mortality as well as mortality in the first year after birth were highest in those who were born during the famine.[37] Therefore, selection effects could have influenced the results of studies investigating the influence of maternal malnutrition.

It could be argued that, besides genetic factors, intrauterine factors in monozygotic twins may also be different from those in dizygotic twins. Around two thirds of monozygotic twins are monochorionic (i.e. share a placenta), whereas all dizygotic twins are dichorionic (i.e. have separate placentas). We do not have data on chorionicity in our group of monozygotic twins, but we consider it unlikely that differences in zygosity between dizygotic and monozygotic twins can fully explain our results. Intrapair differences in birth weight were related to differences in HDL cholesterol and, as reported previously, to differences in height in monozygotic twins,[33] suggesting that intrauterine factors in monozygotic twins are capable of permanently influencing adult outcome. Regardless of whether or not chorionicity plays a role in explaining the differences between dizygotic and monozygotic twins, our finding that the intrauterine growth retardation experienced by the monozygotic twins with the lowest birth weight from each pair was significantly associated with beneficial levels of major cardiovascular risk factors (such as total cholesterol, LDL cholesterol and apolipoprotein B) strongly contradicts the hypothesis that nongenetic intrauterine factors are the cause of the association between low birth weight and these major cardiovascular risk factors. Although this finding was unexpected, it may be consistent with an earlier observation of lower plasma cholesterol concentrations after maternal undernutrition during pregnancy and lactation in rats.[38]

It has been demonstrated that serum lipid levels in adolescence track into adulthood. Adolescents with an adverse lipid profile at a given age continue to have an adverse lipid profile as they grow and age.[39;40] In addition, an adverse lipid profile in adolescence is strongly associated with atherosclerosis.[41;42] Therefore, our results in adolescent subjects are relevant for the development of cardiovascular disease in adults.

In our study, there was an important interaction between birth weight and current BMI, such that the associations of birth weight with total cholesterol, LDL cholesterol and apolipoprotein B were larger in subjects with a high than in subjects with a low BMI. This is consistent with the results from several studies that investigated the association of birth weight with other cardiovascular risk factors, such as blood pressure,[43;44] diabetes,[45] and coronary heart disease.[1] Previous studies have also shown that adjustment for current size (i.e. weight or BMI) increases the strength of the association between birth weight and cardiovascular risk factors in later life. Therefore, Lucas et al. have suggested that it is the change in size from birth to later life rather than size at birth itself that is implicated.[46] However, in our study, the associations were similar after adjustment for (differences in) current size, suggesting that change in size

from birth to later life plays a minor role in the association between birth weight and serum lipid profile in later life.

It has been suggested that improvement of fetal nutrition and thereby, intrauterine growth may prevent the development of cardiovascular disease.[13] However, if the relationship between low birth weight and elevated levels of total cholesterol, LDL cholesterol and apolipoprotein B is caused by genetic factors, improvement of fetal nutrition may not prevent the development of cardiovascular disease, at least not through improving these constituents of the lipid profile. On the other hand, the association between intrapair differences in birth weight and differences in HDL cholesterol suggests that improvement of fetal growth may also have beneficial effects.

In summary, we found that the association between low birth weight and high levels of total cholesterol, LDL cholesterol and apolipoprotein B persisted in the intrapair analysis in dizygotic twin pairs, but was reversed within monozygotic twin pairs. Furthermore, we found that the association between low birth weight and low levels of HDL-cholesterol tended to persist in the intrapair analysis in both dizygotic and monozygotic twins. These data suggest that genetic factors account for the association of low birth weight with high levels of total cholesterol, LDL cholesterol and apolipoprotein B, whereas intrauterine factors possibly play a role in the association of low birth weight with low levels of HDL cholesterol.

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The association between low birth weight and high levels of cholesterol is not due to an increased cholesterol synthesis or absorption
Analysis in twins

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Abstract

Background Low birth weight may be associated with high levels of cholesterol in later life through genetic factors that affect both birth weight and cholesterol metabolism. Alterations in cholesterol synthesis and absorption may play an important role in this association.

Methods We examined birth weight and plasma ratios of lathosterol (a precursor of cholesterol; an estimate of cholesterol synthesis), and campesterol and β-sitosterol (plant sterols; estimates of cholesterol absorption) to cholesterol in 53 dizygotic and 58 monozygotic adolescent twin pairs.

Results After adjustment for current weight, birth weight was not associated with the ratios of lathosterol, campesterol and β-sitosterol either in the overall sample (+0.07 μmol/mmol per kg [95% confidence interval: -0.11 to 0.25]; P=0.5, +0.02 μmol/mmol per kg [-0.33 to 0.37]; P=0.9 and -0.04 μmol/mmol per kg [-0.23 to 0.15]; P=0.8, respectively) or in the intrapair analysis in dizygotic twins (+0.27 μmol/mmol per kg [-0.28 to 0.82]; P=0.3, -0.03 μmol/mmol per kg [-1.07 to 1.01]; P=1.0 and +0.04 μmol/mmol per kg [-0.56 to 0.64]; P=0.9, respectively) or in the intrapair analysis in monozygotic twins (+0.54 μmol/mmol per kg [-0.09 to 1.18]; P=0.09, -0.60 μmol/mmol per kg [-1.59 to 0.39]; P=0.2 and -0.43 μmol/mmol per kg [-0.99 to 0.14]; P=0.14, respectively).

Conclusions Plasma levels of lathosterol, campesterol and β-sitosterol, which are indicators of cholesterol synthesis and absorption, thus do not explain the association of low birth weight with high levels of total and LDL cholesterol. As an alternative hypothesis, we suggest that a decrease in cholesterol clearance may play an important role.

Introduction

Low birth weight is associated with an increased risk of cardiovascular morbidity and mortality.[1,2] An atherogenic lipid profile may, in part, explain these associations.[3-7] The association between birth weight and an atherogenic lipid profile has been attributed to a programmed response to intrauterine malnutrition that induces permanent changes in the structure and function of organs, which cause increased levels of cholesterol in later life.[8] The alternative view is that genetic factors influencing both birth weight and lipid profile could explain the relationships between these two variables.[9]

Studies in dizygotic and monozygotic (genetically identical) twin pairs offer a unique opportunity to investigate the influence of intrauterine and genetic factors**.** Differences within dizygotic twin pairs are a function of both genetic and nongenetic factors, whereas differences within monozygotic pairs are caused by nongenetic factors. In our cohort of adolescent twin pairs, we have previously shown that low birth weight was associated with high total and LDL cholesterol within dizygotic twin pairs, but with low total and LDL cholesterol within monozygotic twin pairs.[10] These data suggest that the association of birth weight with total and LDL cholesterol is strongly influenced by the elimination of genetic factors. Therefore, these genetic factors play an important role in the association of low birth weight with elevated levels of total and LDL cholesterol.[10]

The metabolic alterations in cholesterol metabolism that underlie these changes in plasma lipids are not known. Direct assessment of cholesterol synthesis is expensive, time-consuming and difficult in large-scale studies, but plasma ratios of lathosterol (a precursor of cholesterol), and campesterol and β-sitosterol (plant sterols) to cholesterol are indicators of whole body cholesterol synthesis and absorption, respectively.[11-16] In a study in children born preterm, Mortaz et al. demonstrated that low birth weight was associated with an increase in cholesterol synthesis, as indicated by an increase in plasma lathosterol, and a compensatory decrease in cholesterol absorption, as indicated by a decrease in plasma campesterol.[17] They interpreted this association as a consequence of intrauterine programming.[17] However, both birth weight [18,19] and indicators of cholesterol metabolism[20,21] are influenced by genetic factors. Therefore, the association between them may also be explained by genetic influences.

To examine the association between birth weight and cholesterol metabolism and the possible influence of genetic factors, we now investigated birth weight and markers of cholesterol synthesis and absorption in our sample of adolescent dizygotic and monozygotic twin pairs.

Methods

Subjects

This study is part of a larger project in which cardiovascular risk factors were studied in 160 adolescent twin pairs and their parents.[22-25] Addresses of twins living in Amsterdam and neighboring cities were obtained from City Council population registries. Twins still living with their biological parents were contacted by letter. A questionnaire was used to gather information on various factors including the use of medication and smoking behavior. The maternal questionnaire included questions regarding birth weight and gestational age of their children. Opposite-sex dizygotic twin pairs were excluded because of the effects of sex differences within a pair on both birth weight and indicators of cholesterol metabolism. Subjects using oral contraceptives were also excluded for these analyses. None of the subjects used any other medication that may affect plasma concentrations of lathosterol, campesterol and β-sitosterol. Thus, 53 dizygotic twin pairs (average age 17.0 years) and 58 monozygotic twin pairs (average age 16.0 years) were eligible for analysis. This study was approved by the Institutional Review Board, and subjects gave their informed consent.

Measurements

Height and weight were measured in a standardized way. After acclimatization EDTA blood was obtained between 8:30 and 10:30 AM by venipuncture after overnight fasting. Plasma was separated from cells after centrifugation for 10 minutes at 3000 rpm. Concentrations of lathosterol, campesterol and β-sitosterol were determined with gas chromatography on a 30-m×0.25mm CP Sil 5CB column in a Chrompack model 438S gas chromatograph, as described previously.[25] Concentrations were expressed as μmol/L. In addition, values were expressed as μmol/mmol of cholesterol, because the measurements of the sterols are influenced by plasma cholesterol levels.[12-15] Cholesterol values of this sample[10,24,25] were determined using enzymatic methods (Boehringer Mannheim, FRG, CHOD-PAP kit number 236691). Although some studies investigating indicators of cholesterol metabolism have used gas chromatography to measure cholesterol, the use of enzymatic methods is in accordance with several studies that validated the use of lathosterol, campesterol and β-sitosterol as indicators of cholesterol metabolism,[11-13] and with the study of Mortaz et al., in which an association between birth weight and indicators of cholesterol metabolism was found in preterm singletons.[17]

Data analysis

In the overall sample, linear regression analysis was used to investigate the influence of birth weight on indicators of cholesterol metabolism after adjustment for age and sex, and after additional adjustment for current weight. An interaction analysis was

performed to investigate whether zygosity or current weight influenced these associations. Intrapair analyses were performed to investigate the influence of intrauterine and genetic factors.[10,23,26-31] As a first intrapair analysis, the paired *t* test was used to compare twins with the lowest birth weight from each pair with their cotwins with the highest birth weight. For this analysis, 2 dizygotic and 2 monozygotic twin pairs had to be excluded because the birth weight of the twins within a pair was equal. In addition, linear regression analysis was used to analyze whether intrapair differences in birth weight influenced intrapair differences in indicators of cholesterol metabolism before and after adjustment for differences in current weight (including the 4 twin pairs in which the birth weight of the twins within a pair was equal). Intrapair differences in birth weight were calculated by randomly subtracting the cotwin with the lowest birth weight from the cotwin with the highest birth weight or vice versa.[32] Interaction analysis was performed to investigate whether zygosity or differences in current weight influenced the associations between intrapair differences in birth weight and indicators of cholesterol metabolism. A two-tailed P-value < 0.05 was considered significant. All analyses were performed on a personal computer using the statistical software package SPSS version 9.0 (SPSS Inc).

Results

Lathosterol, campesterol and β-sitosterol (whether expressed as concentration or as ratio to cholesterol) were not related to birth weight in the overall sample. (Table 1, upper panel). The results were similar after additional adjustment for current weight (Table 1, lower panel). Interaction analysis indicated no effect modification by zygosity or current weight (data not shown).

Comparison between cotwins with the lowest and cotwins with the highest birth weight
Birth weight and gestational age were similar in dizygotic and monozygotic twins (Table 2). The differences in birth weight between the cotwins with the lowest birth weight and those with the highest birth weight from each pair were similar for dizygotic and monozygotic twin pairs (380 and 306 g, respectively; *P* for the difference, 0.2; Table 2). Lathosterol, campesterol and β-sitosterol (whether expressed as concentration or as ratio to cholesterol) were similar in the twins with the lowest and the highest birth weight in both dizygotic and monozygotic twins.

Associations between intrapair differences in birth weight and indicators of cholesterol metabolism
To further explore the relation between birth weight and indicators of cholesterol metabolism, we determined the associations between intrapair differences in birth

Table 1. Associations between birth weight and indicators of cholesterol metabolism in the overall sample of twins

	Beta (95%-CI)†	*P*
Adjusted for age and sex:		
Lathosterol (µmol/L)	0.17 (-0.55 to 0.89)	0.6
Campesterol (µmol/L)	-0.39 (-1.75 to 0.97)	0.6
β-Sitosterol (µmol/L)	-0.40 (-1.15 to 0.35)	0.3
Lathosterol ratio (µmol/mmol)*	0.11 (-0.06 to 0.29)	0.2
Campesterol ratio (µmol/mmol)*	-0.04 (-0.38 to 0.30)	0.8
β-Sitosterol ratio (µmol/mmol)*	-0.06 (-0.24 to 0.13)	0.5
Adjusted for age, sex and current weight:		
Lathosterol (µmol/L)	0.02 (-0.72 to 0.76)	1.0
Campesterol (µmol/L)	-0.11 (-1.50 to 1.28)	0.9
β-Sitosterol (µmol/L)	-0.29 (-1.07 to 0.48)	0.6
Lathosterol ratio (µmol/mmol)*	0.07 (-0.11 to 0.25)	0.5
Campesterol ratio (µmol/mmol)*	0.02 (-0.33 to 0.37)	0.9
β-Sitosterol ratio (µmol/mmol)*	-0.04 (-0.23 to 0.15)	0.8

*indicates µmol/mmol of total cholesterol
†Beta (95%-CI) per kg birth weight.
CI indicates confidence interval.

weight and differences in lathosterol, campesterol and β-sitosterol. Intrapair differences in the indicators of cholesterol metabolism (whether expressed as concentration or as ratio to cholesterol) were not related to intrapair differences in birth weight in the dizygotic twin pairs either before or after adjustment for differences in current weight (Table 3). In the unadjusted intrapair analysis in monozygotic twins, low birth weight was associated with low concentrations of lathosterol and high concentrations of campesterol and β-sitosterol. However, only the association with lathosterol was statistically significant. After controlling for cholesterol levels by using the ratio of lathosterol to cholesterol, the intrapair association of birth weight with lathosterol was smaller. After additional adjustment for differences in current weight, the association was of borderline significance (Table 3). Interaction analysis indicated that the associations between intrapair differences in birth weight and differences in lathosterol, campesterol and β-sitosterol (whether expressed as concentration or as ratio to cholesterol) were not significantly influenced by zygosity or differences in current weight ($P > 0.2$).

Additional analyses

After restricting the analyses to subjects born after a gestational age shorter than 37 weeks, low birth weight was associated with a decreased ratio of lathosterol to cholesterol (+0.30 µmol/mmol per kg [95% confidence interval: 0.04 to 0.56]; $P=0.03$). The results of the intrapair analyses in this subgroup, however, were similar compared

Table 2. Clinical characteristics of the cotwins with the lowest and the highest birth weight in dizygotic and monozygotic twin pairs

	Dizygotic Twin Pairs			Monozygotic Twin Pairs		
	Cotwins with the lowest birth Weight	Cotwins with the highest birth weight	*P*	Cotwins with the lowest birth weight	Cotwins with the highest birth weight	*P*
Birth weight, g	2246±493	2626±558	<0.001	2319±529	2625±485	<0.001
Gestational age, weeks	36±8.4	36±8.4	-	37±2.8	37±2.8	-
n (male/female)	51(30/21)	51(30/21)	-	56(29/27)	56(29/27)	-
Age, years	17.0±1.7	17.0±1.7	-	16.0±1.8	16.0±1.8	-
Current weight, kg	59.9±7.8	61.8±10.1	0.09	57.5±9.7	58.6±9.5	0.03
Current BMI, kg/m^2	20.0±1.9	20.3±2.2	0.5	19.5±2.3	19.7±2.3	0.2
Smoking, n	7	9	-	4	4	-
Total cholesterol (mmol/L)	4.15±0.71	3.99±0.62	0.1	4.23±0.80	4.32±0.79	0.1
Lathosterol (µmol/L)	6.27±2.80	6.43±2.58	0.8	6.37±2.13	7.24±3.21	0.10
Campesterol (µmol/L)	12.76±5.95	12.51±5.75	0.8	12.42±4.48	11.80±4.78	0.4
β-Sitosterol (µmol/L)	7.10±3.56	6.94±2.93	0.8	6.96±2.68	6.36±2.33	0.2
Lathosterol ratio*	1.52±0.58	1.65±0.71	0.3	1.55±0.57	1.71±0.79	0.2
Campesterol ratio*	3.17±1.52	3.17±1.41	1.0	3.02±1.15	2.82±1.21	0.3
β-Sitosterol ratio*	1.77±0.91	1.76±0.72	0.9	1.68±0.64	1.52±0.60	0.12

Mean±SD. BMI indicates body mass index. The association between birth weight and total cholesterol has been investigated previously.[10] *expressed as µmol/mmol of total cholesterol.

Table 3. Associations between intrapair differences in birth weight and differences in indicators of cholesterol metabolism in dizygotic and monozygotic twin pairs

	Dizygotic Twin Pairs		Monozygotic Twin Pairs	
	Beta (95%-CI) †	*P*	Beta (95%-CI)†	*P*
Unadjusted:				
Lathosterol (µmol/L)	0.56 (-1.60 to 2.73)	0.6	3.55 (0.91 to 6.18)	0.01
Campesterol (µmol/L)	-2.3 (-5.98 to 1.38)	0.2	-0.49 (-4.64 to 3.67)	0.8
β-Sitosterol (µmol/L)	-1.24 (-3.41 to 0.92)	0.3	-1.10 (-3.37 to 1.17)	0.3
Lathosterol ratio (µmol/mmol)*	0.40 (-0.11 to 0.91)	0.12	0.75 (0.10 to 1.40)	0.02
Campesterol ratio (µmol/mmol)*	-0.35 (-1.31 to 0.61)	0.5	-0.24 (-1.26 to 0.79)	0.6
β-Sitosterol ratio (µmol/mmol)*	-0.19 (-0.76 to 0.37)	0.5	-0.33 (-0.88 to 0.23)	0.2
Adjusted for differences in current weight:				
Lathosterol (µmol/L)	-0.03 (-2.39 to 2.32)	1.0	2.75 (0.15 to 5.34)	0.04
Campesterol (µmol/L)	-1.04 (-4.99 to 2.92)	0.6	-1.91 (-5.93 to 2.11)	0.3
β-Sitosterol (µmol/L)	-0.27 (-2.56 to 2.02)	0.8	-1.43 (-3.77 to 0.90)	0.2
Lathosterol ratio (µmol/mmol)*	0.27 (-0.28 to 0.82)	0.3	0.54 (-0.09 to 1.18)	0.09
Campesterol ratio (µmol/mmol)*	-0.03(-1.07 to 1.01)	1.0	-0.60 (-1.59 to 0.39)	0.2
β-Sitosterol ratio (µmol/mmol)*	0.04 (-0.56 to 0.64)	0.9	-0.43 (-0.99 to 0.14)	0.14

* indicates µmol/mmol of total cholesterol. †Beta (95%-CI) per kg birth weight. CI indicates confidence interval.

to the results of the intrapair analyses in the total group. In these subjects, birth weight was not associated with campesterol and β-sitosterol. Adjustment for gestational age or (differences in) smoking did not change the results (data not shown). The results were also similar if the associations were adjusted for (differences in) current BMI instead of current weight.

Discussion

We studied 53 dizygotic and 58 monoygotic adolescent twin pairs. We have previously demonstrated in this sample that low birth weight was associated with high total and LDL cholesterol within dizygotic twin pairs, but with low total and LDL cholesterol within monozygotic twin pairs.[10] In addition, indicators of cholesterol metabolism (i.e. lathosterol, campesterol and β-sitosterol) were related to cholesterol and current weight in this sample.[25] Therefore, this sample allowed us to investigate whether the association between birth weight and cholesterol is influenced by cholesterol synthesis or absorption, and the possible influence of genetic factors. We could not demonstrate an association of birth weight with indicators of cholesterol synthesis and absorption either in the overall sample or in the intrapair analyses in dizygotic and monozygotic twin pairs.

Several studies have found an association of low birth weight with high total and LDL cholesterol in singletons[3-5] and we have previously reported this association in the overall sample of twins and in the intrapair analysis in dizygotic twin pairs.[10] In the present study, we could not demonstrate an association between low birth weight and an increased cholesterol synthesis or absorption, as indicated by elevated plasma levels of lathosterol, campesterol and β-sitosterol. We cannot exclude the possibility that low birth weight may be associated with other indicators of cholesterol metabolism, such as squalene, methyl sterols and cholestanol,[33] or with direct measurements of cholesterol metabolism using radioactive isotope techniques [33]. However, many studies have shown that plasma ratios of lathosterol, campesterol and β-sitosterol to cholesterol are useful indicators of cholesterol metabolism.[12-16] Therefore, we propose that the association between low birth weight and high levels of total and LDL cholesterol may be due to a decreased cholesterol clearance. This could be investigated by studying the in vivo kinetics of apolipoprotein B containing lipoproteins using a stable isotope approach.[34]

The intrapair association between differences in birth weight and differences in lathosterol in monozygotic twins may suggest an explanation for our previous finding of an association of low birth weight with low total and LDL cholesterol after the elimination of genetic influences.[10] However, it should be noted that this association was only of borderline significance. Interestingly, the finding of an association of low birth weight with low cholesterol levels is in line with a study in rats that demonstrated lower plasma cholesterol concentrations after maternal undernutrition.[35]

Our results differ from the results from Mortaz et al..[17] They demonstrated, in preterm infants, that low birth weight was associated with an increase in cholesterol synthesis, as indicated by an increase in plasma lathosterol, and a compensatory decrease in cholesterol absorption, as indicated by a decrease in plasma campesterol.[17] In our study, we could not detect these associations. In the total group of subjects, birth weight was not associated with cholesterol synthesis or absorption. After restricting the

analyses to subjects born after a gestational age shorter than 37 weeks, low birth weight was associated with a decreased, not an increased, ratio of lathosterol to cholesterol. However, the results of Mortaz et al. may differ from ours for several reasons. First, the subjects in the study of Mortaz et al. were born very prematurely (average gestational age: 31.1 weeks), whereas our subjects were born after 36.3 weeks, which is considered the term period for a twin pregnancy. Second, subjects in the study of Mortaz et al. were younger than our twin subjects (11.2 years vs. 16.5 years).

It has been suggested that intrauterine growth in twins is not comparable to intrauterine growth in singletons. However, birth weight in twins has been associated with many variables that have been related to birth weight in singletons, such as blood pressure,[23,26] diabetes,[28,29] myocardial infarction[30] and height.[31] These studies suggest that differences in birth weight in twins can be used as a model for differences in birth weight in singletons.

In summary, we found no evidence that plasma levels of lathosterol, campesterol and β-sitosterol, which are indicators of cholesterol synthesis and absorption, can explain the association of low birth weight with high levels of total and LDL cholesterol. As an alternative hypothesis, we suggest that the association between low birth weight and high levels of total and LDL cholesterol is due to a decreased cholesterol clearance.

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The association between birth weight and plasma fibrinogen is abolished after the elimination of genetic influences

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Abstract

Background Low birth weight is associated with an increased risk of atherothrombosis, which may be related in part to the association between low birth weight and high plasma fibrinogen. The association between birth weight and fibrinogen may be explained by intrauterine, socio-economic or genetic factors.

Methods We examined birth weight and fibrinogen in 52 dizygotic and 56 adolescent monozygotic (genetically identical) twin pairs.

Results The dizygotic but not the monozygotic twins with the lowest birth weight from each pair had a fibrinogen level that was higher compared with their co-twins with the highest birth weight (dizygotic twins: 2.62±0.46 g/L vs. 2.50±0.41 g/L [P = .04]; monozygotic twins: 2.42±0.45 g/L vs. 2.49±0.39 g/L [P = .2]).

Conclusions These findings suggest that the association between birth weight and plasma fibrinogen is abolished after the elimination of genetic influences and, therefore, that this association has genetic causes. Improvement of intrauterine nutrition may not lower fibrinogen levels in later life.

Introduction

Indices of fetal growth, such as birth weight, are inversely associated with the prevalence and mortality of coronary heart disease and stroke.[1,2] It has been suggested that increased plasma concentrations of fibrinogen in later life may play a role in these associations.[3-5] The inverse association between birth weight and fibrinogen has been attributed to a programmed response to intrauterine malnutrition that induces permanent changes in the structure and function of organs, which cause increased levels of fibrinogen in later life.[1] This theory may be supported by a study demonstrating that people who were exposed to the Dutch famine in early gestation had slightly elevated levels of fibrinogen in later life.[5] If the association between birth weight and fibrinogen is due to intrauterine nutrition, improvements in intrauterine nutrition may lower fibrinogen levels in later life. However, the alternative view is that some factors other than intra-uterine nutrition may influence both growth in utero and levels of fibrinogen. Environmental causes, particularly those associated with socio-economic status, as well as genetic factors have been proposed as alternative explanations.[6] If the association between birth weight and fibrinogen is due to socio-economic or genetic causes, improvement of intrauterine nutrition may not lower fibrinogen levels in later life. Studies in dizygotic and monozygotic twin pairs still living with their parents offer a unique opportunity to distinguish between intra-uterine, socio-economic and genetic influences.[7-9] Studying differences in twin pairs avoids socio-economic factors that could confound the association between size at birth and cardiovascular disease in later life. Furthermore, investigating differences in monozygotic (genetically identical) twin pairs allows elimination of the influence of genotype on this association.

We here investigated the association between birth weight and fibrinogen in a group of adolescent dizygotic and monozygotic twin pairs still living with their parents. The underlying hypotheses for the within pair analyses were that if intrauterine nutrition is responsible for the association between birth weight and fibrinogen, the association would be present within both dizygotic and monozygotic twin pairs. If socio-economic factors are responsible, the association would be absent within both dizygotic and monozygotic twin pairs. If a genetic predisposition is responsible, birth weight would be associated with fibrinogen within dizygotic twin pairs, but not within monozygotic twin pairs.

Methods

Subjects

This study is part of a larger project in which cardiovascular risk factors were studied in 160 adolescent twin pairs and their parents.[8-10] Addresses of twins living in Amsterdam and neighbouring cities were obtained from City Council population registries. Twins

still living with their biological parents were contacted by letter. A questionnaire was used to gather information on various factors including the use of medication and smoking behaviour. The maternal questionnaire included questions regarding birth weight and gestational age of their children. This questionnaire was sent to the mothers a few weeks ahead of their visit to our department, allowing them to obtain birth data from birth certificates.[8,9] Opposite-sex dizygotic twin pairs (n=28) were excluded from the analyses because of the effects of sex differences within a pair on both birth weight and fibrinogen. Subjects using oral contraceptives were also excluded (6 dizygotic twin pairs and 7 monozygotic twin pairs). None of the subjects used any other medication that may affect plasma concentrations of fibrinogen. Thus, 52 dizygotic and 56 monozygotic twin pairs were available for analysis.

Measurements

Height and weight were measured in a standardized way. After acclimatization blood was obtained between 8:30 and 10:30 AM by venipuncture after overnight fasting. Total fibrinogen antigen in EDTA plasma was determined with an enzyme immuno assay that uses a pool of rabbit anti human fibrinogen IgG's as catching antibodies[11] and peroxidase conjugated monoclonal antibodies against fragment DD (DD13)[12] as tagging antibodies. Pooled plasma of healthy volunteers was used as a standard (100%) from which the fibrinogen content was determined additionally with a gravimetric method to express fibrinogen in g/L.[13]

Statistical Methods

The paired t test was used to compare twins with the lowest birth weight from each pair with their cotwins with the highest birth weight. For this analysis, 2 dizygotic twin pairs and 1 monozygotic twin pair had to be excluded because the birth weight of the twins within a pair was equal. Differences in dizygotic twin pairs and in monozygotic twin pairs were compared using the independent samples t-test. In addition, linear regression analysis was used to analyse whether intrapair differences in birth weight influenced intrapair differences in fibrinogen before and after adjustment for differences in current weight or body mass index (including the 3 twin pairs in which the birth weight of the twins within a pair was equal). Interaction analysis was performed to investigate whether zygosity or differences in current weight influenced the associations between intrapair differences in birth weight and fibrinogen. Linear regression analysis was used to analyse the association between birth weight and fibrinogen in the overall sample. Interaction analysis was performed to investigate whether zygosity influenced this association. A two-tailed P-value < 0.05 was considered significant. All analyses were performed on a personal computer using the statistical software package SPSS version 9.0 (SPSS Inc).

Results

As a first intrapair analysis, we compared co-twins with the lowest birth weight from each pair with their co-twins with the highest birth weight. The dizygotic but not the monozygotic twins with the lowest birth weight from each pair had fibrinogen levels that were higher compared with their co-twins with the highest birth weight (Table 1). The differences in fibrinogen between the cotwins with the lowest and the cotwins with the highest birth weight were significantly different in dizygotic compared with monozygotic twin pairs (p = 0.02). In both the dizygotic and the monozygotic twins, current BMI and smoking habits were similar in cotwins with the lowest and the cotwins with the highest birth weight. In an additional analysis, intrapair differences in birth weight were associated with differences in fibrinogen in dizygotic twins (regression coefficient, -0.25 g/L per kg [95%-CI: -0.49 to -0.01], p<0.05) suggesting that the larger the difference in birth weight, the higher the fibrinogen in the twin with the lowest birth weight compared with the co-twin with the highest birth weight. In monozygotic twins, however, intrapair differences in birth weight were not associated with differences in fibrinogen (+0.16 g/L per kg [95%-CI: -0.12 to 0.45], p = 0.3). Interaction analyses confirmed that these associations were significantly different between dizygotic and monozygotic twins (p = 0.03). In the overall sample of twins, birth weight was not associated with fibrinogen (regression coefficient: 0.01 g/L per kg [95%-CI: -0.10 to 0.13], p = 0.8, adjusted for age and sex).The results were similar after adjustment for (differences in) current weight, body mass index or smoking. In addition, the results were similar after the exclusion of smokers. Interaction analyses demonstrated that the (intrapair) associations between birth weight and fibrinogen were not different between men and women (P for interaction always >0.4).

Table 1. Clinical characteristics of the co-twins with the lowest and the highest birth weight in dizygotic and monozygotic twin pairs

	Dizygotic Twin Pairs			Monozygotic Twin Pairs		
	Co-twins with the lowest birth weight	Co-twins with the highest birth weight	p	Co-twins with the lowest birth weight	Co-twins with the highest birth weight	p
Birth weight, g	2226±477	2604±540	<0.001	2339±524	2637±475	<0.001
Gestational age, weeks	37±2.8	37±2.8	-	36±8.4	36±8.4	-
n (male/female)	50(29/21)	50(29/21)	-	55(29/26)	55(29/26)	-
Age, years	17.0±1.7	17.0±1.7	-	16.0±1.8	16.0±1.8	-
Current BMI, kg/m^2	20.0±1.9	20.3±2.2	0.5	19.5±2.3	19.8±2.3	0.2
Smoking, n	7	9	-	4	4	-
Fibrinogen, g/L	2.62±0.46	2.50±0.41	0.04	2.42±0.45	2.49±0.39	0.2

Mean±SD. BMI indicates body mass index.

Discussion

We found that low birth weight was associated with high fibrinogen within dizygotic twin pairs, but not within monozygotic twin pairs. These data provide the first evidence that the association between birth weight and fibrinogen is abolished after the elimination of genetic influences. Importantly, these findings contradict the hypothesis that improvement of intrauterine nutrition may lower fibrinogen levels in later life.

It could be argued that, besides genetic factors, intrauterine factors may also differ between dizygotic and monozygotic twins and may be the cause of the difference in the intrapair association between birth weight and fibrinogen. However, previous studies have demonstrated that the associations between low birth weight and increased cardiovascular risk factors in overall samples of twins were similar in dizygotic and monozygotic twins.[8,9,14] In addition, intrapair differences in birth weight were related to differences in diabetes,[15] HDL cholesterol,[9] and height[16,17] in both dizygotic and monozygotic twins. These studies suggest that intrauterine differences between dizygotic and monozygotic twins do not explain the differences in the intrapair association between birth weight and fibrinogen in dizygotic and monozygotic twins.

It could be suggested that twin pairs cannot be used as a model to study the association between birth weight and cardiovascular risk factors in singletons. However, birth weight in twins has been associated with many variables that have been related to birth weight in singletons.[8,9,14-17] In addition, fibrinogen levels in our adolescent twins were not different from levels found in studies in singletons.[18,19]

The absence of an association between birth weight and fibrinogen in the overall sample is consistent with several studies in singletons,[20-22] but not all.[3,4] Our results in dizygotic twin pairs demonstrate that the association between birth weight and fibrinogen may be strengthened after the elimination of socio-economic factors. In contrast, this association is abolished after the elimination of genetic factors. Genetic and socio-economic influences may be different across populations and may explain the contradictory findings of studies in singletons. Interestingly, it has been demonstrated that, although size at birth was not associated with plasma levels of fibrinogen in people born around the Dutch famine, people who were exposed to famine in early gestation had slightly elevated levels of fibrinogen in later life.[5]

The results from the Dutch famine birth cohort could be interpreted as a specific effect of the intrauterine environment on fibrinogen levels.[5] However, an alternative explanation is that this finding reflects a selective survival advantage of fetuses genetically susceptible to an increased cardiovascular risk.[23] During the famine, the number of conceptions was about 50% lower than the pre-famine level and perinatal mortality as well as mortality in the first year after birth were highest in those who were born during the famine.[24]

Our findings suggest that genetic factors account for the association between birth weight and fibrinogen. However, the genetic factors that may be responsible are not known. Interestingly, it has recently been demonstrated that several inherited risk factors for thrombophilia were related to low birth weight in Caucasian children.[25] Therefore, further research into the genetic factors responsible for the association between birth weight and fibrinogen is warranted.

In summary, we found that low birth weight was associated with high levels of fibrinogen within dizygotic twin pairs, but not within monozygotic twin pairs. These data suggest that genetic factors account for the association between birth weight and fibrinogen. Therefore, improvements in intrauterine nutrition may not lower fibrinogen levels in later life.

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Intra-uterine and genetic influences on the relationship between size at birth and height in later life Analysis in twins

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Abstract

Background Epidemiological studies have consistently shown a positive association between size at birth (i.e. birth weight or birth length) and height in children, adolescents and adults. To examine whether this association is explained by genetic or non-genetic (intra-uterine) factors, we investigated birth weight, birth length and height in 60 dizygotic and 68 monozygotic adolescent twin pairs still living with their parents.

Methods Birth weight of the twins was obtained from their mothers. Height was measured in a standardised way.

Results The mean age was 17±1.7 years for the dizygotic twins and 16±1.8 years for the monozygotic twins. Both dizygotic and monozygotic twins with the lowest birth weight from each pair had a height that was lower compared to their co-twins with the highest birth weight (dizygotic twins: 172.2±7.9 vs. 173.8±9.4 cm [P=0.05]; monozygotic twins: 171.1±9.4 vs. 171.8±9.5 cm [P=0.01]). Similarly, both dizygotic and monozygotic twins with the shortest birth length from each pair had a height that was lower compared to their co-twins with the longest birth length (dizygotic twins: 172.3±7.9 vs. 174.9±9.7 cm [P<0.05]; monozygotic twins: 168.9±10.6 vs. 169.9±10.2 cm [P<0.01]). In addition, intra-pair differences in birth weight and birth length were significantly associated with differences in height in both dizygotic twins (regression coefficient: 4.3 cm/kg [95% confidence interval: 1.0 to 7.5] and 0.96 cm/cm [0.17 to 1.74], respectively) and monozygotic twins (2.8 cm/kg [1.4 to 4.1] and 0.73 cm/cm [0.40 to 1.06], respectively). These associations were stronger in dizygotic than in monozygotic twins, but this difference was not statistically significant (for birth weight P=0.4; and for birth length P=0.6). However, genetic model fitting indicated that models incorporating a genetic source of the covariance gave a better description of the observed association of birth weight and length with height in later life than models not incorporating this genetic source. The results were similar for data on adult height after 12 years of follow-up in a subgroup of these twin pairs.

Conclusions These data suggest that the association between size at birth and height in later life is influenced by non-genetic intra-uterine and by genetic factors.

Introduction

Infants with a small size at birth are at increased risk of impaired postnatal growth and development.[1] Although most affected infants show some degree of catch-up in growth during the first years of life,[2,3] epidemiological studies have consistently shown a positive association between size at birth (i.e. birth weight or birth length) and height in children,[2,4,5] adolescents[6-10] and adults.[3,11-15] A recent study of approximately 40,000 young men showed that there was a mean difference of more than 7 cm in height between men with a low and a high birth weight (<2500 and >4500 g, respectively), and a mean difference of almost 10 cm in height between men who were short and those who were long at birth (<48 and >55 cm, respectively). [16] One leading theory postulates that intra-uterine programming in response to fetal malnutrition induces permanent changes in structure and function, which may cause shorter height in later life.[1] This theory is supported by a study in East Java that showed that energy supplementation during pregnancy promoted postnatal growth in children.[17] However, human exposure to famine in utero during World War II did not result in a decreased height in later life.[18,19] Alternatively, it has been put forward that some factors other than intra-uterine nutrition may influence both growth in utero and during childhood. Environmental causes, particularly those associated with socio-economic status,[11,20] as well as genetic factors have been proposed.[3,8,11] In other words, the socio-economic status or genotype responsible for short stature in later life may also cause retarded fetal growth in utero.

Twin pairs still living with their parents offer a unique opportunity to distinguish between intra-uterine, socio-economic and genetic influences.[21] Specifically, studying dizygotic twin pairs avoids socio-economic factors that could confound the association between size at birth and height in later life. Furthermore, investigating monozygotic (genetically identical) twin pairs allows almost complete elimination of the influence of genotype on the association between the variance in size at birth and that in height.[21] Therefore, if socio-economic factors are responsible for the association between size at birth and height in later life, it could be expected that the dizygotic twins with the smallest size at birth from each pair will not have a shorter height in later life compared to their co-twins with the largest size at birth. In addition, it could be expected that intra-pair differences in size at birth are not associated with intra-pair differences in height in dizygotic twins. If, however, genetic factors are responsible, these intra-pair associations would hold true for dizygotic twins, but not for monozygotic twins. To examine these issues, we investigated birth weight, birth length and height in a group of adolescent dizygotic and monozygotic twin pairs still living with their parents. To investigate whether these associations persisted into adulthood, we also analysed follow-up data on adult height in a subgroup of these twin pairs.

Methods

This study is part of a larger project in which cardiovascular risk factors were studied in 160 adolescent twin pairs and their parents.[22-24] Zygosity was determined as described in detail previously.[22] The maternal questionnaire included questions regarding birth weight, birth length and gestational age of their children.[25] Height was measured in a standardised way in all subjects.

In the total group, linear regression analysis was used to investigate the influence of birth weight and birth length on height in later life after adjustment for age and sex. Opposite-sex dizygotic twin pairs were excluded because of the effects of sex differences within a pair on both size at birth and height in later life. Birth weight data were available in 60 dizygotic and 68 monozygotic twin pairs, whereas birth length was available in 50 dizygotic and 61 monozygotic twin pairs. An interaction analysis was performed to investigate whether zygosity influenced the associations between size at birth and height. As in previous twin studies investigating the association between birth weight and adult health,[25-29] we compared the twins with the lowest birth weight from each pair with their co-twins with the highest birth weight. For this analysis, the paired Student t-test was used and 2 dizygotic and 2 monozygotic twin pairs had to be excluded because the birth weight of the twins within a pair was equal. In addition, twins with the shortest birth length from each pair were compared with their co-twins with the longest birth length. For this analysis, 10 dizygotic and 19 monozygotic twin pairs had to be excluded because the birth length of the twins within a pair was equal. The differences in dizygotic twins and the differences in monozygotic twins were compared using the independent samples t-test, and MANOVA was used to adjust for differences in birth weight. Linear regression analysis was used to analyse whether intra-pair differences in birth weight and length influenced intra-pair differences in height in dizygotic and monozygotic twins. Intra-pair differences in size at birth were calculated by randomly subtracting the twin with the smallest size at birth from the co-twin with the largest size at birth or vice versa.[30] Interaction analysis was performed to investigate whether zygosity or sex influenced the associations between intra-pair differences in size at birth and differences in height.

To investigate whether these associations persisted into adulthood, we also analysed data on adult height after 12 years of follow-up. Data on adult height could be obtained in 31 dizygotic and 30 monozygotic twin pairs. Of these twin pairs, birth length was available in 26 dizygotic and 25 monozygotic twin pairs. In the total group, linear regression analysis was used to investigate the influence of birth weight and birth length on height in later life after adjustment for age and sex. In addition, linear regression analysis was used to analyse whether intra-pair differences in birth weight and length influenced intra-pair differences in height in dizygotic and monozygotic twins. A two-tailed P-value < 0.05 was considered significant. The above analyses were performed on a personal computer using the statistical software package SPSS version 9.0 (SPSS, Chicago, IL, USA).

Table 1. Association of birth weight and length with height (cm) in the total group of twins after adjustment for age and sex

Variable	Beta	95%-CI	*P*
Birth weight (per kg)	3.6	1.9 to 5.4	<0.001
Birth length (per cm)	0.92	0.62 to 1.22	<0.001

Beta is the slope of the linear regression line. CI indicates confidence interval

Finally, we used genetic model fitting to determine the relative magnitudes of genetic, common environmental and unique environmental influences on the relationship between size at birth and height in later life. Genetic model fitting uses maximum likelihood-based path analysis. Genetic model fitting was done with Mx, a computer program specifically designed for the analysis of genetically informative data.[31] Although less transparent than regression analyses, it is very sensitive in determining genetic and environmental influences. In addition, genetic model fitting allows the inclusion of the data of opposite-sex dizygotic twins (28 pairs). The covariance of birth weight and length with height was decomposed into a genetic and a non-genetic part. Estimates of the relative importance of these genetic and non-genetic influences were allowed to differ between men and women.

Results

In the total group of twins, positive associations of birth weight and birth length with height were found after adjustment for age and sex (Table 1). Interaction analysis indicated that these associations were not significantly modified by sex or zygosity (data not shown).

Comparison between twins with the smallest size at birth and their co-twins with the largest size at birth

The differences in birth weight between the twins with the lowest birth weight and their co-twins with the highest birth weight were larger for dizygotic compared to monozygotic twin pairs (378 and 283 g, respectively; P for the difference, 0.07; Table 2). The mean age was 17.4±1.9 years for the dizygotic twins and 16.4±2.0 years for the monozygotic twins. Both dizygotic and monozygotic co-twins with the lowest birth weight from each pair were shorter in later life than their co-twins with the highest birth weight. The differences in height between the twins with the lowest and the co-twins with the highest birth weight were larger in dizygotic than in monozygotic twins, but this difference was not statistically significant (*P* for the difference 0.3; after adjustment for differences in birth weight, *P*=0.4).

Table 2. Dizygotic and monozygotic co-twins according to birth weight

	Dizygotic twin pairs			Monozygotic twin pairs		
Variable	Co-twin with the lowest birth weight	Co-twin with the highest birth weight	*P*	Co-twin with the lowest birth weight	Co-twin with the highest birth weight	*P*
Birth weight (kg)	2.27±0.49	2.65±0.55	<0.001	2.34±0.51	2.62±0.47	<0.001
N (male/female)	58(30/28)	58(30/28)	-	66(32/34)	66(32/34)	-
Age (years)	17.4±1.9	17.4±1.9	-	16.4±2.0	16.4±2.0	-
Height (cm)	172.2±7.9	173.8±9.4	0.05	171.1±9.4	171.8±9.5	0.01

Table 3. Dizygotic and monozygotic co-twins according to birth length

	Dizygotic twin pairs			Monozygotic twin pairs		
Variable	Co-twin with the shortest birth length	Co-twin with the longest birth length	*P*	Co-twin with the shortest birth length	Co-twin with the longest birth length	*P*
Birth length (cm)	45.9±3.3	48.2±3.2	<0.001	45.8±3.2	47.3±3.1	<0.001
N (male/female)	40(24/16)	40(24/16)	-	42(19/23)	42(19/23)	-
Age (years)	17.1±1.9	17.1±1.9	-	16.0±1.9	16.0±1.9	-
Height (cm)	172.3±7.9	174.9±9.7	<0.05	168.9±10.6	169.9±10.2	<0.01

The differences in birth length between the twins with the shortest birth length and their co-twins with the longest birth length were larger for dizygotic compared to monozygotic twin pairs (2.3 and 1.5 cm, respectively; *P* for the difference, <0.05, Table 3). The mean age was 17.1±1.9 years for the dizygotic twins and 16.0±2.0 years for the monozygotic twins. Both dizygotic and monozygotic twins with the shortest birth length from each pair were significantly shorter in later life than their co-twins with the longest birth length**.** The differences in height between the twins with the shortest and the co-twins with the longest birth length were larger in dizygotic than in monozygotic twins, but this difference was not statistically significant (*P* for the difference, 0.2; after adjustment for differences in birth length, *P*=0.3).

Associations between intra-pair differences in size at birth and differences in height

Table 4 shows that intra-pair differences in birth weight and length were positively associated with differences in height in both dizygotic and monozygotic twins. For example, a positive difference in birth weight of 1 kg within pairs was associated with a positive difference in height of 4.3 cm in dizygotic twin pairs and 2.6 cm in monozygotic twin pairs. These associations were stronger in dizygotic than in monozygotic twins, but this difference was not statistically significant (for birth weight *P*=0.4; and for birth length *P*=0.6).

If subjects with a gestational age shorter than 37 weeks (for the analysis with birth weight, 24 dizygotic and 28 monozygotic twin pairs; for the analysis with birth length, 20 dizygotic and 25 monozygotic twin pairs) were excluded, the results were similar.

Table 4. Associations of intra-pair differences in birth weight and length with differences in height (cm)

	Dizygotic twin pairs			Monozygotic twin pairs		
Variable	Beta	95%-CI	*P*	Beta	95%-CI	*P*
Birth weight (per kg)	4.3	1.0 to 7.5	<0.05	2.8	1.4 to 4.1	<0.01
Birth length (per cm)	0.96	0.17 to 1.74	<0.05	0.73	0.40 to 1.06	<0.01

Beta is the slope of the linear regression line. CI indicates confidence interval

Adjustment for gestational age also did not importantly change the results (data not shown). Interaction analysis indicated that the intra-pair association between size at birth and height in later life was not influenced by sex in either dizygotic or monozygotic twins.

Associations between size at birth and height during follow-up

Birth weight and birth length were significantly associated with adult height at follow-up after adjustment for age and sex (regression coefficient: 3.7 cm/kg [95%-confidence interval: 1.35 to 6.1] and 0.71 cm/cm [0.28 to 1.15], respectively). In addition, intra-pair differences in birth weight and birth length were positively associated with differences in final height in both dizygotic twins (regression coefficient: 3.4 cm/kg [95-% confidence interval: -0.32 to 7.1] and 0.41 cm/cm [-0.63 to 1.45], respectively) and monozygotic twins (2.1 cm/kg [0.13 to 4.2] and 0.25 cm/cm [-0.21 to 0.70], respectively).

Genetic model fitting

Table 5 summarises the genetic model fitting analyses for birth weight and height and for birth length and height. The full model in Table 5 specifies influences of additive genes (A), common -or shared- environment (C) and unique -or not shared- environment (E) for size at birth and height, and for the covariance between them. Compared to this full model, a model that specifies common environmental influences for size at birth only (model 2) does not show a significant deterioration in fit (for birth weight: χ^2=4.985, *df*=4, *P*=0.3; for birth length: χ^2=5.476, *df*=4, *P*=0.2) indicating that shared familial factors do not contribute to height later in life. Models 3 and 4, however, both fit the data significantly worse (Model 3: for birth weight, χ^2=18.012, *df*=6, *P*<0.01; for birth length, χ^2=28.927, *df*=6, *P*<0.001, and Model 4: for birth weight, χ^2=28.464, *df*=6, *P*<0.001; for birth length χ^2=27.588, *df*=6, *P*<0.001). In model 3, the covariance between birth weight and height is due solely to environmental factors and genetic effects do not contribute to the covariance. In model 4, genetic factors are the only source of covariation between the two measures. The fact that these more parsimonious models do not describe the data as well as model 2 indicates that the association between size at birth and height is due both to genetic and unique environmental (i.e. intrauterine) factors.

Table 5. Genetic model fitting for birth weight and height and for birth length and height

	Birth weight		Birth length	
Model	-2log-likelihood	df	-2 log-likelihood	df
1. full model (ACE)	2989.476	750	3829.144	710
2. only C for size at birth	2994.461	754	3834.611	714
3. no genetic correlation	3017.940	756	3858.071	716
4. no environmental correlation	3007.488	756	3856.732	716

The full ACE model included Additive genetic, Common environmental and unique Environmental influences. Model 2 included only C for size at birth and models 3 and 4 specified no genetic or no environmental association between size at birth and height. All models also included Age as a factor that could explain part of the variance.

Table 6. Standardized parameter estimates for size at birth and height in later life. Proportions of (co-) variance explained by genetic and environmental influences and age

	Additive genetic factors		Common environment		Unique environment		Age	
	Men	Women	Men	Women	Men	Women	Men	Women
Variables								
BW	0.25	0.49	0.49	0.24	0.24	0.20	0.01	0.06
BL	0.26	0.35	0.60	0.49	0.13	0.07	0.01	0.10
Height	0.96	0.52	0.00	0.00	0.03	0.02	0.01	0.45
Associations								
BW - height	0.83	0.56	0.00	0.00	0.14	0.06	0.03	0.38
BL - height	0.81	0.51	0.00	0.00	0.16	0.03	0.03	0.46

BW indicates birth weight; BL, birth length.

Table 7. Genetic and environmental correlation between size at birth and height in later life

	Genetic correlation		Environmental correlation	
	Men	Women	Men	Women
BW-height	0.49	0.49	0.45	0.39
BL-height	0.46	0.55	0.68	0.33

BW indicates birth weight; BL, birth length.

Table 6 (upper panel) shows the proportion of variance in birth weight, birth size and height explained by additive genetic factors, common environment, unique environment and age for men and women separately. Table 6 (lower panel) shows the proportion of co-variance between size at birth and height in later life explained by genetic and environmental factors. Genetic and environmental covariation can also be expressed as genetic and environmental correlations (Table 7). These correlations may be conceptualised in a simplified manner as an indication of the extent to which genetic (or environmental) influences for two measures are 'the same' or 'overlap'. These correlations differ from the proportion of phenotypic covariation in that the heritability of each variable is not included in calculations. Even though genetic influences on two variables may be slight, it could be that the influences are identical. This would lead to a high genetic correlation, but a low proportion of phenotypic covariation due to the small

individual genetic influences. For example, in males, the genetic correlation between birth weight and height in later life was 0.49, suggesting that about half of the genetic effects on birth weight and height are the same. The environmental correlation between birth weight and height was 0.45, indicating that about half of the environmental influences on birth weight and height are the same.

Discussion

In accordance with previous studies in singletons, we found positive associations between size at birth and height in later life in twins. In both dizygotic and monozygotic twin pairs, the twins with the lowest birth weight from each pair had a shorter height in later life compared to their co-twins with the highest birth weight. Furthermore, both dizygotic and monozygotic twins with the shortest birth length from each pair had a shorter height in later life compared to their co-twins with the longest birth length. In addition, significant positive associations of intra-pair differences in birth weight and length with intra-pair differences in height were observed in both dizygotic and monozygotic twin pairs. These data suggest that the association between size at birth and height in later life is independent of socio-economic factors and, to some extent, of genetic factors. Intra-uterine factors are therefore likely to be important. However, the comparison of monozygotic twins with dizygotic twins demonstrates that elimination of genetic factors diminishes the size of the association between size at birth and height in later life, suggesting that genetic factors also play a role. This is supported by the genetic model fitting that indicates that models incorporating a genetic source of the variance gave a more accurate description of the association between size at birth and height in later life than models not incorporating a genetic source. Taken together, these data suggest that the association between size at birth and height in later life is influenced by intra-uterine and genetic factors, whereas socio-economic factors do not play a role. Our findings during follow-up suggest that these influences persist into adulthood and are important in the determination of adult height.

Our results in monozygotic twins are consistent with a study that showed a positive association between intra-pair differences in birth weight and adult height in a group of adult monozygotic twins.[32] However, this study did not examine intra-pair associations in dizygotic twins and therefore, the importance of socio-economic and genetic influences on the association between birth weight and height could not be investigated. Furthermore, data on birth length were not available.

It has been suggested that improvement of fetal nutrition and thus, intra-uterine growth, may prevent the development of short stature in later life.[1] Our twin study indeed demonstrated that intra-uterine factors play a role in the association between size at birth and height in later life, suggesting that improvement of intra-uterine growth may increase height in later life. This is in accordance with a study in East Java that showed

that energy supplementation during pregnancy promoted postnatal growth in children,[17] but in contrast to studies in Europe that demonstrated that human exposure to famine in utero did not result in a decreased height in later life.[18,19] It should, however, be noted that the demonstrated associations between intrapair differences in size at birth and differences in height cannot be due to maternal nutrition, which is the same for both twins. In addition, the genetic model that specifies common environmental influences for size at birth only did not show a significant deterioration in fit, indicating that shared familial factors (including maternal nutrition) do not contribute to the association between size at birth and height. Therefore, maternal nutrition is not important in the association between size at birth and height in later life. The intrauterine influences on this association may be related to differences in delivery of nutrients to the twins.

Recently, several studies have reported that exogenous growth hormone administration is an effective option to normalise the childhood growth of low birth weight children.[33-35] However, it is unknown whether growth hormone induced a catch-up in growth towards the genetic target level or an acceleration of growth on top of a genetically determined short height. Our results suggest that the association between low birth weight and short height in later life is in part due to intra-uterine factors. Therefore, exogenous growth hormone may influence childhood growth by inducing a catch-up in growth towards the genetic target level.

We conclude that both non-genetic intra-uterine and genetic factors influence the association between size at birth and height in later life. This suggests that improvement of the intra-uterine delivery of nutrients may in part prevent the development of short stature in later life.

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Summary, general conclusions and future perspectives

Summary

In this thesis we have investigated the relationships among birth weight, microvascular function and cardiovascular risk factors (figure 1). The microcirculation is important in determining the peripheral vascular resistance as well as the delivery of nutrients to the tissues. An impaired microvascular function may therefore be important in the development of high blood pressure and insulin resistance, both of which are associated with increased cardiovascular risk. Low birth weight is also associated with an increased cardiovascular risk. This association is probably mediated by the association of low birth weight with high blood pressure, insulin resistance and other cardiovascular risk factors. In addition, it has been suggested that an impaired microvascular function is related to a lower birth weight. In view of these findings, a better understanding of the relationships among birth weight, microvascular function and cardiovascular risk factors may contribute to prevention of cardiovascular disease and reveal new therapeutic targets.

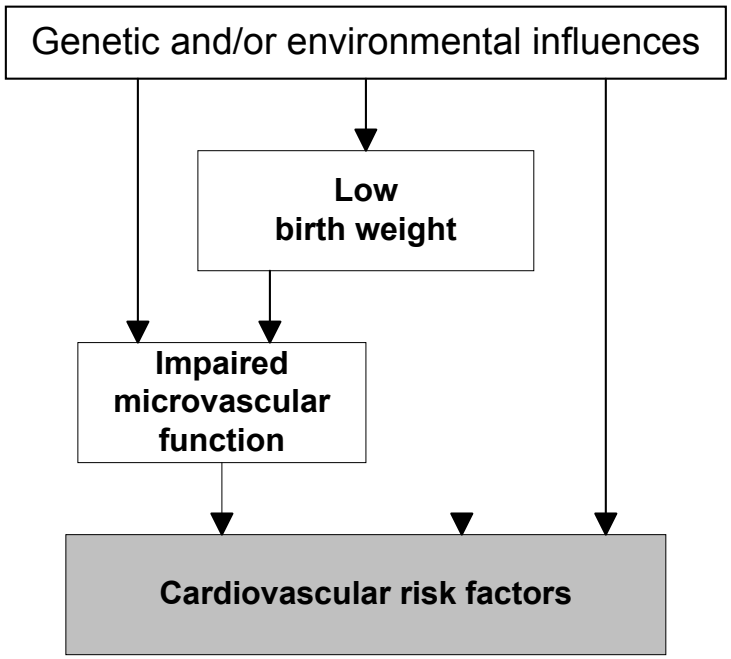

Figure 1. The postulated relations among birth weight, microvascular function and cardiovascular risk factors. Both birth weight and microvascular function are related to cardiovascular risk factors, and microvascular function may link birth weight with cardiovascular risk. All of these variables may be influenced by genetic and/or environmental influences.

Part 1 Birth weight and microvascular function

Epidemiological studies have consistently demonstrated that weight at birth is negatively associated with blood pressure and insulin resistance in adult life. Regardless of whether or not the origin of these relationships is genetic or environmental, alterations in microvascular function may be a possible mechanism explaining these associations. We have previously demonstrated that capillary recruitment during post-occlusive reactive hyperaemia was related to birth weight, blood pressure and insulin sensitivity in adults, but it could not be resolved whether the impaired capillary recruitment in subjects with a low birth weight was a cause or a consequence of the higher blood pressure and/or insulin resistance. In a study of prepubertal children, we found that birth weight was associated with capillary recruitment, but not with blood pressure or insulin sensitivity (Chapter 2). These data suggest that an impairment in capillary recruitment is a primary disturbance in individuals with a low birth weight, and is not secondary to higher blood pressure and/or insulin resistance. Changes in microvascular function may be a potential mechanistic explanation for the association of birth weight with blood pressure and insulin resistance.

Part 2 Microvascular function and cardiovascular risk factors

Insulin-mediated changes in muscle perfusion have been proposed to modulate insulin-mediated glucose uptake. We have investigated the effect of insulin on the microcirculation which may permit such modulation. Systemic hyperinsulinaemia induced recruitment of capillaries in skin, augmented nitric-oxide-mediated vasodilatation and influenced vasomotion in skin (Chapter 3). In addition, locally administered insulin induced a rapid increase in total skin blood flow, independent of systemic effects. These findings offer a potential physiological framework for further study of the functional coupling between insulin's metabolic and vascular actions.

Microvascular function has also been proposed as a possible mechanism explaining the association of acute smoking with an increased blood pressure and a decreased insulin sensitivity. We have examined the acute effects of smoking on skin microcirculatory function (Chapter 4). Acute smoking was associated with an impaired recruitment of capillaries and an impaired microvascular endothelium-dependent vasodilatation, whereas endothelium-independent vasodilation was not influenced. These findings are consistent with the hypothesis that the association of acute smoking with an increased blood pressure and a decreased insulin sensitivity is due to changes in microvascular function.

The inflammatory cytokine tumour necrosis factor α (TNF-α) has been reported to play an important role in insulin resistance. The mechanism by which TNF-α may cause insulin resistance is not clear. It has been suggested that TNF-α causes defects in capillary function, with a decreased access of insulin and glucose to tissues. To test this hypothesis, we assessed plasma TNF-α levels, skin capillary recruitment during post-occlusive reactive hyperaemia and insulin sensitivity in healthy adult individuals

(Chapter 5). In addition, to investigate whether these associations are already present in prepubertal children, we measured these variables in 21 of their children. TNF-α was associated with capillary recruitment during post-occlusive hyperaemia in adults. In addition, this capillary recruitment could partly explain the relationship between TNF-α and insulin resistance. Our findings thus provide support for a vascular component of TNF-α-induced insulin resistance. These associations, however, were not present in the prepubertal children. Our findings therefore suggest that the relationships among TNF-α, vascular function and insulin sensitivity are initiated during growth from childhood to adulthood.

Coronary microvascular disease may explain the occurrence of myocardial ischaemia without overt coronary artery blockage, as well as heart failure and mortality after myocardial infarction. However, methods to assess the coronary microcirculation are invasive and applicable only in experimental settings. The skin microcirculation offers an opportunity to noninvasively explore the relation of systemic microvascular dysfunction to (risk factors for) coronary heart disease. In our study, coronary heart disease risk was assessed with the use of the coronary heart disease risk score according to the Framingham Heart Study. We found that an increased coronary heart disease risk was associated with a lower endothelium-dependent vasodilatation and capillary recruitment in skin (Chapter 6). Our findings thus suggest that microvascular function in skin may be a valid model for the study of the relationships between cardiovascular risk factors on the one hand and microvascular function on the other.

Part 3 Birth weight and cardiovascular risk factors in twins

In Chapter 7 we have postulated that twin studies offer a unique opportunity to distinguish between intrauterine and genetic origins of the association between birth weight and cardiovascular risk factors in later life. We have discussed several advantages and limitations of the use of twin studies to investigate the influence of intrauterine and genetic factors. We have also emphasized that the comparison of within-pair analyses with unpaired analyses cannot be used to identify maternal influences on the association between birth weight and cardiovascular risk factors.

Many epidemiological studies have shown an inverse association between birth weight and blood pressure. To examine whether this association is explained by intrauterine or genetic factors, we have investigated birth weight and blood pressure in dizygotic and monozygotic adolescent twin pairs (Chapter 8). Intrapair differences in birth weight were negatively associated with differences in blood pressure in dizygotic twins, but not in monozygotic twins. This difference in the birth weight-blood pressure relationship between dizygotic and monozygotic twin pairs suggests that genetic factors may play an important role in the association between birth weight and blood pressure. Alterations in sympathetic and parasympathetic activity may be important mechanisms

explaining this birth weight-blood pressure relationship. We showed that low birth weight is associated with increased sympathetic activity, and that a large part of the association between birth weight and blood pressure is explained by this increase (Chapter 9). In addition, the within-pair analyses demonstrated that the association between birth weight and sympathetic activity was present in dizygotic twins but not in monozygotic twins, suggesting that the association between low birth weight and an increased sympathetic activity also depends on genetic factors. Birth weight was not associated with parasympathetic activity.

In a subgroup of this twin cohort, we found that intrapair differences in birth weight were negatively associated with differences in insulin resistance in both dizygotic twins and monozygotic twins (Chapter 10). This association was significant within monozygotic twins. These data suggest that the association between low birth weight and insulin resistance is not entirely due to a common genetic factor. Therefore, the association between these two variables appears, at least in part, due to intrauterine factors.

Low birth weight was associated with high total and LDL cholesterol within dizygotic twin pairs, but with low total and LDL cholesterol within monozygotic twin pairs (Chapter 11). In addition, low birth weight was associated with high fibrinogen within dizygotic twin pairs, but not within monozygotic twin pairs (Chapter 13). These data suggest that the association of birth weight with these cardiovascular risk factors is strongly influenced by the elimination of genetic factors (as achieved by using differences within monozygotic twin pairs). On the other hand, intrapair differences in birth weight were positively associated with differences in HDL cholesterol in both dizygotic and monozygotic twins. These data suggest that the association between birth weight and levels of HDL cholesterol may be independent of genetic factors. Plasma levels of lathosterol, campesterol and β-sitosterol, which are indicators of cholesterol synthesis and absorption, did not explain the association of low birth weight with high levels of total and LDL cholesterol. (Chapter 12), suggesting that these associations may be due, instead, to a decrease in cholesterol clearance.

Finally, intrapair differences in birth weight were significantly associated with differences in height in both monozygotic and dizygotic twins in adolescence (Chapter 14). The results were similar for data on adult height after 12 years of follow-up in a subgroup of these twin pairs. These data suggest that the association between size at birth and height in later life is in part due to intrauterine factors.

General discussion and future perspectives

1. Methodological considerations

1.1 Birth weight as a measure of intrauterine growth

In several chapters in this thesis, birth weight is used as a measure of intrauterine growth. The reliability of the birth weight data is therefore important. All children described in Chapter 2 had been born at the VU University Medical Center in Amsterdam. Therefore, birth weight data could easily be obtained from hospital records, which is the most reliable way to obtain birth data. However, in older individuals that have been born at home or in different hospitals, obtaining birth records is very labour-intensive and the recovery rate is usually low. As an alternative, several studies have relied upon self-reported birth weight data, but the accuracy of self-reported birth weight in adults is moderate (correlations with birth weight obtained from birth records ranged from 0.64 to 0.82).[1-4] In our studies of adolescent twins (Chapter 8-14), we have used information on birth weight data obtained from the mothers, which has been shown to be very accurate and reliable (correlations with birth weigh obtained from birth records ranged from 0.89 to 0.96).[3,5-7] The accuracy of maternal recall is not surprising. When asked, mothers are usually quick to remember their child's birth weight, because this piece of information is usually eagerly awaited at the time of delivery and also repeatedly relayed to friends and family, thereby imprinting it to memory.[6] Nevertheless, in our twin study, the questionnaires regarding birth weight were sent to the mothers a few weeks ahead of their visit to the department of Biological Psychology. This allowed the mothers to obtain the birth data from birth certificates, further increasing the accuracy of the data.

It has been proposed that birth weight is only a crude marker of intrauterine growth. The same birth weight may be the result of many different paths of growth, and more detailed measurements of body size at birth may give insights into the adaptations made by the fetus. This may sound reasonable, but alternatives do not clearly solve this problem either, as illustrated by the fact that numerous estimates of intrauterine growth have been used, for example birth length,[8-21] crown-heel length,[12] ponderal index,[8,13-16,21-29] abdominal circumference,[12,30-32] chest circumference,[30] head circumference,[8,11-13,24,28,30,32] placental weight,[19,27,33-36] maternal weight[37] and weight gain,[38] maternal pelvic size,[12,13] maternal triceps skinfold thickness,[38] birth weight in relation to head circumference,[13,39] birth length in relation to head circumference,[26,33,39] birth weight in relation to placental weight,[10,13,21,24,28,35,40,41] ponderal index in relation to placental weight,[37] placental weight in relation to head size,[13] head circumference in relation to abdominal circumference,[27] and the length of the second finger in relation to the length of the fourth finger.[42] Selective emphasis on different estimates of size at birth is in addition likely to have biased the strength of the association between size at birth and cardiovascular risk (factors) in these studies.[43]

1.2 Measurement of microvascular function

1.2.1 Skin microcirculation versus muscle microcirculation

Since muscle is considered the main peripheral site of insulin-mediated glucose uptake,[44] insulin-mediated vasodilatation,[45] and vascular resistance,[46] it might have been more straightforward to study muscle rather than skin microcirculatory function. However, the study of muscle capillary density requires invasive techniques, and muscle capillary recruitment cannot be studied by currently available methods. It should be appreciated that microvascular dysfunction appears to be a systemic process.[47-49] Several studies have suggested that microcirculation in skin resembles the microcirculation in other tissues. Although skin microvascular resistance does not make a major contribution to the total peripheral vascular resistance, an association between skin microvascular function and blood pressure could nevertheless be demonstrated in several studies.[50-54] In addition, in individuals with hypertension, microvascular defects can be demonstrated in heart,[49] skeletal muscle[55] and skin.[56,57] Similarly, although muscle is the main peripheral site of insulin-mediated glucose uptake, an association of diabetes and/or insulin resistance with microvascular function has been reported in heart,[49] skeletal muscle[58,59] and skin[50-52,54] Moreover, metabolic and vascular effects of insulin could be demonstrated in skin.[60,61] Taken together, these studies suggest that microvascular function in skin resembles microvascular function in other tissues in many ways. Nevertheless, direct comparisons between skin and muscle microvascular function have not been reported, and it seems worthwhile to investigate whether our findings in skin can be extrapolated to muscle. Recent developments of the laser Doppler technique allow the assessment of blood flow in deeper tissues, such as the human skeletal muscle, with a small laser Doppler probe.[62,63] With this technique vasomotion and post-occlusive reactive hyperaemia can be investigated. It has recently been shown that the heterogeneity of laser Doppler flowmetry in perfused muscle was indicative of nutritive and nonnutritive flow.[64] In addition, insulin stimulated laser Doppler signal by rat muscle in vivo, consistent with nutritive flow recruitment.[65] Therefore, it is of interest to investigate the effect of hyperinsulinaemia on microvascular perfusion in human muscle as assessed with laser Doppler.

1.2.2 Skin microcirculation versus cardiac microcirculation

Coronary microvascular disease may explain the occurrence of myocardial ischaemia without overt coronary artery blockage [66-70], as well as heart failure [71,72] and mortality.[72] We have demonstrated that individuals at an increased risk for coronary heart disease are characterized by an impaired microvascular function in skin. However, our data do not provide direct evidence of an association between coronary microvascular disease and risk of coronary heart disease, as there was no assessment of coronary microcirculation. As described above, microvascular defects could not only be demonstrated in skeletal muscle[55,58,59] and skin,[50,56,57] but also in heart muscle[49] of individuals with hypertension, diabetes and/or insulin resistance. Nevertheless,

comparisons between microvascular function tests in skin and heart should be performed.

1.2.3 Microvascular endothelium-dependent vasodilation

Iontophoresis of acetylcholine and sodium nitroprusside has been widely used to investigate microvascular endothelial and smooth muscle cell function.[50,51,54,73-78] However, two important issues should be discussed.

First, the mechanism of acetylcholine-mediated vasodilatation in the skin microcirculation is not clear. Some studies are consistent with an important role for nitric oxide.[79,80] Consequently, sodium nitroprusside, as an nitric oxide-donor, may be an appropriate endothelium-independent control of the acetylcholine-mediated vasodilation. Other studies have suggested that prostaglandins mediate the vasodilatory effect of acetylcholine,[81,82] but this could not be found in a recent study.[83] If prostaglandins are important, iontophoresis of sodium nitroprusside is not an appropriate endothelium-independent control of the acetylcholine-mediated vasodilation. In other words, although sodium nitroprusside is an endothelium-dependent vasodilator, it may not be an appropriate control for possible prostaglandin-dependent pathways of the acetylcholine-mediated vasodilation. Tests with intra-arterial infusion of a nitric oxide synthase inhibitor, N(G)-monomethyl-L-arginine (L-NMMA) and acetylsalicylic acid are necessary to elucidate the role of nitric oxide and prostaglandins, respectively. If prostaglandins are important in the skin microvascular vasodilation, it would be appropriate to develop prostaglandin-dependent, endothelium-independent procedures as a control. For example, it may be of interest to investigate the possibility to iontophorese prostaglandin E2 and F2α, which have been shown to mediate vasodilatation.[84,85]

Second, the interpretation of the response during iontophoretic administration of sodium nitroprusside may be biased by non-specific vasodilation in response to the current and/or the water used as the drug vehicle. During anodal iontophoresis these non-specific effects are weak,[86] but several studies have reported that during cathodal iontophoresis the non-specific effects may be substantial.[76,86] Previous studies in our department with the use of the Perimed iontophoresis system have shown that both acetylcholine and nitroprusside vehicle elicited a small but significant increase in blood flow (median: from 14.7 to 16.2 PU ($P<0.01$) and from 15.4 to 17.7 PU ($P<0.01$), respectively).[51] However, correction for vehicle response did not importantly affect any of the associations among microvascular function, birth weight, blood pressure and insulin sensitivity. In Chapter 3 of this thesis, the vehicle responses were unaltered during hyperinsulinaemia (absolute changes in microcirculatory flow before and during hyperinsulinaemia were: -2.1±7.4 vs. +1.0±5.0 PU, $P=0.6$, and +6.9±16.3 vs. +4.6±13.8, $P=0.9$ during iontophoretically applied acetylcholine vehicle and sodium nitroprusside vehicle, respectively). Taken together, these data suggest that the non-

specific effects do not play an important role in the relationships among birth weight, microvascular function, blood pressure and insulin's vascular and metabolic actions.

1.3 Measurement of cardiovascular risk factors

1.3.1 Measurement of blood pressure

Because blood pressure measurements are notoriously variable, measuring blood pressure only once will result in an inaccurate estimation of the true blood pressure and an underestimation of the strength of the true associations of blood pressure with other variables. For example, imprecision resulting from a single measurement of diastolic blood pressure resulted in a 60% attenuation of the relative risk for the effect of elevated blood pressure on stroke and MI.[87] Repeating the blood pressure measurement is an effective method to get a more precise estimate of the true blood pressure.[88] In our twin studies, the means of six blood pressure measurements were used in the analyses.

The accuracy of the estimation of blood pressure may be further improved by ambulatory measurements. In Chapters 2 and 6, ambulatory monitoring (Spacelabs 90207; Redmond, Washington, USA) was used to obtain 24-h recordings of blood pressure. The readings were downloaded onto a computer spreadsheet and individually edited into daytime and night-time periods from the subjects' diaries.[89] Several studies have shown that ambulatory blood pressure is more closely related to target organ damage than office or home blood pressures.[90-92] In addition, long-term follow-up showed that ambulatory blood pressure was a better predictor of cardiovascular risk than was office blood pressure.[93,94]

1.3.2 Measurement of insulin sensitivity

It is generally believed that the euglycaemic hyperinsulinaemic clamp technique is the best available standard for the measurement of insulin-mediated glucose uptake.[95]We have used this technique to investigate insulin sensitivity in the children in Chapter 2. We have also used this technique to investigate the effect of hyperinsulinaemia on microvascular function (Chapter 3). However, the technique is invasive and time-consuming, and, therefore, difficult to perform in large-scale studies. As an alternative, insulin sensitivity can be estimated with homeostatic model assessment (HOMA). Homeostatic model assessment (HOMA) relies on the product of fasting plasma glucose and fasting plasma insulin.[96] Although reasonable correlations can be observed between HOMA and clamp-derived insulin sensitivity,[97-99] it should be realised that from a pathophysiological standpoint, HOMA and clamp-derived insulin sensitivity may provide different information.[95,97,100] HOMA essentially explores hepatic insulin sensitivity,[97] whereas the clamp technique measures insulin-mediated glucose disposal in peripheral tissues, mainly muscle.[95]

1.3.3 Measurement of TNF-α

In chapter 5, human TNF-*α* was measured in plasma by a sandwich enzyme immunoassay. It is not known to what extent circulating TNF-α-levels are biologically active. Nevertheless, several studies have shown an inverse relationship of plasma levels of TNF-α with insulin sensitivity.[101-103] These relationships are similar to studies demonstrating associations between TNF-α mRNA and insulin resistance.[104,105] In addition, these relationships are similar to animal studies demonstrating that administration and/or neutralisation of TNF-α has direct effects on insulin resistance.[106-109] Taken together, these data suggest that circulating TNF-α-levels provide information that is useful to study the link between TNF-α and insulin resistance. Circulating TNF-α may either be a marker of the amount of TNF-α that is produced locally or act synergistically with locally produced TNF-α.

1.3.4 The Framingham coronary heart disease risk score as a measure of coronary heart disease risk

In Chapter 6, coronary heart disease (CHD) risk was estimated with the use of the coronary heart disease risk score derived from the Framingham Heart Study. The CHD risk score can be calculated for men and women from risk factors that can easily be obtained: age, blood pressure, cigarette smoking, total cholesterol, HDL-cholesterol and diabetes.[110] Although this risk score has been widely used to estimate CHD risk in middle-aged white populations,[111-114] it should be noted that it is only a surrogate marker of coronary heart disease. Not all individuals with a high risk score will develop coronary heart disease, and many individuals with a low risk score will develop coronary heart disease. To investigate whether microvascular function is involved in the development of coronary heart disease, longitudinal studies are necessary. For example, it could be investigated whether microvascular function predicts disease progression and coronary event rates in individuals with an increased risk for coronary heart disease.

1.3.5 Measurement of cardiac autonomic nervous system activity

Several techniques have been developed as indicators of the activity of the autonomic nervous system, such as muscle sympathetic nerve activity,[115,116] plasma levels of norepinephrine,[117,118] spectral analysis of heart rate,[119,120] bedside cardiovascular reflex tests as described by Ewing,[121,122] 24-h heart rate variability,[123,124] and cardiac pre-ejection period and respiratory sinus arrhythmia.[125-128] However, it is unknown which test (or combination of tests) should be considered as the gold standard technique. It is very possible that there is no single gold standard technique, and that all these techniques may provide relevant information about different aspects of the autonomic nervous system. In Chapter 9, we have used the cardiac pre-ejection period and respiratory sinus arrhythmia as indicators of cardiac sympathetic and parasympathetic activity, respectively. The pre-ejection period is an index of cardiac contractility that indicates the effect of the beta-adrenergic inotropic drive on the left ventricle:[128-130] the

shorter the pre-ejection period, the stronger the sympathetic control of heart rate. It should be emphasized that the absolute cardiac pre-ejection period is not a direct measure of the sympathetic outflow at the postganglionic sympathetic nerve endings. Instead, it is a reflection of the effect of the sympathetic outflow on the heart. For example, the effect of the sympathetic nervous system on the heart is also influenced by the number of beta-receptors in the heart: down-regulation and up-regulation of the number of receptors will thus have an important influence on the effect of the sympathetic nervous system on the heart. With respect to the determination of blood pressure levels, this property is actually an advantage, because it is not the sympathetic outflow to the heart itself that is important in the determination of blood pressure, but the effect of the sympathetic nervous system on the heart.

1.3.6 Measurement of cholesterol metabolism

In Chapter 12, we have demonstrated that birth weight was not associated with plasma ratios of lathosterol (a precursor of cholesterol), and campesterol and β-sitosterol (plant sterols) to cholesterol either in the overall sample or in the intrapair analyses in dizygotic and monozygotic twin pairs. Plasma ratios of lathosterol (a precursor of cholesterol), and campesterol and β-sitosterol (plant sterols) to cholesterol are indicators of whole body cholesterol synthesis and absorption, respectively. [131-136] It should be emphasized that this technique allows only the indirect assessment of cholesterol metabolism. Therefore, we cannot exclude the possibility that low birth weight may be associated with direct measurements of cholesterol metabolism as assessed by isotope techniques.[137] However, such direct assessment of cholesterol metabolism is expensive, time-consuming and difficult in large-scale studies. We also cannot exclude the possibility that birth weight is associated with other indicators of cholesterol metabolism, such as squalene, methyl sterols and cholestanol.[137] Our results differ from the results from Mortaz et al., [138] who demonstrated, in preterm infants, that low birth weight was associated with an increase in cholesterol synthesis, as indicated by an increase in plasma lathosterol, and a compensatory decrease in cholesterol absorption, as indicated by a decrease in plasma campesterol.

2. Birth weight and microvascular function endothelium-dependent vasodilation

We have shown in prepubertal children that birth weight was positively and significantly associated with capillary recruitment, but not with microvascular endothelium-(in)dependent vasodilation (Chapter 2). Studies investigating the association between birth weight and endothelial function have shown conflicting results. A significant relationship between birth weight and endothelium-dependent vasodilation in the macrocirculation in children[139,140] and young adults[141] has been demonstrated. In addition, Martin et al. have reported a diminished skin microvascular endothelium-dependent vasodilation in low birth weight babies[142] and children.[143] However, in our previous study in adults, we observed a positive relationship between

birth weight and acetylcholine-induced skin vasodilation, but this was no longer significant after correction for age, sex and body mass index.[78] In addition, in our study in prepubertal children (Chapter 2) and in a study by Goh et al. in 3-month old babies,[144] no relationship between birth weight and microvascular endothelium-dependent vasodilation could be found.

As put forward by Virdis and Schiffrin,[145] the picture is further complicated when we consider that the endothelium might also play a role in capillary recruitment. In our department, we have demonstrated that, in essential hypertensive patients, impaired skin capillary recruitment is caused by both functional and structural rarefaction.[57] Although the issue remains controversial, it has been hypothesised that some endothelium-dependent relaxing factors might play a role in the functional component of capillary recruitment.[146] Virdis and Schiffrin suggest that more definitive evidence on the role of the endothelium as a functional component of capillary recruitment would clarify the discrepant results concerning cutaneous endothelial function in low birth weight individuals.[145] However, it should be emphasized that capillary recruitment is not exclusively determined by endothelial function. This may explain why the association between birth weight and characteristics of the capillary network (i.e. capillary recruitment) is more pronounced than the association between birth weight and microvascular endothelium-dependent vasodilation. The influence of endothelial-derived nitric oxide on capillary recruitment can be investigated by intra-arterial infusion of a nitric oxide synthase inhibitor, N(G)-monomethyl-L-arginine (L-NMMA); the influence of endothelium-mediated prostanoids can be examined by blocking these prostanoids with (intra-arterial) acetylsalicylic acid.

3. Microvascular function and cardiovascular risk factors

3.1 Microvascular function: cause or effect?

It is important to realise that the reported association of a diminished microvascular function with high TNF-α levels and elevated cardiovascular risk factors, because of the observational character of these studies, does not prove any cause-and-effect-relationship. As described in Chapter 1, an impaired microvascular function may be both the cause and consequence of elevated blood pressure, insulin resistance and dyslipidaemia. With respect to the association between TNF-α levels and microvascular function, experiments with the infusion of TNF-α and the infusion of TNF-α antagonists may be worthwhile. In addition, further studies of the relationships between cardiovascular risk factors and microvascular function in young individuals at risk for hypertension, insulin resistance or obesity may elucidate important aspects of the temporal relationships among these variables. Specifically, studying children offers the possibility to identify factors present in subjects at risk before overt high blood pressure, insulin resistance and obesity have emerged. Individuals at high risk can be identified by low birth weight or a family history of hypertension, insulin resistance or obesity.

For example, our results in Chapter 2 are consistent with the hypothesis that microvascular dysfunction is a primary disturbance in subjects with a low birth weight. However, observational studies cannot distinguish factors that cause hypertension and/or insulin resistance from factors which antedate these diseases but are not involved in their aetiology. Temporal relationships are also not sufficient evidence for causality and it is possible that the relationships can be explained by other variables. This is an important area for future research.

3.2 Alternative explanations for the relationship between microvascular function and cardiovascular risk factors

An important candidate that may explain the relationships between microvascular function and cardiovascular risk factors is obesity and/or body fat distribution. Insulin resistance and hypertension are both accompanied by changes in body fat distribution, and changes in body fat distribution may be linked to low birth weight.[147-150] In addition, the current (Chapter 5) and previous studies[51,54] in our department have shown that measures of obesity are related to microvascular function. Although body mass index and waist-to-hip ratio were taken into account in the statistical analyses, this does not fully exclude the possibility that obesity or fat distribution plays an important role in the relationship of various variables with microvascular function. In future studies, it may be of interest to use more precise methods to assess visceral and peripheral obesity, such as magnetic resonance imaging. In addition, to elucidate the influence of obesity on microvascular function, blood pressure and insulin resistance, studies of weight reduction in obese individuals may be helpful. Another possible candidate that may explain the associations among impaired microvascular function, low birth weight, elevated blood pressure, insulin resistance and dyslipidaemia is an increased tissue sensitivity to cortisol, amplified by an enhanced secretion of cortisol.[151] Glucocorticoids have been shown to be associated with obesity, blood pressure and insulin sensitivity.[151,152] In addition, glucocorticoids have been shown to influence vascular reactivity[152] and angiogenesis.[153] Human studies have demonstrated that low birth weight babies have higher plasma cortisol levels in adult life.[151,154,155] It has been hypothesised that fetal overexposure to endogenous glucocorticoids might underpin the link between early life events and the development of hypertension and insulin resistance in later life. It has not been investigated whether or not the association between birth weight and cortisol levels is independent of genetic factors. It would be of interest to investigate this issue in twin studies. In addition, the association of cortisol levels with measures of microvascular function, blood pressure and insulin resistance needs to be explored, preferably in different age groups. To further elucidate whether these associations are causal, the influence of cortisol administration or cortisol antagonists on measures of microvascular function, blood pressure and insulin resistance needs to be explored.

3.3 Chronic smoking and microvascular function

We have demonstrated that acute smoking was associated with impaired recruitment of capillaries and impaired microvascular endothelium-dependent vasodilatation, whereas endothelium-independent vasodilation was not influenced (Chapter 4). To investigate the effect of chronic smoking on microvascular function, smokers and (very carefully matched) non-smokers should be compared. We have not investigated a group of matched non-smokers. However, compared with non-smokers that have been investigated in our previous studies,[51,54,57,60,78] skin capillary recruitment, as well as endothelium-dependent and endothelium-independent vasodilation were impaired. Impaired skin endothelium-dependent and endothelium-independent vasodilation in smokers is in accordance with a recent study in chronic smokers,[156] but capillary recruitment was not investigated in this study. In addition, it is of interest to investigate the effect of smoking cessation on microvascular function.

4. Birth weight and cardiovascular risk factors

4.1 Is the association of birth weight with blood pressure relevant?

A review of 80 studies in more than 444,000 singletons demonstrated that, on average, a 1 kg higher birth weight is associated with a 2 mmHg lower blood pressure.[157] In clinical practice this may seem a small difference, but these are relevant differences between the mean values of populations.[158] For example, lowering mean systolic blood pressure in a population by 2 mmHg corresponds to a 8% reduction in the risk of stroke.[87] Huxley et al. have recently concluded that the size of the association between birth weight and blood pressure may be overestimated due to publication bias and be of little relevance,[43] but this conclusion was largely based on findings in very large population studies, in which birth weight data and/or blood pressure levels were self-reported, probably causing attenuation of the associations in these studies. Interestingly, in two of their three largest studies weighting the meta-analysis, adjusted odds ratios for hypertension in men of less than 5.5 lb at birth were nevertheless significant at 1.26 (95% confidence interval 1.11–1.44),[159] and for women in the two Nurses' Health Studies,[160] 1.39 (1.29–1.50) and 1.43 (1.31–1.56). The discrepancy between the weak association of birth weight with self-reported blood pressure and the strong association with self-reported hypertension may be due to the fact that self-reported diagnosis of hypertension in these studies was more reliable than self-reported actual blood pressure levels. In addition, it should be appreciated that, in epidemiological studies, associations of birth weight with determinants of blood pressure are likely to be stronger than with blood pressure itself due to compensatory mechanisms that tightly regulate blood pressure. For example, the associations between birth weight and measurements of vascular function are more pronounced than the associations between birth weight and blood pressure.[139,143,161,162] The same phenomenon is likely to play a role in our twin study, in which the association between birth weight and sympathetic activity

(standardized β: 0.24, P<0.0001) was much more pronounced than the association between birth weight and blood pressure (standardized β: 0.12, P=0.05).

4.2 Differences in birth weight in twins as a model for differences in birth weight in singletons

It could be argued that differences in birth weight in twins are a poor model for differences in birth weight in singletons. Intrauterine growth in twins is different from that in singletons.[46] However, the association between birth weight and blood pressure in the overall sample of twins (-1.9 mmHg per kg increase of birth weight) was remarkably similar to the well-established association in singletons (approximately -2 mmHg per kg increase of birth weight).[157] The same holds true for the size of the association of birth weight with serum lipids[163] and later height in the overall sample of twins, which were similar to the size of the associations of birth weight with serum lipids[30,30,30,147,164,164-170] and height[168,171-184] in singletons. In addition, differences in birth weight within twin pairs have been associated with differences in many variables that have been related to birth weight in singletons, such as blood pressure,[185,186] diabetes,[187,188] serum lipids,[163] fibrinogen,[189] myocardial infarction[190] and height.[191,192] Although intrauterine growth in twins may be different from that in singletons, the associations between birth weight and cardiovascular risk in twins suggest that birth weight in twins is relevant for the development of cardiovascular disease, and that differences in birth weight in twins can be used as a model for differences in birth weight in singletons.

On the basis of the results of psychological tests, it has been suggested that the reliability of differences between two measurements in one person depends not only on the reliability of the measurements, but also on the correlation between them. A higher correlation is associated with a lower reliability. If this reasoning applies also to differences in cardiovascular risk factors within twin pairs, this may potentially influence the interpretation of our results. Especially within monozygotic twin pairs, substantial correlations have been observed for the cardiovascular risk factors investigated. This could potentially affect the reliability of the intrapair differences in monozygotic twins. Although we cannot fully exclude the possibility that this results in an underestimation of the associations in monozygotic twins, several findings support the reliability of the differences in monozygotic twins. First, the reliability of the differences in birth weight, HDL cholesterol, insulin resistance and height in monozygotic twins is supported by our findings that differences in birth weight are associated with differences in HDL cholesterol, insulin resistance and height within monozygotic twins. We have also shown that intrapair differences in birth weight were positively associated with differences in total cholesterol LDL cholesterol and apolipoprotein B within monozygotic twins. Others have reported associations between intrapair differences in birth weight and differences in osteoporosis[193] and child problem behaviour in monozygotic twins,[194] and have confirmed the associations of differences

in birth weight with differences in insulin resistance[195] and height in monozygotic twins.[191] Second, the reliability of the differences in blood pressure and preejection period is supported by the fact that_the association between differences in blood pressure and differences in the preejection period within monozygotic twins was strong and significant, and, importantly, somewhat more pronounced than the association between differences within dizygotic twins. Third, associations among intrapair differences in many other variables, such as visceral fat,[196-198] glucose tolerance,[196] total body fat,[198,199] plasma leptin,[197] body mass index,[200] fasting insulin, [198,200] lumbar disc height,[201] low back pain,[201] and physical fitness[199] have been reported in monozygotic twins. Taken together, the differences within monozygotic twin pairs appear reliable, and are unlikely to importantly influence the interpretation of our results.

4.3 Comparison between dizygotic and monozygotic twins

It could be argued that the association between intrapair differences in dizygotic twins cannot be compared to intrapair differences in monozygotic twins to study the influence of genetic factors. Around two thirds of monozygotic twins are monochorionic (i.e. share a placenta), whereas all dizygotic twins are dichorionic (i.e. have separate placentas). Therefore, besides genetic factors, intrauterine factors may also differ between dizygotic and monozygotic twins and may be the cause of any observed differences in the intrapair association of birth weight, e.g. with blood pressure, sympathetic activity, total and LDL cholesterol and fibrinogen. However, we consider it unlikely that the differences in the intrapair associations in dizygotic versus monozygotic twins are due to intrauterine differences. First, the overall associations of birth weight with all investigated variables were similar in dizygotic and monozygotic twins. Second, although a study in 6 twin pairs with twin-twin transfusion syndrome suggested that this syndrome may influence pulse wave velocity in the conduit arteries, chorionicity did not influence blood pressure.[202] In addition, a large prospective twin study (418 twin pairs) demonstrated that chorionicity did not influence the intrapair association between birth weight and blood pressure.[203] Furthermore, it should be noted that intrapair differences in birth weight in monozygotic twins have been related to within-pair differences in HDL cholesterol,[163] insulin sensitivity,[195] diabetes[187,188] and height,[191,192] demonstrating that the twin study design in general is quite capable of showing that intrauterine factors can influence adult outcome.

4.4 Alternative models to investigate the origin of the association between impaired intrauterine growth and adult disease

Alternative methods to investigate the association between impaired intrauterine growth and adult disease include animal studies and studies of maternal undernutrition. Important evidence for the programming hypothesis has been derived from studies in animals. According to Barker in his book “Mothers, babies and disease in later life”, a

remarkable example of programming is the effect of temperature on the sex of reptiles.[158] If the eggs of the American alligator are incubated at 30°C, all the offspring are female. If incubated at 33°C, all the offspring are male. At temperatures between 30 and 33°C, there are varying proportions of females and males. Although we agree that this is a remarkable example of programming, it is highly doubtful whether the prenatal growth of alligators -which are super twins with up to 50 twin brothers and sisters- is comparable to the intrauterine growth of singleton human babies. The same criticism applies to the numerous studies in sheep and rats that have shown that maternal undernutrition during pregnancy is related to disease in her offspring.[204] Human studies of maternal undernutrition have shown much less clear effects, suggesting that criticism to the use of maternal undernutrition in animals as a model for intrauterine growth retardation in humans is justified. In the Leningrad Siege study, no association between intrauterine starvation and glucose intolerance, dyslipidaemia, hypertension or cardiovascular disease in adult life could be found.[205,206] In rural Gambia, moderate-to-severe fetal and childhood malnutrition caused no detectable change in the glucose/insulin axis, nor in blood pressure.[207] In contrast to these studies, in the Dutch Hunger Winter study, people who had been exposed to famine in late or mid gestation had reduced glucose tolerance,[208] but blood pressure levels were unaffected.[209] It should, in addition, be emphasised that these human studies of maternal nutrition may be influenced by selection bias.[210] For example, during the Dutch famine, the number of conceptions was about 50% lower than the pre-famine level and perinatal mortality as well as mortality in the first year after birth were highest in those who were born during the famine.[211] It should also be noted that it is not known to what extent the impaired intrauterine growth during periods of extreme famine is comparable to the impaired intrauterine growth as encountered in the modern Western society.

As an alternative model of intrauterine malnutrition, animal models with ligation of the uterine artery have been developed. The undernutrition in this model may be more relevant to the human situation than animal models of maternal undernutrition.[212] The combination of the results of twin studies with those of undernutrition in animals and humans may further elucidate the complex role for intrauterine nutrition in the development of cardiovascular disease.

4.5 Conclusions from twin studies and a comparison with other models

In our twin studies, low birth weight was associated with insulin resistance, lower HDL and shorter height within monozygotic twin pairs, suggesting that these associations are, at least in part, independent of genetic factors. In contrast, low birth weight was not associated with blood pressure, total and LDL cholesterol, fibrinogen and sympathetic activation within monozygotic twin pairs, suggesting that these associations are, at least in part, due to genetic factors. The association of birth weight with blood pressure, glucose metabolism and height has been investigated in other cohorts of monozygotic twin pairs. In general, these studies show similar results. As reviewed by Huxley et al.,[43]

all published studies investigating birth weight and blood pressure within twins have demonstrated that differences in birth weight within monozygotic twins were not significantly related to differences in systolic blood pressure.[185,186,203,213,214] In contrast, studies investigating birth weight and glucose metabolism within monozygotic twins all suggest that lower birth weight is associated with insulin resistance and diabetes.[187,188] Similarly, low birth weight within monozygotic twin pairs was associated with shorter height in our cohort, and also in other cohorts.[191,215] The association of birth weight with sympathetic activation, serum lipids and fibrinogen has not been reported in other twin studies. Taken together, our studies and other published twin studies suggest a differential influence of intrauterine and genetic factors on the association of birth weight with glucose metabolism, HDL cholesterol and height on the one hand and the association of birth weight with blood pressure, sympathetic activation, total and LDL cholesterol and fibrinogen on the other. Twin studies investigating the association between birth weight and cardiovascular risk factors thus seem to show consistent results. Although, as described above, the results from the Dutch famine birth cohort may be influenced by selection bias, analyses of glucose metabolism and blood pressure in this cohort were compatible with the results from twin studies. Intrauterine exposure to famine was related to changes in glucose metabolism,[208] but not to changes in blood pressure.[209] Interestingly, a similar pattern has also been observed in the rat model of intrauterine nutrition induced by uterine artery ligation, a model that may be more relevant to the human situation than animal models of maternal undernutrition.[212] Uterine artery ligation was related to a diminished glucose tolerance,[212,216] but not to an elevated blood pressure.[212] In conclusion, the results from twin studies, the Dutch famine study as well as ligation studies in rats provide evidence for an intrauterine non-genetic influence on the association between size at birth and glucose metabolism, whereas genetic factors may, at least in part, explain the link between size at birth and blood pressure.

4.6 Candidate genes linking birth weight with cardiovascular risk factors

The search for genes explaining the association between birth weight and cardiovascular disease has focussed on genes linked to insulin secretion or insulin sensitivity.[217] Insulin has a central role in fetal growth.[218] Any gene related to insulin secretion or resistance may therefore be a candidate to link fetal growth with diabetes and cardiovascular disease in later life. Diabetes caused by a rare mutation of the pancreatic glucose sensing gene, glucokinase, which is a regulator of insulin secretion, has been shown to be associated with a reduction in birth weight.[219] However, this mutation is very rare, and cannot explain the link between birth weight and diabetes in the general population. Another candidate is the IGF-I gene. In a Dutch cohort, it has been shown that absence of the wild-type (192 bp) allele of a polymorphism in the promoter region of the IGF-I gene resulted in low birth weight,[220] reduced height in adulthood, diminished insulin-secreting capacity, and a high risk of type 2 diabetes and myocardial infarction.[221]

However, this finding could not be reproduced in other studies.[222,223] The variable number of tandem repeats locus in the insulin promotor region of the insulin gene is another possible candidate. The III/III genotype has been related to insulin resistance and diabetes,[224,225] but paradoxically, this genotype was related to higher, not lower, birth weight.[225,226] In Danish Caucasian individuals, variability of 4 candidate genes for type 2 diabetes (the insulin receptor substrate-1, hepatocyte nuclear factor-1alpha (HNF-1alpha), HNF-4alpha, and HNF-6 genes) was not related to low birth weight.[227] Recently, it has been shown that several genes affecting insulin resistance (an Ala54Thr polymorphism of the intestinal fatty acid binding protein locus (FABP2); UCSNP-43, a common G to A polymorphism of Calpain 10 (CAPN10); and three polymorphisms in Protein Phosphatase 1 Regulatory Subunit 3 (PPP1R3): Asp905Tyr, Arg883Ser and a length polymorphism in an AT(AU)-rich element (ARE) in the 3' untranslated region of PPP1R3 termed ARE1 or ARE2) were not associated with birth weight in the Pima population.[228] Taken together, none of the investigated genotypes related to insulin secretion or insulin sensitivity is able to explain the association between size at birth and disease in later life. However, as described above, the results from twin studies, the Dutch famine study as well as ligation studies in rats provide evidence that intrauterine nutrition may explain the association between size at birth and glucose metabolism. Therefore, the disappointing results of the search for genes related to birth weight and diabetes are not surprising. Although the mechanisms may not be obvious, further studies should investigate whether genotypes related to hypertension are related to low birth weight, as the association between birth weight and blood pressure is, at least in part, dependent on genetic factors.

4.7 Limitations due to sample size

In the intrapair analyses in our twins, we have found evidence for a genetic influence on the association of birth weight with blood pressure, sympathetic activity, total cholesterol, LDL cholesterol and fibrinogen. However, it should be appreciated that evidence for a genetic influence does not exclude the possibility for a non-genetic intrauterine factor. For example, in Chapter 9, the intrapair analyses in monozygotic twins could not exclude a negative association between birth weight and blood pressure; the confidence interval ranged from -5.4 to +5.2 mmHg per kilogram birth weight. From the confidence intervals it can be seen that a negative association between birth weight and blood pressure of -5.4 mmHg/kg birth weight cannot be excluded. Therefore, the possibility that intrauterine factors also influence the relationship between birth weight and blood pressure cannot be ruled out. Similarly, the confidence intervals, among monozygotic twins, for the association of birth weight with LDL cholesterol, fibrinogen and sympathetic activity included negative values, and the influence of an intrauterine factor on these associations cannot be ruled out. To completely exclude the possibility of a substantial intrauterine influence, very large twin cohorts with thousands of monozygotic twin pairs are necessary. Of course, these

studies will be difficult to perform due to logistical problems. As an alternative, meta-analyses of the published twin studies may be of value. As mentioned above, Huxley et al.[43] have reviewed the published studies investigating birth weight and blood pressure within monozygotic twins. The combined estimate in more than 1400 twin pairs was -0.6 mm Hg per kg higher birth weight, with a confidence interval ranging from -2.2 to 1.0 mmHg/kg, demonstrating that, although the point estimate is close to zero, a non-genetic intrauterine effect of -2.2 mmHg per kilogram birth weight still cannot be excluded.

A similar reasoning applies to our findings of evidence for an intrauterine influence on the association of birth weight with insulin resistance, HDL cholesterol and height. Although the results within the monozygotic twins provide evidence for a non-genetic influence, the confidence intervals of the relationships are wide. For example, due to the small number of individuals in which information was available on fasting insulin and glucose levels, the association between birth weight and fasting insulin levels within monozygotic twins (-50.2 pmol/L per kg birth weight) had a confidence interval ranging from -100.2 to -0.2 (after adjustment for differences in body mass index). Although this study provides evidence for an intrauterine environmental influence, we cannot exclude that the size of this effect is quite small (i.e. -0.2 units per kilogram birth weight). In addition, we cannot exclude the influence of genetic factors on the association between birth weight and insulin resistance. Although the intrapair association between birth weight and insulin resistance was not significantly different between dizygotic and monozygotic twin pairs, the confidence interval of the difference ranged from -9.0 to 94.5. In other words, our study could not exclude the possibility that the association between birth weight and insulin resistance was 9 pmol/L per kg birth weight stronger in dizygotic than in monozygotic twin pairs, which would be indicative of genetic influences.

In conclusion, although our studies have demonstrated evidence for genetic influences on several of the associations between birth weight and cardiovascular risk factors, our findings cannot exclude intrauterine influences on these associations, and, vice versa, although our studies have demonstrated evidence for intrauterine influences on several associations between birth weight and other cardiovascular risk factors, our findings cannot exclude genetic influences on these associations. Larger studies and meta-analyses are necessary to investigate the relative contributions of genetic and intrauterine influences more precisely.

4.8 Role for genetic and environmental factors is only valid in a particular setting

Besides the limitations due to the sample size discussed above, there is another issue that should be discussed. We have provided evidence for genetic factors that may, at least in part, explain the link of birth weight with blood pressure, sympathetic activity, total and LDL cholesterol and fibrinogen. As with all studies demonstrating genetic influences, it should be realised that these genetic influence have been found in a

particular environment. In this specific environment, we did not find evidence that variation in environment played a role in the association of birth weight with blood pressure, sympathetic activity, total and LDL cholesterol and fibrinogen. These findings are relevant, because they contradict the hypothesis that intrauterine environmental variation in the normal range is important for blood pressure, sympathetic activity, total and LDL cholesterol, and fibrinogen.[158] However, our findings cannot exclude the possibility that variation in environment outside the range in our population may play a role in these associations. Along the same lines, it should be realised that the evidence for intrauterine environmental factors explaining the association of birth weight with insulin resistance, HDL cholesterol and height has been found in a particular pool of genetic variation. This does not exclude the possibility that genetic variation outside the range in our population may play a role in these associations. The following example of yellow shanks, a disease occurring in certain genetic strains of fowl fed on yellow corn, may be helpful to understand the reasoning above.[229] Both the right set of genes and the yellow corn are necessary to produce the yellow shanks. A farmer with several strains of fowl who feeds them all yellow corn would consider yellow shanks to be a genetic disease, since only one strain would get yellow shanks, despite all strains getting the same diet. A different farmer who owned only the strain liable to get yellow shanks but who fed some of the birds yellow corn and others white corn would consider yellow shanks to be an environmentally determined condition because it depends on diet. In reality, yellow shanks is determined by both genes and environment. This example demonstrates that the estimation of genetic and environmental factors is valid only in a particular genetic population living in a particular environment. In other words, if there is no variation in a genetic or environmental factor that determines a disease in a particular population, this factor will not be noticed in studies in this population. Understanding this concept is especially important for the interpretation of the finding of genetic influences on the association between birth weight and cardiovascular risk factors. Although these studies suggest that, in this particular setting, variation in environment is not a determinant of the association between birth weight and cardiovascular risk factors, they cannot rule out the possibility that manipulation of the environment outside the variation in this particular setting can have an important influence. This knowledge should be kept in mind in further research into possible therapeutic interventions.

5. Microvascular function in twins

The hypothesis that the association between birth weight on the one hand and blood pressure and insulin resistance on the other is explained by an impaired microvascular function may seem in conflict with the hypothesis that there is a differential influence of intrauterine and genetic factors on the association of birth weight with glucose metabolism on the one hand and blood pressure on the other. However, as described

above, it should be appreciated that our finding of a genetic influence on the association between birth weight and blood pressure does not exclude the possibility that there are also intrauterine factors that play a role. In addition, our finding of an intrauterine influence on the association between birth weight and insulin resistance does not exclude a genetic influence. Therefore, our findings of a differential influence of intrauterine and genetic factors on the association of birth weight with glucose metabolism and blood pressure may be consistent with the hypothesis that an impaired microvascular function may play a mechanistic role in the association between birth weight on the one hand and hypertension and insulin resistance on the other. Whether the association between birth weight and microvascular function is due to a genetic factor, an intrauterine factor or both is not known. It is worthwhile to investigate the association between these variables within a group of dizygotic and monozygotic twins.

6. Final conclusions

The findings in the present thesis may contribute to a better understanding of the relationships among birth weight, microvascular function and cardiovascular risk factors. The data clearly illustrate the complexity of the relationships among these variables. In addition, both genetic and environmental factors play a role. A better understanding of the role for microvascular function and birth weight in the development of cardiovascular disease may reveal new therapeutic targets. Therapeutic strategies may be developed specifically targeted at improving or preventing deterioration of microcirculatory function. In addition, strategies may be developed targeted at improving or preventing impaired intrauterine growth. However, the effects of interventions that comprise changes in environment within the normal range may be limited due to the demonstrated influence of genetic factors.

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Nederlandse samenvatting

Hart- en vaatziekten zijn nog steeds de belangrijkste doodsoorzaak in westerse landen. Daarnaast komen hart- en vaatziekten in andere delen van de wereld steeds vaker voor. Bekende risicofactoren voor hart- en vaatziekten zijn een verhoogde bloeddruk en een verhoogd LDL-cholesterol (het ”slechte” cholesterol). Ook diabetes (suikerziekte) en een verminderd effect van het hormoon insuline (insulineresistentie) verhogen het risico op hart- en vaatziekten. Een verhoogd HDL-cholesterol (het “goede” cholesterol) beschermt juist tegen het optreden van hart- en vaatziekten. Deze risicofactoren kunnen slechts een klein deel van het voorkomen van hart- en vaatziekten verklaren. Daarnaast is het niet goed bekend wat de oorsprong is van deze risicofactoren. Recent is gesuggereerd dat een verminderde functie van de microcirculatie en een laag geboortegewicht een belangrijke rol spelen in het ontstaan van hart- en vaatziekten.

De microcirculatie is de verzamelnaam voor de kleinste bloedvaatjes in het lichaam. Een verminderde functie van deze bloedvaatjes zorgt ervoor dat het hart tegen een hogere weerstand moet pompen, waardoor de bloeddruk omhoog kan gaan. Daarnaast zorgt een verminderde functie van de microcirculatie er mogelijk voor dat insuline niet goed wordt aangevoerd naar de cellen, waardoor het niet goed zijn werk kan doen en insulineresistentie optreedt. Dit kan uiteindelijk resulteren in diabetes (suikerziekte).

Grote epidemiologische onderzoeken hebben laten zien dat personen met een laag geboortegewicht meer kans hebben op hart- en vaatziekten. Een laag geboortegewicht hangt ook samen met risicofactoren voor hart- en vaatziekten, zoals hoge bloeddruk, een hoog LDL-cholesterol, een laag HDL-cholesterol, insulineresistentie en diabetes. Personen met een laag geboortegewicht zijn op latere leeftijd ook kleiner dan personen met een hoger geboortegewicht.

Deel 1

Een aantal onderzoeken in volwassenen heeft laten zien dat een laag geboortegewicht samenhangt met een verminderde functie van de microcirculatie. Maar in deze onderzoeken hing een laag geboortegewicht ook samen met een hogere bloeddruk en/of een verminderde gevoeligheid voor insuline. Daardoor is niet te zeggen of de verminderde functie van de microcirculatie oorzaak of gevolg is van de verhoogde bloeddruk en/of een verminderde gevoeligheid voor insuline. We ontdekten in een groep prepuberale kinderen dat de kinderen met een laag geboortegewicht wel een verminderde functie van de microcirculatie hadden maar nog geen verhoogde bloeddruk en ook nog geen verminderde gevoeligheid voor insuline. Dit suggereert dat de verminderde functie van de microcirculatie een primaire stoornis is in personen met een laag geboortegewicht. Deze verminderde functie van de microcirculatie zou een verklaring kunnen zijn voor het verband van een laag geboortegewicht met een verhoogde bloeddruk en verminderde gevoeligheid voor insuline.

Deel 2

Er is verondersteld dat insuline zelf ook vaatverwijdende effecten heeft. Als deze effecten verminderd zijn, kan daardoor de aanvoer van insuline naar de weefsels verminderd raken. We onderzochten het effect van insuline op de microcirculatie. Insulinetoediening via een infuus gaf duidelijke toename van de functie van de microcirculatie. Ook plaatselijke toediening van insuline gaf verbetering van de microcirculatie.

Een verminderde functie van de microcirculatie zou ook de verklaring kunnen zijn voor de verhoogde bloeddruk en verminderde gevoeligheid voor insuline bij sigarettenrokers. We vonden dat na het roken van een sigaret de functie van de microcirculatie verminderd was. Deze bevinding past dus goed bij de hypothese dat een verminderde microcirculatie de oorzaak is van de verhoogde bloeddruk en de verminderde gevoeligheid voor insuline bij sigarettenrokers.

De ontstekingsstof TNF-α hangt ook samen met een verminderde gevoeligheid voor insuline. Het is niet goed duidelijk wat het mechanisme hiervan is. Het zou kunnen dat TNF-α een verminderde functie van de microcirculatie geeft, waardoor de aanvoer van insuline naar de weefsels verminderd raakt. We vonden in een groep volwassenen dat TNF-α inderdaad samenhing met een verminderde functie van de microcirculatie. Dit ondersteunt de hypothese dat een verminderde functie van de microcirculatie van belang is in het verband tussen TNF-α en een verminderde insulinegevoeligheid. In prepuberale kinderen was dit verband echter niet aanwezig. Mogelijk ontstaat dit verband dus pas op latere leeftijd.

Verminderde doorbloeding van de hartspier kan uiteindelijk leiden tot een hartinfarct en hartfalen. Ook hier zou een verminderde functie van de microcirculatie een rol kunnen spelen. De microcirculatie in het hart is erg moeilijk te onderzoeken. De functie van de microcirculatie in de huid zou een afspiegeling kunnen zijn van de functie van de microcirculatie in andere organen. In ons onderzoek vonden we dat mensen met een verhoogde kans op hart- en vaatziekten een verminderde functie van de microcirculatie in de huid hadden. Dit suggereert dat de meting van de microcirculatie een goed model is voor het onderzoeken van het verband tussen risicofactoren voor hart- en vaatziekten en de functie van de microcirculatie.

Deel 3

Het verband tussen een laag geboortegewicht en hart- en vaatziekten is vaak aangetoond. Over het algemeen wordt aangenomen dat dit verband veroorzaakt wordt door een verminderde groei in de baarmoeder waardoor organen niet goed worden aangelegd en op latere leeftijd eerder ziektes ontstaan. Verbetering van de groei in de baarmoeder zal volgens deze hypothese leiden tot beter aangelegde organen en minder ziekten in het latere leven. Een alternatieve hypothese is echter dat het verband verklaard wordt door genetische factoren die samenhangen met zowel een laag geboortegewicht als hart- en vaatziekten. Anders gezegd, het genotype voor hart- en

vaatziekten heeft ook effect op het geboortegewicht. Als deze alternatieve hypothese juist is, zal verbetering van de groei in de baarmoeder niet leiden tot minder hart- en vaatziekten.

Om onderscheid te maken tussen omgevingsfactoren en genetische factoren analyseerden we een groep twee-eiige en eeneiige tweelingparen. We onderzochten of de helft van de tweeling met het laagste geboortegewicht ook degene was met het hoogste niveau van de risicofactor. De verschillen binnen twee-eiige tweelingparen kunnen, in het algemeen, verklaard worden door zowel omgevingsfactoren als genetische factoren. Omdat eeneiige tweelingen genetisch identiek zijn, kunnen verschillen tussen eeneiige tweelingen echter alleen verklaard worden door omgevingsverschillen. Het effect van genen is dus als het ware geëlimineerd door te kijken naar verschillen binnen eeneiige tweelingparen.

In de twee-eiige tweelingen was de helft van de tweeling met het laagste geboortegewicht gemiddeld ook degene met de hoogste bloeddruk. In eeneiige tweelingen was er echter geen verschil in bloeddruk tussen de helft van een tweeling met het hoogste geboortegewicht en degene met het laagste geboortegewicht. Na eliminatie van genetische factoren was het verband tussen geboortegewicht en bloeddruk dus verdwenen. Blijkbaar zijn de genetische factoren nodig voor het verband tussen geboortegewicht en bloeddruk.

Het onwillekeurige zenuwstelsel, met name het sympathische deel daarvan, zou belangrijk kunnen zijn in het ontstaan van hoge bloeddruk in personen met een laag geboortegewicht. We vonden dat het sympathische zenuwstelsel sterk samenhing met geboortegewicht. Dit verband was echter afwezig als gekeken werd naar verschillen binnen de eeneiige tweelingparen. Ook het verband tussen geboortegewicht en het sympathische zenuwstelsel is blijkbaar afhankelijk van genetische factoren.

Eenzelfde patroon werd gevonden voor het verband tussen geboortegewicht en LDL-cholesterol (het “slechte” cholesterol) en fibrinogeen (een eiwit belangrijk voor de stolling van het bloed). Ook bij deze verbanden spelen genetische factoren mee.

Een heel ander patroon werd gevonden voor het verband van geboortegewicht met insulinegevoeligheid, HDL-cholesterol (het “goede” cholesterol) en lichaamslengte. Deze verbanden werden ook gevonden in de analyses van verschillen binnen de eeneiige tweelingparen. Dus de helft van de tweeling met het laagste geboortegewicht had, gemiddeld, een verminderde insulinegevoeligheid, HDL-cholesterol en lichaamslengte vergeleken met degene met het hoogste geboortegewicht. Bij deze verbanden speelt de omgeving dus wel een aantoonbare rol. Verbetering van de groei in de baarmoeder zou derhalve inderdaad een positief effect kunnen hebben op deze factoren.

Het onderzoek in dit proefschrift draagt bij aan het begrip van de verbanden tussen geboortegewicht, de functie van de microcirculatie en risicofactoren voor hart- en vaatziekten. Deze verbanden zijn erg complex. Zowel omgevingsfactoren als genetische

factoren hebben effect. Omdat de functie van de microcirculatie een belangrijke rol lijkt te spelen in het ontstaan van risicofactoren voor hart- en vaatziekten, kunnen behandelstrategieën gericht op de verbetering van de microcirculatie nuttig zijn. Daarnaast kunnen er strategieën ontworpen worden voor het verbeteren van de groei in de baarmoeder. Hierbij is het belangrijk te weten dat de effecten hiervan op hart- en vaatziekten beperkt kunnen zijn door de aangetoonde invloeden van genetische factoren.

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