

**The Role of a CA Repeat  
Polymorphism in the  
Promoter Region  
of the Insulin like  
Growth Factor-I gene  
in Physiology and the  
Pathophysiology of  
Diabetes Mellitus**

**Ingrid Rietveld**



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**The Role of a CA Repeat Polymorphism in the Promoter Region of the Insulin like Growth Factor-I Gene in Physiology and the Pathophysiology of Diabetes Mellitus**

De rol van een CA repeat polymorfisme in de promoter regio van het Insuline-achtige Groei factor-I gen in de fysiologie en in de pathofysiologie van diabetes mellitus

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# 1

## General introduction





## 1 PHYSIOLOGY IGF-I

Insulin like growth factor-1 (IGF-I) is a ubiquitous » 7.5 kDa polypeptide, which influences cell proliferation, differentiation and survival in many tissues [1, 2]. IGF-I is synthesized by most organs and may act as an endocrine, paracrine and/or autocrine growth factor [3]. The most important function of IGF-I is mediating physiological growth [4]. Historically, it was suggested that the growth promoting effects of GH were mediated by a liver derived factor initially termed “sulphation factor” [5]. Simultaneously, a fraction of non-suppressible insulin-like activity from human serum was discovered, which they called “NSILA” [5]. These factors turned out to be identical and were subsequently called “somatomedin”. Finally, the protein was sequenced and hereafter termed insulin like growth factor I (IGF-I) [5].

Human postnatal growth and development is largely under the influence of the growth hormone (GH) – insulin like growth factor (IGF) axis [6]. GH is not essential for intra-uterine growth and development, as demonstrated by the existence of normal-sized infants with either congenital absence of the pituitary or deletions in the genes encoding GH or the GH receptor [3, 7]. Mice carrying null mutations of the IGF-I gene however, are born small in size and grow poorly in the postnatal stages of development [3]. Also in patients with intra uterine growth retardation and postnatal growth deficit, impairment of the IGF-I gene and IGF-I receptor gene has been found [7-10]. Postnatal growth is dependent on GH as GH deficiency or deletions in the GH receptor result in growth retardation [3]. The effects of GH on growth are mediated via intermediate factors of which IGF-I is the most important [3]. Tall children have higher IGF-I and IGFBP-3 levels compared to short children [11].

IGF-I levels increase with age and pubertal development and decline after puberty throughout adulthood [12, 13]. Serum IGF-I levels peak at puberty and occurs earlier in girls than in boys [12, 13]. The relation of age with IGF-I was the same for IGFBP-3 and GH [13, 14]. However, GH response to a combination of GHRH and GHRP-6 or GHRH ± arginine remains the same throughout adult life, suggesting that the pituitary GH reserve is preserved even in older adults [6]. Factors that contribute to the age-related decline of GH and IGF-I include sex steroids, malnutrition, higher body mass index, changes in sleep pattern and physical inactivity [6].

IGF-I has structural and functional homology with insulin, although action takes place by its own receptor activation [1, 2]. IGF-I can interact with the insulin receptor although with a lower affinity than that of insulin [15]. High concentrations of IGF-I are likely to cross-activate with the insulin receptor [16]. The role of IGF-I in connection with glucose metabolism was first postulated by Froesch, who described that NSILA has a hypoglycemic action and is more active on muscle than in adipose tissue when compared to insulin [16]. In a study with healthy volunteers, recombinant human IGF-I administration had a different effect on counter regulatory response compared with insulin [17]. IGF-I infusion reduced glucagon levels and attenuated GH release [17]. Under euglycemic conditions, recombinant IGF-I infusion resulted

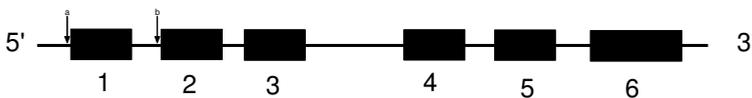
in a reduction of plasma insulin, C-peptide and glucagon levels comparable to insulin infusion [18]. Moreover, it lowered plasma glucose levels by stimulation of peripheral glucose uptake and inhibition of hepatic glucose production, even though the liver has almost no IGF-I receptors [18]. Possible mechanisms for this inhibitory effects include IGF-I mediated effects via the insulin receptor, via IGF-I/insulin hybrid receptors or via IGF-I mediated changes in substrate delivery for hepatic gluconeogenesis [18].

## 2 REGULATION OF IGF-I

Like stated before, IGF-I is one of the factors that are involved in the regulation of somatic growth and cellular proliferation [4, 19]. The liver is the main source of plasma IGF-I as proven by gene targeting studies [20]. Its action is determined by the availability of free IGF-I to interact with the IGF-I receptor (IGF-IR) [19]. The rate of IGF-I production, clearance and degree of binding to the IGF-BPs, modulate the levels of free IGF-I [2, 19].

Twin studies demonstrated that circulating IGF-I levels have a marked genetic component, which may account for large variation between persons [5]. Most circulating IGF-I is under GH control [4]. Its concentration is also influenced by nutritional status, sex steroids, insulin and glucocorticoids [1, 4]. Effects of IGF-I are regulated by the IGF-I receptor, but are also modulated by IGF-I binding proteins [4].

The most important regulators of plasma concentration of IGF-I are separately described below.



**Figure 1:** Schematic presentation of the IGF-I gene. The IGF-I gene contains six exons (boxes), separated by five introns (black lines). The gene contains two independent promoters, promoter 1 (a) and promoter 2 (b). The (CA)<sub>n</sub> polymorphism is located near promoter 1.

### *a IGF-I gene*

The human IGF-I gene, located on the long arm of chromosome 12, is a complex, multi-component gene with six exons, with the mature peptide being encoded by exons 3 and 4 [21]. Alternative leader exons are exons 1 and 2 [21, 22]. Several forms of IGF-I mRNA are transcribed, including the 6 kb form that is regulated by GH [22, 23]. Specific residues of the A chain are critical for recognition by four of the six IGF binding proteins. Specific amino acids in the B chain are required for binding to any of the six forms of IGF binding proteins. Tyrosines 24, 60 and to some extent 31 are critical for IGF-I receptor recognition. The IGF-I gene contains two independent promoters, which are regulated in a cell-type dependent way [22, 24].

Over the years, several polymorphisms in the IGF-I gene have been studied in relation to IGF-I serum levels. In children small for gestational age (SGA), polymorphisms of the IGF-I gene studied are: a cytosine-adenine repeat near the promoter region, a microsatellite marker (CA repeat) lying at the 3' part of the gene, an intronic cytosine-thymidine (CT) repeat lying between exon 2 and 3 [25, 26] and two single nucleotide polymorphism (SNP) markers [26]. In these children, only significant differences in IGF-I serum levels were related with the CT repeat polymorphism. The wild type allele (in this study an allele with a PCR product of 189-bp), was transmitted most often from parent to child [25, 26]. Children homozygous for the 189-bp allele had significantly lower levels of IGF-I [25].

Since Rosen found an association between a CA repeat variation near the promoter region of the IGF-I gene and IGF-I serum levels, more studies were performed to examine the functionality of this polymorphism. The length of this CA repeat sequence ranges from 10 to 24 CA repeats [27, 28]. In the Caucasian population, the most frequent allele contains 19 CA repeats [27-31]. In African-Americans the most frequent allele is shorter and contains 18 CA repeats [32-34]. The (CA)<sub>n</sub> polymorphism was associated with serum IGF-I levels in several study cohorts [27-29, 31, 35, 36], although in different directions. In other studies, no relation was found [30, 33, 37, 38]. Furthermore, this polymorphism was also related to body height, type 2 diabetes, myocardial infarction [28] and birth weight [39] in the Rotterdam study. Also a significant relation with BMD [40] and risk for fragility at old age in women and with bone structure in both genders was observed [41]. Other studies also found a relation with cardiovascular disease [42] or stroke [43].

### ***b IGF binding proteins***

In all extracellular tissues, most of the IGF-I is bound to a family of insulin-like growth factor binding proteins (IGFBPs), which prolong the half life in the circulation; the half life of unbound IGF-I is less than 10 minutes, but of bound IGF-I approximately 12 hours [1, 19]. There are six IGFBPs, which are produced in a variety of biological tissues and are found in various biologic fluids like plasma [44]. The majority of the IGF-I is bound in a trimeric complex, composed of IGFBP3 or IGFBP5 and a liver derived glycoprotein known as acid labile subunit (ALS) [1, 19]. Approximately 90% of IGFBP3 and 55% of IGFBP5 circulates in these complexes in healthy individuals [19, 45]. All three components of the trimeric complex are induced by GH and therefore are affected by GH excess or deficiency [19]. It is assumed that complex formation occurs in the liver as IGF-I and ALS are derived from hepatocytes, whereas IGFBP3 is produced in the Kupffer cells. The ternary complex with IGFBP5 is considered an IGF reservoir [4]. About 25% of circulating IGFs are bound in binary complexes, which can leave the circulation [4], whereas less than 1% of IGF-I circulates in the free form [19].

IGFBPs have several effects on IGF-I and different functional properties:

1. prolongation of IGFs half life in the circulation
2. prevention of IGF induced hypoglycemia

3. regulation of the passage of IGFs from the intravascular to the extra vascular space
4. limitation of the bioavailability of free IGFs to interact with the IGF receptors
5. enhancement of IGF actions by the formation of a pool of slow release IGFs
6. direct cellular actions mediated through their own receptors, acting independently of IGFs

IGFBP3 is the predominant circulating IGFBP [4]. IGFBP3 forms, as stated before, a ternary complex with IGF-I [19]. In the last few years, it has become apparent that especially IGFBP3 has IGF independent effects [45].

IGFBP1 has a RGD sequence in its structure, a recognition sequence for membrane integrin receptors, suggesting the possibility of an IGF independent action via these receptors [19]. IGFBP1 is metabolically regulated and its expression is inhibited by insulin [4]. IGFBP3 levels in the circulation do not change acutely while IGFBP1 and IGFBP2 levels depend on the metabolic state and the insulin level [19]. IGFBP2 is a major BP in cerebrospinal fluid [19]. IGFBP2 and IGFBP4 have mainly inhibitory effects on IGF mediated functions by competitively binding to IGF-I [19]. IGFBP4 is regulated by vitamin D and PTH [19]. IGFBP5 inhibits growth since it is expressed inversely with renal tissue growth status but stimulates actions of IGF-I particularly in bone [19]. In some circumstances some IGFBPs, especially IGFBP3 and IGFBP5, may potentiate IGF action [4]. IGFBP6 which is mainly found in cerebrospinal fluid and serum preferentially binds to IGF-II by over two orders of magnitude better than to IGF-I [19].

IGFBP proteases are proteolytic enzymes that can catalyze the limited hydrolysis of IGFBPs, causing release of free IGFs to interact with its receptor [19, 45]. There are three classes of IGFBP proteases: kallikreins, cathepsins and matrix metalloproteinases proteases [19].

### ***c IGF-I receptor***

IGF-I preferably binds to the IGF-I receptor, but under certain conditions also binds to the IGF-II receptor, the insulin receptor and hybrid receptors. Actions of IGF-I as a potent mitogen, anti-apoptotic factor and modulator of differentiation are mediated mainly through the IGF-IR [46]. IGF-I receptors are found on most tissues except, notably, liver and adipose tissue [15, 16]. The number of IGF-I receptors is regulated by GH and thyroxin and is tightly controlled within a range of 20,000 to 35,000 receptors per cell. The IGF-I receptor (IGF-IR) and insulin receptor are tyrosine kinase receptors and are composed of two extracellular alpha subunits and two intracellular beta subunits [4, 19, 46, 47]. The alpha subunits contains the IGF-binding domain with an affinity constant of  $10^{-9}$  M for IGF-I; the affinity constant is sixfold lower for IGF-II and 200- to 300-fold lower for insulin [15, 46]. The beta subunits have intrinsic tyrosine kinase activity [19, 46, 47]. Binding of IGF-I to the receptor results in phosphorylation of the insulin receptor substrates 1 and -2 (IRS-1 and -2) [4, 46]. Eventually the mitogen activated protein (MAP) kinase pathway is activated, which is important for stimulation of cell growth by IGF-I [4]. Activation of PI-3 kinase is important for stimulation of protein synthesis and glucose transport [4]. This pathway is also important for IGF-I stimula-

tion of cell motility and inhibition of apoptosis [2, 46]. The presence of IGF-I prevents cells in culture to undergo apoptosis [46].

Targeted disruption of the IGF-IR results in cell refractoriness to viral and cellular oncogenes, increasing the probability of apoptotic cell death and can inhibit neoplastic proliferation [46]. Deletions of IGF-IR result in decreased IGF-I binding and intrauterine growth retardation [2].

#### ***d Growth hormone***

The regulation of the GH/IGF-I system is dependent on the integrity of the hypothalamus, the pituitary and the liver. GH is released in pulses under the influence of growth hormone releasing hormone (GHRH) and somatostatin. GH release is inhibited by antibodies to GHRH, by GH itself and by IGF-I via a direct effect of IGF-I on the IGF-I receptor in the pituitary [6]. GH action is mediated by the binding of GH to the GH receptor, resulting in its dimerization and the auto-phosphorylation of the tyrosine kinase, Janus kinase 2 (JAK2). This kinase stimulates phosphorylation of signaling proteins, STAT proteins STAT1, STAT3 and STAT5. An intact JAK2-STAT5b signaling pathway is essential for GH stimulation of IGF-I gene expression [48]. The effects of GH on plasma IGF-I concentrations are complex [3]. Although it stimulates IGF-I gene transcription and secretion by the liver, part of the GH-mediated increase in circulating IGF-I levels is controlled by concurrent stimulation of IGFBP-3 and ALS [49]. Together, these three proteins form a stable ternary complex; any factor that attenuates the relative increase in any of the three components will produce a major reduction in serum IGF-I [2].

Postnatal growth is dependent on normal pulsatile secretion of GH from pituitary somatotrophs [50]. Most growth promoting effects of GH are believed to be mediated by IGF-I [3]. In states of GH deficiency, levels of IGF-I are also reduced [3, 51]. Similarly, excess GH results in acromegaly with concomitant increase in circulating IGF-I levels [3, 50, 52].

#### ***e Insulin***

IGF-I has 48% structural homology with pro-insulin; the A and B domains have 60-70% homology, but there is no homology with the C domain [16]. Delivery of insulin by the portal system influences hepatic IGF-I production and inversely regulates IGFBP-1 hepatic production [5]. Therefore, normal hepatic insulin action is required for normal rates of IGF-I synthesis [53]. During hyperinsulinaemic clamping, free IGF-I increased while total IGF-I was unaltered [5]. Furthermore, 60 hours of fasting decreased free IGF-I levels [54]. IGF-I can bind to the insulin receptor, but with an affinity of only 1-5% of that of insulin [44].

#### ***f Nutritional status***

Nutritional status is an important determinant of plasma IGF-I [5]. A minimum intake of 20 kcal/kg per day of energy and 0.6 g/kg of protein are necessary to maintain normal plasma values. IGF-I levels are low in patients with protein-calorie malnutrition, but can be normalized by adequate nutritional rehabilitation [55]. Serum GH concentrations are often high in

malnourished patients, suggesting that they are resistant to the action of GH, whereas basal insulin concentrations are normal or low [55]. A decrease in levels is also seen in diseases associated with malnutrition such as hepatic failure, inflammatory bowel disease and renal failure [55]. Overfeeding causes serum IGF-I to increase by 19% in a few days [55].

### **3 DIABETES MELLITUS**

#### *a Epidemiology and classification of Diabetes mellitus*

Diabetes mellitus is the result of less or insufficient insulin production or action. Type 1 diabetes is characterized by absolute insulin deficiency due to beta cell destruction caused by an auto immune process and acute onset, usually before 25 years of age [56, 57]. Type 2 diabetes is caused by disturbances in insulin action and beta cell dysfunction [56, 58] and increases with age [59]. In the Rotterdam Study, prevalence of diabetes increased from 5.9 percent at ages < 60 years to 19.8 percent at ages >85 years in males and from 3.8 at ages < 60 years to 18.9 percent at ages > 85 years in women. Impaired glucose tolerance increased from 8.8 and 11.0 percent to 24.3 in men and women aged < 60 years to 34.7 and 24.3 percent in men and women aged > 85 years [59].

The number of people with type 2 diabetes mellitus is increasing due to population growth, aging, urbanization and increasing prevalence of obesity [60]. Most patients with type 2 diabetes are obese and obesity itself causes some degree of insulin resistance [61, 62]. Intensive glucose control has delayed the development and progression of retinopathy, nephropathy and neuropathy in patients with type 1 [63, 64] and those with type 2 diabetes [65, 66], and decreased diabetes –related mortality or myocardial infarction in a group of newly diagnosed type 2 diabetes [66, 67]. Impaired glucose tolerance has been associated with cardiovascular disease risk factors and events, whereas impaired fasting glucose was much less strongly associated with CVD events and mortality [68].

In 1997 recommendations for the classification and diagnosis of diabetes were released by a collaboration between members of the American Diabetes Association (ADA) and the World Health Organization (WHO) [56]. Diabetes is defined as a random glucose  $\geq 11.1$  mmol/l or fasting plasma glucose (FPG)  $\geq 7.0$  mmol/L or 2-hour post-load glucose  $\geq 11.1$  mmol/L after a 75-g glucose tolerance test [56]. FPG is the preferred diagnostic test because it predicts adverse outcomes and is more reproducible. When FPG is above 5.6 mmol/L, risk of retinopathy greatly increases [69]. The ability of baseline levels of FPG to predict diabetes have been recently analyzed by the Expert committee in four populations and it was concluded that lowering the IFG cut point to 5.6 mmol/L optimizes its sensitivity and specificity [68]. The lower limit for IFG was then set to 5.6 mmol/L [61] and it still is [62]. Impaired glucose homeostasis is defined as a FPG from 5.6 to <7.0 mmol/L (impaired fasting glucose, IFG) and/or 2 hour post-load glucose from 7.8 to <11.1 mmol/L (impaired glucose tolerance, IGT) [62]. IFG

and IGT are referred as having pre-diabetes and are associated with the metabolic syndrome [62].

### ***b Pathophysiology diabetes mellitus***

Peripheral insulin resistance is often considered the first step in the development of type 2 diabetes [58, 70]. Insulin resistance is found in the majority of patients with type 2 diabetes and it is present in the early prediabetic stage of impaired glucose tolerance [70]. People with impaired glucose tolerance have approximately 50% of normal beta cell function; people with diabetes have less than 15% of normal beta cell function [71].

Insulin resistance results in insufficient insulin action and as a consequence hyperinsulinemia, eventually leading to glucose intolerance and hyperglycemia because of defective adaptation of beta cells [56, 58]. Hyperinsulinemia is thought to contribute to the development of atherosclerosis and hypertension [70]. Deficient action of insulin also results in abnormalities in carbohydrate, fat and protein metabolism [72]. Toxicities from chronic hyperglycemia and elevated free fatty acids (FFA) may directly affect beta cells and aggravate insulin resistance, further worsening hyperglycemia. Glucotoxicity begins to impair beta cell function when fasting blood glucose exceeds 8.9 mmol/l [73]. Even in patients with normal beta cell function, hyperinsulinemia may fail to compensate for insulin resistance if FFA levels are high. Reducing FFA supply through weight loss is then recommended [73].

### ***c Role of IGF-1 in the development of diabetes***

IGF-1 levels are decreased in children with newly diagnosed insulin-dependent diabetes [5]. In both adult type 1 and type 2 diabetes, significantly lower IGF-1 levels are observed compared to controls [74-76]. Also IGFBP3 levels are significantly lower as IGFBP1 levels are significantly higher [77]. Currently it is believed that the low IGF-1 concentrations are caused by a combination of decreased hepatic release of IGF-1 and hepatic hypersecretion of IGFBP-1, an inhibitor of IGF action, due to low portal insulin concentrations [78]. Reduced IGF-1 levels lead to elevated pulsatile secretion of GH, secondary increased lipolysis and elevated free fatty acid levels, which results in insulin resistance [50].

In recent years, it has become clear that GH and IGF-1 not only control somatic growth, but are also significantly involved in carbohydrate, lipid and protein metabolism [50]. In GH receptor knock out mice, IGF-1 restored islet cell mass and improved insulin secretion and glucose tolerance [50]. In humans, GH deficiency is associated with hypoglycemia in infants [50]. GH excess in adults causes insulin resistance and hyperglycemia because of GH's anti-insulin actions [79]. Reduction of the elevated GH levels by pegvisomant (a GH-receptor antagonist) results in a marked improvement in carbohydrate metabolism and the diabetic state [80]. IGF-1 has different effects on carbohydrate metabolism [50]. In healthy volunteers, rhIGF-1 improved whole body insulin sensitivity as demonstrated with a hyperinsulinemic-euglycemic clamp [81]. In other studies, infusion of rhIGF-1 increased stimulated glucose uptake and

glucose disposal [82]. IGF-I causes hypoglycemia despite a significant reduction in circulating insulin levels, which suggests that IGF-I works through the IGF-IR in muscle and liver [50]. IGF-I is expressed by beta cells throughout life [83]. It stimulates growth, maturation and functions of the islet beta cells and plays a dominant role in increasing the population of islet beta cells in the developing and regenerating pancreas. IGF-I is only effective in regenerating the pancreatic beta cells in the physiologically relevant glucose concentration range between 6 and 18 mM [84, 85]. In addition, at glucose concentrations > 18 mM, beta cell proliferation by IGF-I was reduced [85]. Prolonged hyperglycemia contributes to reducing cell mass by inhibiting beta cell growth [85].

Studies reported a much higher IGF-IR expression on muscle, hypothesizing that under normal circumstances IGF-I might influence glucose homeostasis largely through its insulin like effects on muscle [86]. In the presence of insulin resistance, there is upregulation of insulin/IGF-I hybrid receptor expression in both muscle and fat tissue [86].

A significant positive correlation between insulin sensitivity and circulating IGF-I concentration among patients with varying degrees of glucose tolerance have been reported [87]. Individuals with IGF-I concentration above the mean in the Ely study (>152 ug/l) had a significantly lower risk of developing impaired glucose tolerance or type 2 diabetes [88]. Individuals in the highest tertile of baseline IGF-I had significantly lower follow-up 2h glucose concentrations than those in the lowest tertile [88].

In type 2 diabetes, first phase insulin secretion is lost, resulting in excessive rise of postprandial glucose and as a result to a hyperinsulinemic second-phase insulin response [73]. In animal models, ablation of the IGF-I receptor from pancreatic beta cells results in the absence of the first phase of glucose stimulated insulin secretion and a strong reduction in second phase insulin secretion [89]. Beta cell specific deletion of the IGF-IR leads to hyperinsulinemia and absent first phase insulin response after glucose stimulation and a blunted second-phase insulin secretion [90]. In these mice, there was a normal islet beta cell mass [90].

In type 1 diabetes, rhIGF-I reduced insulin requirements by 50% and a decrease in blood glucose levels, which suggested an increase in insulin sensitivity [91]. Treatment with recombinant IGF-I led to significant reductions in HbA1c [92]. Furthermore, patients who had increased IGF-I levels had the greatest reductions in HbA1c [92]. In type 2 diabetes, administration of rhIGF-I resulted in significant improvement in insulin sensitivity and a decrease in blood glucose levels [93]. Insulin treatment also normalized the reduced free IGF-I and total IGF-I, but it showed a slower pattern of normalization [5].

### *d IGF-I in relation to micro-angiopathic complications of diabetes mellitus*

#### *Retinopathy*

Pituitary ablation by hypophysectomy or yttrium-90 implantation prevented and improved diabetic retinopathy, suggesting that GH might play a central role in the pathogenesis of diabetic complications [94]. Likewise, GH deficient dwarfs with glucose intolerance rarely developed severe proliferative retinopathy [94]. Intravitreal IGF-I concentrations have been found to be higher in diabetes patients, where it promotes chemotaxis of retinal endothelial cells, an essential step in vascular neogenesis [95]. In retinopathy of prematurity, IGF-I is critical for normal retinal vascular growth in knockout mice [96] and infants [97].

The existing literature on the role of IGF-I in the development of diabetic retinopathy is conflicting [98]. Proliferative diabetic retinopathy has been associated with low, normal and high total IGF-I levels. Also free IGF-I levels were not exclusively associated with retinopathy. No significant differences between total IGF-I serum levels were found in diabetics with varying degrees of diabetic retinopathy, although patients without retinopathy had significantly higher levels compared to controls and those with retinopathy [99]. In a prospective study, however, total IGF-I levels were raised significantly in patients with active proliferative retinopathy, while IGF-I levels did not differ in patients with preproliferative and active proliferative retinopathy [100]. Another study observed a positive relation between IGF-I levels and diabetic retinopathy [74]. However, lower IGF-I levels were observed in the group with minimal retinopathy [74].

Free IGF-I levels were significantly lower in patients with diabetic retinopathy in both type 1 and type 2 diabetics [94]. In addition, free IGF-I levels were significantly negative associated with HbA1c levels [94]. Other studies found no relation between free IGF-I levels and diabetic retinopathy [75, 76].

#### *Nephropathy*

Micro albuminuria is an early marker of renal disease in type 1 and type 2 diabetes, but is also related with cardiovascular disease in the diabetic and general population. Macroalbuminuria is a marker for progressive diabetic kidney disease [101]. Insufficient diabetic control in diabetes is an important contributor to the overall risk of developing diabetic nephropathy [102]. Interaction between haemodynamic and metabolic factors leads to development of diabetic nephropathy. Hyperglycemia leads to increased oxidative stress, renal polyol formation and accumulation of advanced glycation end products (AGEs). Formation of AGEs results from the reaction between the reduction of sugars and amino residues on proteins, lipids and nucleic acids. Binding of AGEs to its receptor leads to activation of a number of pathways, implicated in the development of diabetic complications, especially diabetic renal disease, because of accumulation of glycosylation products on proteins such as vessel wall collagen and crystallins. Inhibition of AGEs reduced albuminuria in a type 1 diabetic rodent

model. Other factors involved in the development of diabetic nephropathy are protein kinase C, epithelial mesenchymal transition (leading to invasion into tubulointerstitium through disrupted basement membranes and contribute to increased matrix synthesis), transforming growth factor  $\beta$ 1 and connective tissue growth factor [103].

IGF-I has also been implicated in the development of diabetic nephropathy. Most of the IGF-I is released from the liver, but the kidneys also synthesize relatively large amounts of the peptide [104]. IGF-I is synthesized by the glomeruli and considerable concentrations are present in the collecting ducts [104]. In the nephron, IGF-I receptors are expressed at anatomic sites different from where IGF-I is synthesized [104]. IGF-I enhances renal plasma flow, glomerular filtration rate (GFR) and creatinine clearance [104]. In the kidney, IGF-I has been associated with renal hypertrophy and compensatory renal growth in a number of conditions, including diabetes mellitus [104]. In animal models, IGF-I stimulates proliferation and migration of mesangial cells by increasing IGF-I sensitivity during hyperglycemia. Also migration and survival of podocytes is altered by inhibiting apoptosis of the podocytes under hyperglycemic conditions. Progressive renal failure in diabetic nephropathy results from chronic interstitial fibrosis due to increased activity of connective tissue growth factor by IGF-I [105].

Chronic kidney disease (CKD) in children results in growth retardation due to amongst others resistance to hormones mediating growth [48, 106]. In children and adults with CKD, the half life of GH is prolonged, likely due to attenuated IGF-I feedback [48, 106]. In uremia a defect in post receptor GH activated JAK2 signal transducer and STAT transduction is described as one of the mechanisms of GH resistance [48]. Raised renal concentrations of IGF-I are thought to protect diabetic kidney cells from ischemic injury and to accelerate tissue repair and recovery of renal function [1, 107]. In a study by Zandbergen et al, free IGF-I levels increased insignificantly by 6% after ten weeks of treatment with the angiotensin receptor antagonist losartan [108]. There was a significant reduction of the urinary albumin excretion rate by 43% and a significant reduction of blood pressure [108]. Recombinant human IGF-I therapy improved GFR in patients with end-stage renal failure [92, 109, 110]. Unfortunately, this effect could not be replicated by investigators in a recent study [111].

## **4 SCOPE OF THIS THESIS**

The aim of this thesis was to investigate the influence of a variation in the promoter region of the IGF-I gene on physiologic endpoints like the age-related decline of IGF-I and body height. Second, we studied the association between this IGF-I polymorphism and the beta cell function and the development of micro- and macrovascular complications in patients with type 2 diabetes.

In chapter 2 we examined the influence of this IGF-I polymorphism on the age-related decline of IGF-I.

In chapter 3 we investigated the relation between the length of the IGF-I alleles and body height. Furthermore, we investigated whether the IGF-I gene influenced the secular trend in body height.

In chapter 4, the role of the IGF-I gene on the beta cell function, stratified by glucose level, was determined.

In chapter 5, we studied the association between the IGF-I polymorphism and microvascular complications of type 2 diabetes; retinopathy and development of micro-albuminuria in patients with normal as well as impaired glucose tolerance.

In chapter 6 the role of the IGF-I polymorphism and mortality in type 2 diabetes patients was investigated.

Finally, conclusions and the general discussion are presented in chapter 7.

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# 2

**A polymorphism in the IGF-I gene influences  
the age-related decline in circulating total  
IGF-I levels**



Recent studies have demonstrated an association between a 192-bp polymorphism of the insulin-like growth factor-I (IGF-I) gene and total IGF-I serum levels, birth weight, body height and the risk to develop diabetes and cardiovascular diseases later on in life. This IGF-I gene polymorphism in the promoter region of the IGF-I gene may directly influence the expression of IGF-I. In the present study we evaluated the role of this polymorphism in the age-related decline in serum IGF-I levels.

All subjects were participants of the Rotterdam Study, a population based cohort study of diseases in the elderly. We studied a total group of 346 subjects, which comprised two subgroups; a randomly selected population-based sample of 196 subjects, and a group of 150 subjects selected on IGF-I genotype. In the total group of 346 individuals the relationship between this 192-bp polymorphism and the age-related decline in circulating total IGF-I levels was studied.

Homozygous carriers of the 192-bp allele demonstrated significant decline in serum IGF-I with age ( $r = -0.29$ ,  $p = 0.002$ ). This decline is similar to that seen in the general population. An age-related decline in serum total IGF-I was not observed in heterozygotes ( $r = -0.06$ ,  $p = 0.48$ ) and non-carriers ( $r = -0.12$ ,  $p = 0.32$ ). Interestingly, the relationship between age and serum IGFBP-3 levels showed the same pattern.

We observed only in homozygous carriers of the 192-bp alleles of the IGF-I gene an age-related decline in circulating total IGF-I levels, but not in heterozygotes and non-carriers of the 192-bp allele. We hypothesize that this IGF-I gene polymorphism directly or indirectly influences GH-mediated regulation of IGF-I secretion.

## INTRODUCTION

Insulin-like growth factor-I (IGF-I) is a peptide, which stimulates skeletal growth, cell differentiation and metabolism and influences body composition. Its secretion is regulated amongst others by growth hormone (GH), the nutritional status, liver function and insulin, whereby circulating IGF-I is mainly synthesized by the liver [1]. Most of the IGF-I is bound to one of the six IGF binding proteins, of which IGFBP-3 is the most abundant. This binding protein forms a ternary complex with IGF-I and acid labile subunit (ALS), which inhibits the functional properties of IGF-I [1, 2]. Most of the effects of GH on linear growth are mediated by IGF-I [1]. Deficiency of GH leads to a change in body length and body composition. [1, 3, 4]. The degree of impaired growth and body size composition depends on age of onset of GH deficiency [5]. GH deficiency in children is characterized by lowered serum IGF-I levels, short stature and low body weight [6, 7]. In patients who become GH-deficient later in life, circulating IGF-I concentrations are often normal in relation with age- and sex-related normal values [5, 8]. This suggests that circulating IGF-I levels later in life become less and less GH-dependent. Furthermore, IGF-I levels are regulated by several variables other than growth hormone [9]. Elderly men and women secrete GH less frequently and at lower amplitude than young individuals [10]. GH secretion declines to approximately 20% of that in puberty and serum IGF-I and IGFBP-3 levels decline in parallel [11, 12].

Recently, a polymorphism in the promoter region of the IGF-I gene has been identified, which was associated with IGF-I serum levels, birth weight and body height [13, 14]. Non-carriers of the most frequent allele (length 192-bp) were demonstrated to have low total serum IGF-I levels and lower height [13] as well as lower birth weight [14]. In the present study we investigated the relationship between this polymorphism in the IGF-I gene and age-dependent decline of circulating IGF-I levels.

## SUBJECTS AND METHODS

### *Study population*

All subjects included for the present study were participants from the Rotterdam Study, a population-based cohort study of diseases in the elderly. The Rotterdam Study is a single-Center prospective follow-up study in which all residents aged 55 years and over of the Rotterdam suburb Ommoord were invited to take part. The study was approved by the Medical Ethics Committee of Erasmus Medical Center Rotterdam and written informed consent was obtained from all participants. The aim of the Rotterdam study is to investigate determinants of chronic and disabling cardiovascular, neurodegenerative, locomotor and ophthalmologic diseases. The design of the study has been described previously [15]. The

baseline examination of the Rotterdam Study was conducted between 1990 and 1993. A total of 7,983 participants (response rate 78 percent) were examined.

A group of 346 subjects aged between 55 and 75 years was studied for the present study, which comprised two different subsamples from the Rotterdam Study: a) The first study group consisted of 196 subjects which was randomly selected. b) The second study group of 150 healthy subjects had been selected based on their IGF-I genotype (50 homozygous carriers of the 192-bp allele, 50 heterozygotes and 50 non-carriers) as described earlier by Vaessen et al. [13].

Twenty-two participants who had diabetes, diagnosed on a history of medication, a fasting glucose level of 7.8 mmol/L or above and / or a random glucose of 11.1 mmol/L or above, were excluded since diabetes mellitus and its treatment may affect the GH-IGF-I axis. Also three subjects using estrogen replacement therapy medication were excluded, because this type of medication is known to influence IGF-I concentrations [16]. In three individuals, no IGF-I genotype could be determined. After exclusion we studied the remaining group of 318 subjects which comprised a population based sample of 168 subjects and a sample selected on genotype consisting of 150 subjects.

### ***Measurements***

Blood sampling and storage have been described elsewhere [17]. Serum was separated by centrifugation and quickly frozen in liquid nitrogen. Blood measurements were performed on fasting blood samples, unless otherwise specified. Total IGF-I was determined by a commercially available radioimmunoassay (Medgenix Diagnostics, with intra-assay and inter-assay variation of 6.1% and 9.9%). Because of financial restrictions measurements of IGFBP-1, IGFBP-3, glucose and insulin were only done in the first study group consisting of 168 subjects. Commercially available immunoradiometric assays were used for the measurement of IGFBP-1 and IGFBP-3 (Diagnostic System Laboratories Inc, intra-assay and inter-assay C.V. for IGFBP-1: 4.0% and 6.0%, respectively; and for IGFBP-3: 1.8% and 1.9% respectively). Serum glucose levels were determined using a standard glucose hexokinase method. Insulin was measured by a commercially available assay (IRMA, Medgenix Diagnostics, intra-assay and inter-assay variation of 3-6% and 5-12%).

Genotypes of the 192-bp IGF-I promoter polymorphism were determined as described earlier [13]. This resulted in three possible genotypes: carriers homozygous for the 192-bp allele, carriers heterozygous for the 192-bp allele and non-carriers of the 192-bp allele. At the baseline examination height and weight were measured wearing indoor clothes and without shoes. Body mass index was defined as weight (kilograms) divided by the square of height (meters).

### ***Statistical analysis***

Mean age, serum total IGF-I, BMI and body height between the study groups were compared using an independent samples t-test. Distribution of sex between the two study groups was

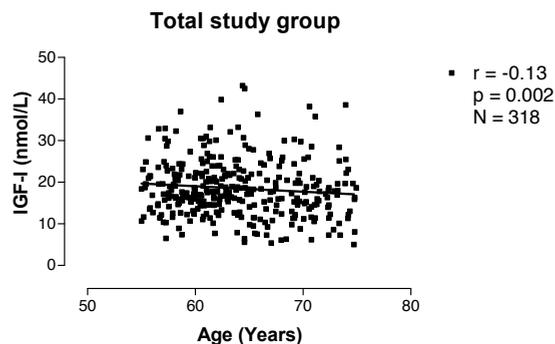
compared using a chi-square test. Body height and serum IGF-I were compared between genotypes using analyses of variance. Serum IGF-I values were logarithmically transformed for the analyses to achieve normal distribution and adjusted for the possible confounders age, sex and BMI. Because of ease of interpretation non-transformed IGF-I data are presented. Data given on IGF-I levels and height are presented as mean  $\pm$  SEM.

A linear regression was used to study the correlation between serum IGF-I and age and partial correlation coefficients are given. In all these analyses we adjusted for the same possible confounders, except age. All analyses were performed using the SPSS for Windows software package, version 10.0.5 (SPSS Inc., USA).

## RESULTS

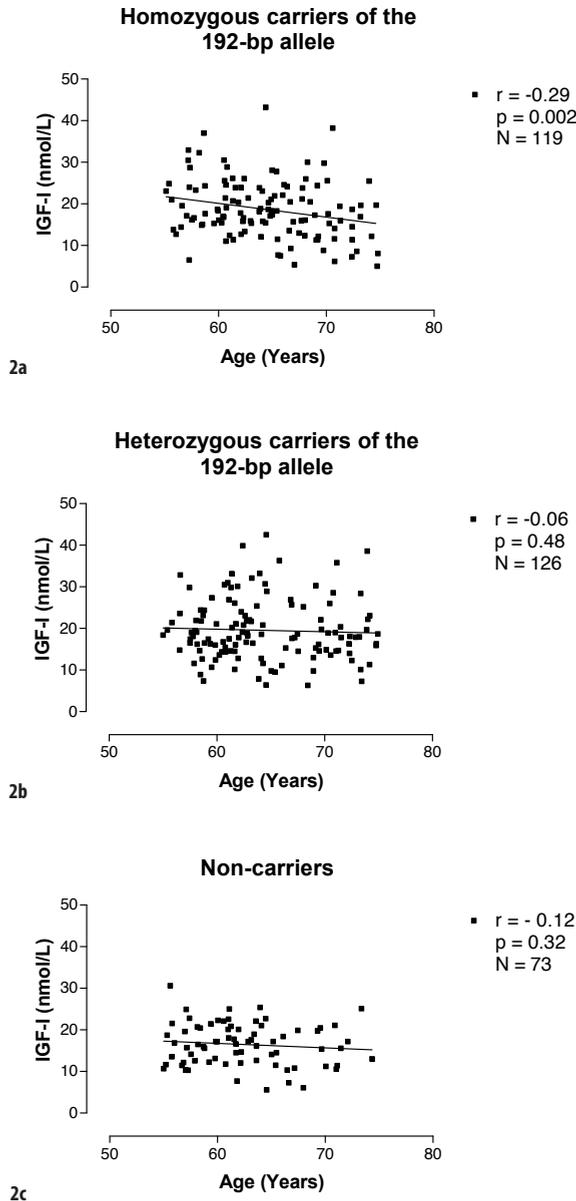
We studied the differences in the two study groups with regard to mean age, serum total IGF-I, BMI, body height and the distribution of sex. The subgroup of 150 individuals were significantly younger (60.7 years  $\pm$  0.3 vs. 67.0 years  $\pm$  0.4;  $p \leq 0.001$ ) and comprised less men (36.8% vs. 47.6%;  $p = 0.03$ ) than the subgroup of 168 subjects. There were no significant differences in mean serum total IGF-I, BMI and body height.

The total study group comprised 73 non-carriers of the 192-bp allele (23%), 126 heterozygous carriers of the 192-bp allele (39.6%) and 119 homozygous carriers of the 192-bp allele (37.4%). In the group as a whole, non-carriers had lower levels of IGF-I (16.5  $\pm$  0.6 nmol/L vs. 18.7  $\pm$  0.6 nmol/L;  $p = 0.01$ ) and shorter height compared to homozygous carriers (167.0  $\pm$  0.9 cm vs. 169.7  $\pm$  0.8 cm;  $p = 0.03$ ). With regard to fasting IGFBP-3, IGFBP-1, insulin and glucose no significant differences were observed between the three IGF-I genotypes. (data not shown).



**Figure 1.** Relation between serum total IGF-I and age in the whole study group. Regression line is presented and partial correlation coefficient is calculated from the data after adjustment for gender and BMI.

Figure 1 shows the age-related decline of IGF-I in the total study group of 318 subjects. A significant inverse relation between IGF-I and age was observed ( $N = 318$ ,  $R = -0.14$ ,  $p = 0.002$ ). This relation remains after adjustment for gender and BMI. Stratification of the relationship between serum IGF-I level and age according to genotype showed that there was only a



**Figure 2.** Relation between serum total IGF-I and age according to the IGF-I genotype in (a) homozygous carriers of the 192-bp allele, (b) heterozygous carriers of the 192-bp allele and (c) non-carriers of the 192-bp allele. Regression lines are presented and partial correlation coefficients are calculated from the data after adjustment for gender and BMI.

highly significant relation between IGF-I and age in homozygous carriers of the 192-bp allele. This was not significant in heterozygous carriers and in non-carriers. Figure 2 shows the relation between IGF-I levels and age in the three IGF-I genotype strata. Adjustment for gender and BMI did not change these relationships.

IGFBP-3 levels were only measured in a sample of 168 subjects. In this sample, IGFBP-3 levels decreased with age ( $r = -0.18$ ,  $p = 0.02$ ) and stratification per genotype showed again only a highly significant correlation in homozygous carriers ( $N = 72$ ;  $r = -0.35$ ;  $p = 0.002$ ), which was not observed in heterozygous carriers ( $N = 79$ ;  $r = -0.07$ ;  $p = 0.57$ ) and in non-carriers ( $N = 24$ ;  $r = 0.06$ ;  $p = 0.77$ ) (data not shown). These correlation coefficients remained after adjustment for gender and BMI. Fasting insulin, glucose and IGFBP-1 were not related to the presence of the 192-bp allele of the IGF-I gene.

## DISCUSSION

GH secretion, circulating IGF-I and IGFBP-3 levels in normal individuals demonstrate a gradual continuous decline during aging [12]. In the present study we observed that this well-known relationship with regard to IGF-I and IGFBP-3 was highly influenced by the presence of two 192-bp alleles in the IGF-I gene.

GH is secreted in a pulsatile way, while serum IGF-I and IGFBP-3 levels are constant over the day. The principal factor enhancing the production and secretion of IGF-I and IGFBP-3 is growth hormone [18] and IGF-I and IGFBP-3 levels correlate in children and adolescents well with the 24 hours secretion of GH [18]. In adults, a significant relation between 24-hours GH levels and IGF-I levels has been reported in normal and GH deficient (GHD) subjects [19]. However, in adults there is also an important influence of non-GH factors on the IGF-I levels [9]. In accordance with this observation, in previous studies of GH deficient adults, it was demonstrated that determination of serum IGF-I and IGFBP-3 levels become increasingly less discriminative as a diagnostic measurement for GHD as patients get older [5, 8]. This suggests that the actual level of circulating IGF-I and IGFBP-3 becomes less and less GH dependent with aging, ultimately leading to a situation in which circulating IGF-I concentrations becomes more and more influenced by nutrition, liver function, sex steroid levels and insulin [1].

In all clinical situations a close correlation between IGF-I and IGFBP-3 levels has been observed [20]. GH production and secretion decreases with increasing age.

In our study a genotype-specific pattern of age-related decrease in circulating IGF-I and IGFBP-3 levels was only observed in homozygous carriers of the 192-bp allele. For heterozygotes and non-carriers no relationship between circulating IGF-I and IGFBP-3 levels with age was observed. This suggests that only in the presence of two 192-bp alleles of the IGF-I gene the circulating IGF-I levels are influenced by GH secretion, but in the presence of only one or none of these alleles this relationship in elderly subjects is absent. In

heterozygotes and non-carriers, circulating IGF-I levels seems less GH-dependent and more influenced by other factors such as nutrition, liver function, sex steroids and insulin levels. To test our hypothesis, a study should be performed to investigate whether there is a difference in IGF-I response to recombinant human GH (rhGH) administration between homozygotes compared to heterozygote and non-carriers of the 192-bp allele. In this way, the effect of other non-GH factors on the IGF-I level can be minimized.

A shortcoming of our study is that we did not measure GH secretion and that we did not investigate a population-based sample. In fact, the sample was oversampled with a higher than expected number of non-carriers of the 192-bp allele. This was done in order to maximize the statistical power in the rare subgroup of non-carriers. Genotyping a population-based sample would represent homozygous carriers of the 192-bp allele as the most common allele. In conclusion, in healthy elderly individuals aged between 55 and 75 years, we observed that the age-related decline in circulating IGF-I and IGFBP-3 levels was only present in homozygous carriers of the 192-bp allele of the IGF-I gene. This suggests that this particular polymorphism, which is located 1 kb upstream of the promoter region of the IGF-I gene might, directly or indirectly, be responsible for the GH-driven regulation of IGF-I levels.

## **ACKNOWLEDGEMENTS**

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# 3

**A polymorphic CA repeat in the IGF-I gene is associated with gender-specific difference in body height, but has no effect on the secular trend in body height**



A polymorphism near the promoter region of the IGF-I gene has been associated with serum IGF-I levels, age related decline of serum IGF-I levels, body height, birth weight and intima media thickness in hypertensive subjects.

We investigated the association between the length of the IGF-I alleles of this promoter polymorphism and IGF-I levels and body height. Furthermore, we investigated the potential influence of this polymorphism on final height in relation to the secular trend of individuals born between 1917 and 1945. All subjects were participants of the Rotterdam Study.

We observed, in analyses including only homozygous carriers, the highest IGF-I levels in homozygous carriers of the 192-bp allele ( $18.7 \text{ nmol/L} \pm 0.6$ ) and homozygous carriers of the 194-bp allele ( $17.7 \text{ nmol/L} \pm 1.4$ ). IGF-I levels were significantly lower in individuals with homozygous longer alleles ( $>194\text{-bp}$  ( $12.0 \text{ nmol/L} \pm 1.2$ ;  $p < 0.001$ )) and homozygous shorter alleles ( $<192\text{-bp}$  ( $15.6 \text{ nmol/L} \pm 1.4$ ;  $p < 0.05$ )) compared to homozygous carriers of the 192-bp and the 194-bp allele. In males and females separately, an optimum for serum IGF-I was also observed in homozygous carriers of the 192-bp and 194-bp allele. Only in males, homozygous carriers of the 192-bp allele were significantly taller than homozygous carriers of the shorter alleles ( $174.9 \text{ cm} \pm 0.2$  vs.  $171.5 \text{ cm} \pm 1.4$ ;  $p = 0.01$ ). When all subjects genotyped for the IGF-I promoter polymorphism were included in the analysis, a clear optimum for IGF-I levels and body height was observed in carriers of the 192-bp and/or 194-bp allele in the total population.

Between 1917 and 1945, a secular trend in body height was observed in our Dutch population. Mean final body height was significantly higher in carriers of the most frequent alleles (192-bp and/or the 194-bp), than carriers of the remaining shorter and longer genotypes ( $p\text{-trend} < 0.01$ ).

In conclusion, we observed an optimum in IGF-I levels and final body height for the 192-bp and 194-bp allele of the IGF-I gene. A gender-specific effect of the IGF-I alleles on body height was observed. The secular trend in body height observed in our elderly Dutch population was similar for the different genotypes; carriers of the 192-bp and/or the 194-bp allele remained significantly taller throughout time.

## INTRODUCTION

A polymorphism in the Insulin-like Growth Factor-I (IGF-I) gene has been identified near the promoter region, comprising a variable length of a CA-repeat sequence. The number of CA-repeats ranges between 10 and 24 with the most common allele containing 19 CA-repeats in the Caucasian population [1-5]. This 19-CA repeat allele equals the 192-bp allele described in previous studies, where we observed an association between this polymorphism and circulating serum total IGF-I levels, body height and birth weight [2, 6], as well as with age related decline of serum IGF-I levels [7] and intima media thickness in hypertensive individuals [8]. However, other investigators have reported different associations between this polymorphism and circulating IGF-I levels: homozygous carriers of the 192-bp allele were reported to have higher [2], lower [3] or similar IGF-I levels [1] compared to non-carriers of this allele. An inverse correlation was observed between the number of CA repeats in the IGF-I gene and circulating IGF-I levels in two previous studies [4, 9].

Also for other genes an inverse relation between transcriptional activity and length of alleles has been suggested e.g. between the number of CAG repeats in the androgen receptor (AR) gene and functionality of the AR protein [10, 11]. Furthermore, severity of disease has been associated with the deletion of two octapeptide repeats of the prion protein gene in Creutzfeldt-Jakob disease [12] as well with increasing length of the CGG repeat in the fragile X syndrome [13].

IGF-I is not only a candidate for influencing body height, but determines also the response to famine in animals [14, 15]. Secular trends in body composition have been observed for body height, body weight and BMI and seem primarily related to lifestyle, environmental and socio-economic factors [16-18]. Genetic factors might also play a role, but so far no major genes have been found to be related to secular trends in body composition.

The aim of the present study was to investigate the relationship between the number of CA repeats in the IGF-I gene and total circulating IGF-I serum levels and body height. We also studied the influence of this  $(CA)_n$  polymorphism on the secular trend in body height in a sample of the Dutch population.

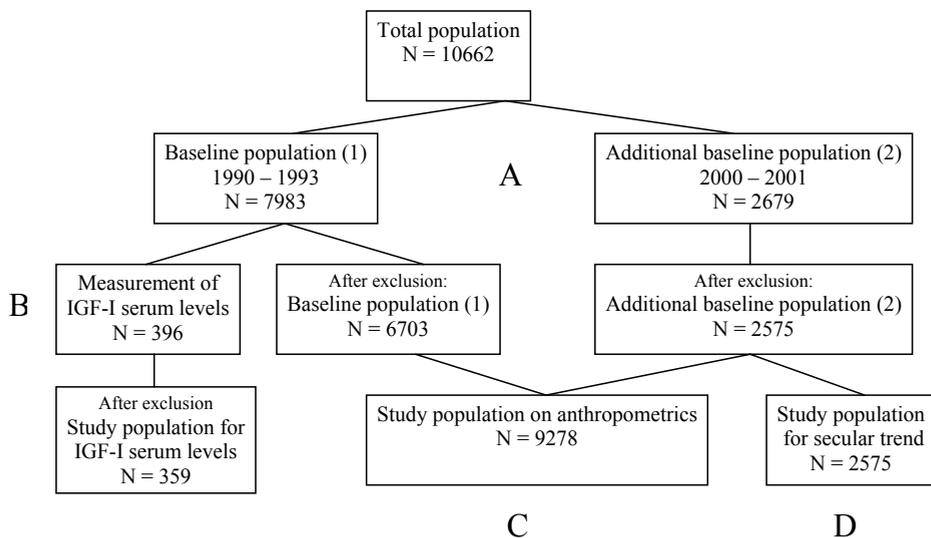
## SUBJECTS AND METHODS

### *Study population*

All subjects included in the present study were participants of the Rotterdam Study. The Rotterdam Study is a single-center prospective follow-up study in which all residents aged 55 years and over of the Rotterdam suburb Ommoord were invited to participate. The study was approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam and written informed consent was obtained from all participants. The aim of the Rotterdam study

is to investigate determinants of chronic and disabling cardiovascular, neurodegenerative, locomotor and ophthalmologic diseases. The design of the study has been described previously [19]. Baseline examination of the Rotterdam Study was conducted between 1990 and 1993. A total of 7983 participants (response rate 78 percent) were examined. Between 2000 and 2001, an additional baseline population within the Rotterdam Study of 2679 residents (response rate 67 percent), who were mainly between 55 and 65 years of age, participated (see flow-chart below at A). In total, the study population consisted of 10662 individuals, mean age  $69.1 \pm 9.7$ , 40.4% males, height  $167.0 \pm 9.5$  cm, weight  $74.1 \pm 12.6$  kg and BMI  $26.6 \pm 3.9$  kg/m<sup>2</sup>. Mean body height in males was  $174.8 \pm 6.9$  cm and in females  $161.4 \pm 6.7$  cm. Overall, the individuals in the second baseline survey were younger ( $64.7 \pm 7.8$  years;  $p < 0.001$ ). The flow-chart below represents which study populations were used to investigate our study objectives.

### Study populations of the Rotterdam Study



**Figure 1:** Study populations of the Rotterdam Study

#### **Measurement of IGF-I levels**

We measured circulating total IGF-I levels in blood samples of a subsample from the first baseline cohort consisting of 396 subjects aged between 55 and 75 years (see flowchart below at B). The samples were derived from the following study groups: a) 196 healthy subjects, selected at random [20]; b) 150 healthy subjects were selected on the basis of their IGF-I genotype (50 homozygous carriers of the 192-bp allele, 50 heterozygotes and 50 individuals with both alleles shorter or longer than 192-bp) [2]; c) finally, for the present study we selected an additional 50 subjects on the basis of the following IGF-I genotypes: homozygous

carriers with less than 192-bp and homozygous carriers with more than 194-bp. This group of 50 subjects included all individuals in the age range between 55 and 75 years with these particular genotypes found among the 7983 participants of the first baseline examination of the Rotterdam study. Measurements of IGF-BP's were not performed in all 396 participants and therefore were not used for the analyses.

Thirty-one participants who had diabetes, were excluded from the analyses since diabetes mellitus and its treatment may affect the GH-IGF-I axis [21]. Also three subjects using estrogen replacement therapy were excluded, because this type of medication is known to influence IGF-I concentrations [22]. In three individuals, no IGF-I genotype could be determined. After exclusion we studied the remaining group consisting of 359 individuals. Since subjects participating in the second survey were examined approximately 10 years later, we didn't measure circulating IGF-I levels in this cohort, because variations in the characteristics of the immuno-assays used for the determination of the IGF-I levels had to be excluded.

### ***Anthropometric studies***

The relation between the IGF-I genotypes and body height was studied in the pooled population of the first and second baseline examination together. Of the 10662 subjects, 9587 subjects of them were successfully genotyped for the promoter polymorphism in the IGF-I gene. Data on anthropometrics were missing for 309 subjects and were excluded, leaving 9278 subjects for the analyses (see flowchart on previous page at C). The influence of this polymorphism on the secular trend on final body height in relation to the secular trend was after exclusion only studied in the second baseline cohort of 2575 individuals (see flowchart on previous page at D). To study the influence on final body height, we divided the birth cohorts in quartiles. For this part of the study we selected individuals born between 1917 and 1945. The year 1917 was chosen, because the number of participants born before this year was very small (N=37).

### ***Determination of the IGF-I genotypes***

IGF-I genotypes were determined as described previously [2]. The most common allele contains 19 CA-repeats, which equals a length of 192-bp as described in previous studies [2, 6]. Four different divisions for the IGF-I genotypes were made to investigate the role of the different length of alleles of the IGF-I gene and functional parameters.

- a) Analyses were first performed on the genotypes based on the presence of the most frequent allele (192-bp) as described by Vaessen et al. [2], resulting in homozygous, heterozygous and non-carriers of the 192-bp allele.
- b) Secondly, to analyze the relation between the length of the alleles and IGF-I serum levels and body height, we selected only subjects homozygous for one of the most frequent alleles (192-bp or 194-bp) or who were homozygous for alleles shorter than 192 bp or longer than 194 bp. This resulted in homozygous carriers of the 192-bp allele, homozygous carriers of the

194-bp allele, homozygous carriers of alleles <192 bp (homozygous short) and homozygous carriers of alleles >194 bp (homozygous long). We analyzed the relation between the length of the IGF-I alleles and IGF-I serum levels in 189 homozygous carriers and for body height in 4486 homozygous carriers.

c) The relation between length of the alleles and IGF-I serum levels and body height was also analyzed including all subjects genotyped for the IGF-I polymorphism. The approach used was based on our findings from the homozygous (non-) carriers of the 192-bp and 194-bp allele (see method b) and comprised the following six groups of the IGF-I genotypes: 1) <192-bp/<192-bp; 2) <192-bp/192-bp and <192-bp/194-bp; 3) 192-bp/192-bp, 194-bp/194-bp and 192-bp/194-bp; 4) >194-bp/192-bp; 5) >194-bp/192-bp and >194-bp/194-bp; and 6) >194-bp/>194-bp

d) The last approach comprised only two IGF-I genotype groups: 1) homozygous 192-bp, homozygous 194-bp and heterozygous 192-bp/194-bp, 2) all other genotype groups (192/-, 194/-, -/- where (-) represents non-carriers of the 192-bp and 194-bp allele). This approach was used to analyze the relation between the IGF-I gene and the secular trend in body height.

**Table 1:** Allele distribution of the IGF-I promoter polymorphism in 9278 subjects

Length PCR product	(CA)n	Baseline 1 & 2		
		Total N = 9278	Females N = 5386	Males N = 3892
<b>198 bp</b>	<b>22</b>	1,5%	1,5%	1,5%
<b>196 bp</b>	<b>21</b>	6,9%	6,9%	6,9%
<b>194 bp</b>	<b>20</b>	19,3%	19,4%	19,2%
<b>192 bp</b>	<b>19</b>	65,5%	65,3%	65,8%
<b>190 bp</b>	<b>18</b>	4,5%	4,6%	4,3%
<b>188 bp</b>	<b>17</b>	1,8%	1,9%	1,8%
<b>Other rare alleles<sup>a</sup></b>	<b>-</b>	0,5%	0,4%	0,5%

All Rotterdam Study participants genotyped for the IGF-I promoter polymorphism  $\zeta$ 174-bp CA<sub>10</sub>, 176-bp CA<sub>11</sub>, 186-bp CA<sub>16</sub> and 200-bp CA<sub>23</sub>. N indicates number of people; percentages are based on allele frequencies of the pooled populations.

### Measurements

At baseline examinations height and weight were measured wearing indoor clothes and without shoes. Blood sampling and storage have been described elsewhere [23]. Serum was separated by centrifugation and quickly frozen in liquid nitrogen and kept at -80° C. Blood measurements were performed on non-fasting blood samples. Total IGF-I was determined by a commercially available radio immunoassay (Medgenix Diagnostics, with intra-assay and inter-assay variation of 6.1% and 9.9%).

### Statistical analysis

Age between genotypes was compared using analyses of variance and distribution of sex by a chi-square test. Body height and body weight were compared between genotypes by

general linear model and were adjusted for age, sex and cohort. Body weight was additionally adjusted for body height, since weight correlates with height. After stratification by gender, analyses were adjusted for age and cohort. Serum IGF-I values were compared by a general linear model after logarithmically transformation to achieve normal distribution and adjusted for age, sex and BMI. Because of ease of interpretation non-transformed IGF-I data are presented. In all analyses we checked the use of pooled variance. Adjusted values are presented and data given on all above-mentioned parameters are presented as mean  $\pm$  standard error of mean (SEM) or number with percentages. Analyses regarding body height in different birth cohorts were performed using a linear regression. Data are presented as mean  $\pm$  SEM. The secular trend in body height was analyzed by comparing mean body height between the IGF-I genotypes in the birth cohort quartiles using a general linear model. Interaction between year of birth and body height was also tested in the general linear model. All analyses were performed using the SPSS for Windows software package, version 10.0.5 (SPSS Inc., USA).

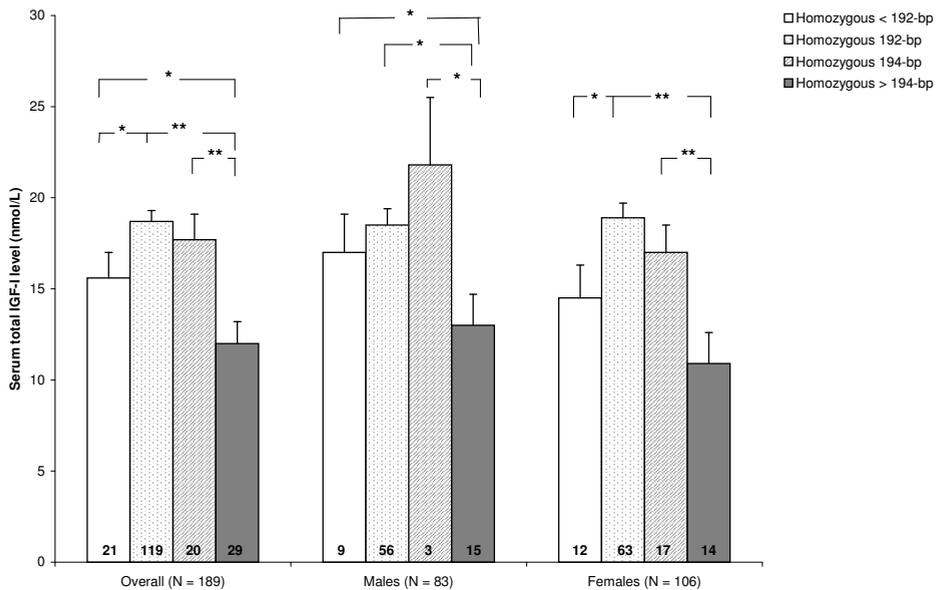
## RESULTS

Table 1 shows the allele frequencies of the (CA)<sub>n</sub> polymorphism in the promoter region of the IGF-I gene for all the finally included subjects (N = 9278). Allele frequencies were similar for the first and second baseline cohort.

First we analyzed in this population the relation between IGF-I genotypes, based on the division made by Vaessen et al., and body height [2]. As previously observed, homozygous carriers of the 192-bp allele were significantly taller (167.3 cm  $\pm$  0.1;  $p < 0.01$ ) compared to non-carriers of the 192-bp allele (166.6 cm  $\pm$  0.2). In the subsample from the first baseline cohort, in which total circulating IGF-I levels were measured (N = 359), homozygous carriers of the 192-bp allele also had significantly higher IGF-I levels (18.7 nmol/L  $\pm$  0.6;  $p < 0.001$ ) compared to non-carriers of the 192-bp allele.

Next, we selected only subjects homozygous for one of the most frequent alleles (192-bp or 194-bp) or who were homozygous for alleles shorter than 192-bp or longer than 194-bp and whose circulating IGF-I levels had been measured. In this group we analyzed the relation between the length of the alleles and serum IGF-I levels. Figure 2 shows mean serum total IGF-I levels based on the length of the allele in all 189 homozygous carriers, as well as in men and women separately. We observed the highest IGF-I levels for homozygous carriers of the 192-bp allele (N = 119, 18.7 nmol/L  $\pm$  0.6) and homozygous carriers of the 194-bp allele (N = 20, 17.7 nmol/L  $\pm$  1.4) in the total group. Both the short alleles (both alleles <192-bp) and the long alleles (both alleles >194-bp) were associated with significantly lower serum total IGF-I levels (N = 21, 15.6 nmol/L  $\pm$  1.4;  $p < 0.05$  respectively N = 29, 12.0 nmol/L  $\pm$  1.2;  $p < 0.001$ ), compared to the 192-bp allele and the 194-bp allele. Homozygous long alleles (>194-bp) had

significant lower IGF-I levels than homozygous short alleles (<192-bp) ( $p \leq 0.05$ ). In males and females separately, IGF-I serum levels were comparable between homozygous carriers of the 192-bp allele and the 194-bp allele ( $p = \text{NS}$ ). In females, the highest IGF-I levels were observed in homozygous carriers of the 192-bp allele ( $N = 56$ ) and in males in homozygous carriers of the 194-bp allele ( $N = 3$ ). In both males and females, the lowest IGF-I levels were observed in homozygous short and long alleles. In males, homozygous long alleles had significantly lower IGF-I levels than homozygous short alleles ( $p \leq 0.05$ ). After adjustment for age, BMI and sex (only in the total group) the differences remained statistically significant.



**Figure 2:** Mean IGF-I levels in nmol/L  $\pm$  SEM in 189 individuals (left; overall), 83 males (middle) and 106 females (right). This selection was based on homozygosity for the 192-bp, 194-bp, <192-bp and >194-bp alleles. Number of subjects per IGF-I genotype is indicated in the bars. \*  $p \leq 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

Subsequently, we studied the relation between the length of alleles and some anthropometric data in subjects homozygous for one of the most frequent alleles (192-bp or 194-bp) or who were homozygous for alleles shorter than 192-bp or longer than 194-bp. Of the total population of 9178 participants, 4486 subjects were homozygous carriers. Table 2 shows the results of the relation between the length of alleles and body height and body weight in all 4486 homozygous carriers of the first and second survey, as well as in men and women separately. Overall, homozygous carriers of the 192-bp allele were taller ( $167.3 \text{ cm} \pm 0.1$ ) than homozygous shorter alleles (<192-bp ( $165.7 \text{ cm} \pm 0.9$ )) and homozygous longer alleles (>194-bp ( $166.0 \text{ cm} \pm 0.7$ )), but the difference didn't reach statistical significance ( $p = 0.1$  and  $p = 0.1$ , respectively). Body height was significantly higher in homozygous carriers of the

192-bp alleles pooled with homozygous carriers of the 194-bp alleles than homozygous carriers <192-bp alleles pooled with homozygous >194-bp alleles ( $p=0.02$ ). In males, homozygous carriers of the 192-bp allele were significantly taller ( $174.9 \text{ cm} \pm 0.2$ ) than homozygous < 192-bp allele ( $171.5 \text{ cm} \pm 1.4$ ;  $p = 0.01$ ). In females, no significant differences in body height were observed between the genotypes. Analyses in all subjects showed a significant higher body weight for homozygous carriers of the 192-bp allele compared to homozygous >194-bp allele ( $p = 0.03$ ). This relation was not observed in males and females

**Table 2:** Relation length alleles IGF-I gene and functional parameters in pooled populations of homozygous carriers

		<i>Homozygous &lt;192-bp allele</i>	<i>Homozygous 192-bp allele</i>	<i>Homozygous 194-bp allele</i>	<i>Homozygous &gt;194-bp allele</i>	<i>p*</i>	<i>p**</i>	<i>p***</i>	<i>p****</i>
Overall	Number	53	4011	343	79				
(N=4486)	Height (cm)	$165.7 \pm 0.9$	$167.3 \pm 0.1$	$167.0 \pm 0.3$	$166.0 \pm 0.7$	0.1	0.1	0.8	0.02
	Weight (kg)	$71.8 \pm 1.5$	$74.3 \pm 0.2$	$74.0 \pm 0.6$	$70.9 \pm 1.3$	0.1	0.01	0.7	0.002
Males	Number	22	1692	144	37				
(N=1896)	Height (cm)	$171.5 \pm 1.4$	$174.9 \pm 0.2$	$175.2 \pm 0.5$	$173.9 \pm 1.1$	0.01	0.4	0.2	0.02
	Weight (kg)	$77.3 \pm 2.2$	$79.8 \pm 0.3$	$79.9 \pm 0.9$	$76.6 \pm 1.7$	0.3	0.07	0.8	0.04
Females	Number	31	2318	199	42				
(N=2590)	Height (cm)	$161.5 \pm 1.1$	$161.7 \pm 0.1$	$160.9 \pm 0.4$	$160.2 \pm 1.0$	0.9	0.1	0.3	0.2
	Weight (kg)	$67.8 \pm 2.1$	$70.3 \pm 0.2$	$69.8 \pm 0.8$	$66.7 \pm 1.8$	0.2	0.05	0.7	0.03

IGF-I divided in homozygous carriers of alleles shorter than 192-bp, homozygous carriers of 192-bp, homozygous carriers of 194-bp allele and homozygous carriers of alleles longer than 194-bp. Overall adjusted for age, sex and cohort; males and females adjusted for age and cohort.

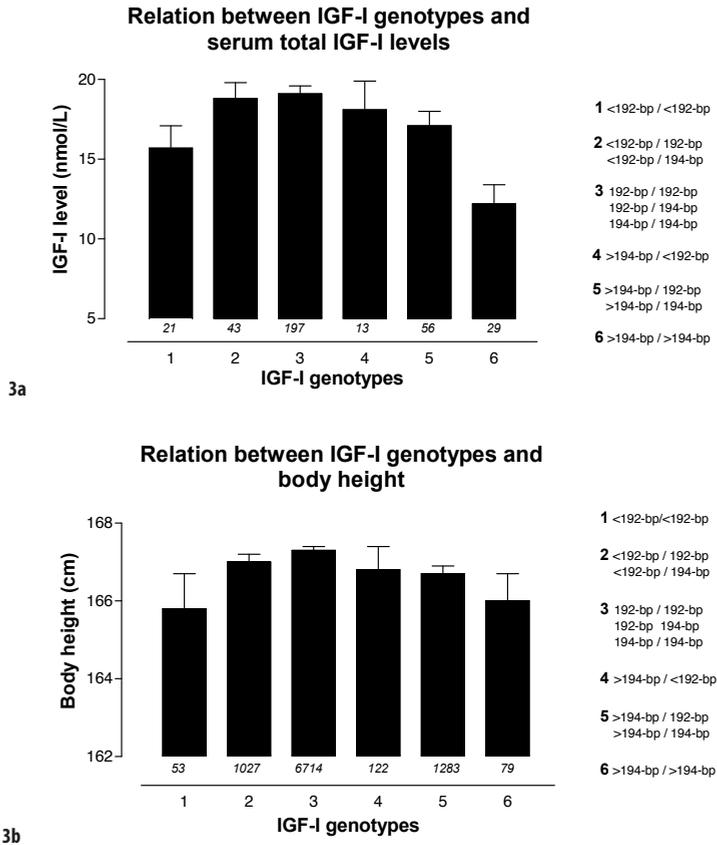
Body weight was additionally adjusted for body height. All data are presented as mean  $\pm$  SEM.

$p^*$  between homozygous 192-bp allele and homozygous < 192-bp allele;  $p^{**}$  between homozygous 192-bp allele and homozygous > 194-bp allele;  $p^{***}$  between homozygous <192-bp allele and homozygous > 194-bp allele,  $p^{****}$  between homozygous 192-bp and 194-bp versus homozygous <192-bp and >194-bp

separately.

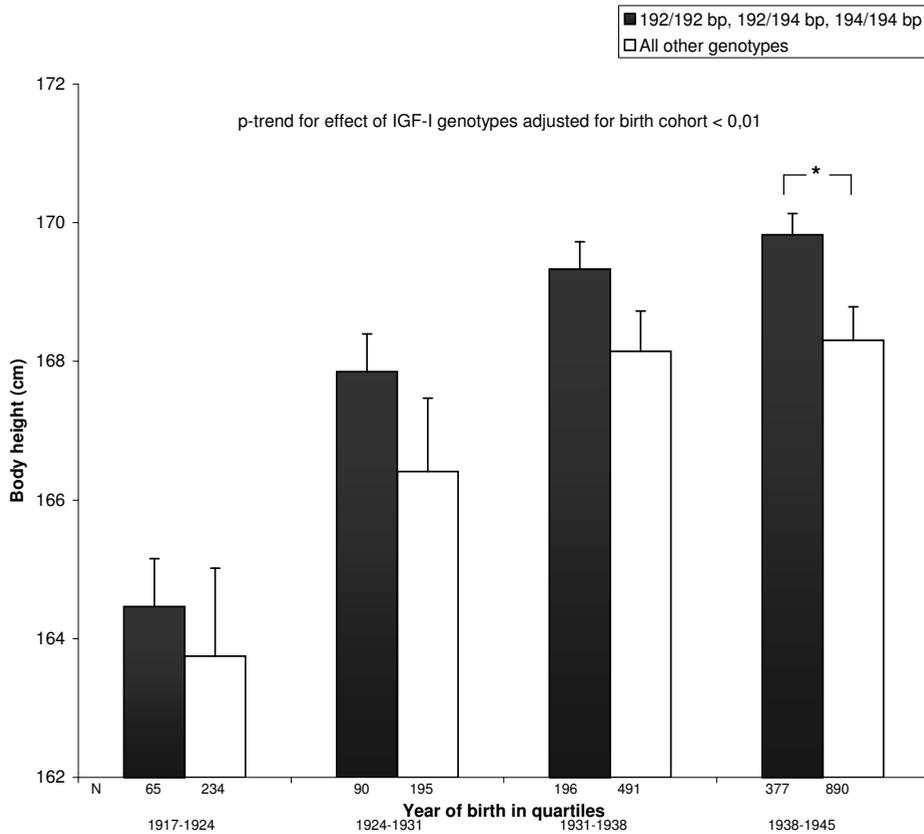
Figure 3A shows the relation between the IGF-I genotype and IGF-I serum levels in the sample of 359 subjects after adjustment for age, sex and BMI. Figure 3B shows the relation between the IGF-I genotypes and body height in all 9278 participants after adjustment for age, sex and cohort. All IGF-I genotypes are included and groups were made based on our previous findings. An optimum for the group consisting of the 192-bp/192-bp, 194-bp/194-bp and 192-bp/194-bp genotypes is observed for both serum total IGF-I levels and body height. IGF-I serum levels and body height increased with increasing length of the IGF-I alleles shorter than 192-bp and decreased with increasing length of the IGF-I alleles longer than 194-bp. The same optimum for the 192-bp allele and the 194-bp allele in IGF-I serum level was also observed in males and females separately. For body height, this optimum was only observed in males (data not shown).

Between 1917 and 1945, a significant increase in body height with increasing year of birth is observed ( $b = 0.23 \pm 0.03 \text{ cm/year}$ ;  $p < 0.001$ ). Mean body height increased approximately 5 cm over this period. After stratification per IGF-I genotype, we observed a significantly higher



**Figure 3:** (a) Relation between IGF-I genotypes and IGF-I levels in 359 subjects after adjustment for age, sex and BMI. (b) Relationship between IGF-I genotypes and body height in 9278 persons after adjustment for age, sex and cohort. All IGF-I genotypes are included and 6 genotype groups are made: 1) <math><192\text{-bp}/<192\text{-bp}</math>; 2) <math><192\text{-bp}/192\text{-bp}</math> and <math><192\text{-bp}/194\text{-bp}</math>; 3) <math>192\text{-bp}/192\text{-bp}</math>, <math>192\text{-bp}/194\text{-bp}</math> and <math>194\text{-bp}/194\text{-bp}</math>; 4) <math>>194\text{-bp}/<192\text{-bp}</math>; 5) <math>>194\text{-bp}/192\text{-bp}</math> and <math>>194\text{-bp}/194\text{-bp}</math>; 6) <math>>194\text{-bp}/>194\text{-bp}</math>. Number of subjects is indicated in italic below the bars.

body height in the group consisting of 192-bp/192-bp, 194-bp/194-bp and 192-bp/194-bp compared to the group consisting of the remaining genotypes as shown in figure 4 ( $p$ -trend < 0.01). In all groups the difference in body height was approximately 1.5 cm between the two genotype groups, but reached only statistical significance in the period between 1938 and 1945. Interaction between year of birth and body height was not significant ( $p = 0.9$ ). When the data were examined by gender, the trend was seen in both males and females.



**Figure 4:** Relation between mean body height and year of birth after stratification by IGF-I genotype in second baseline cohort comprising 2538 individuals after excluding 37 individuals, who were born before 1917. IGF-I genotypes made in two groups, one group consisted of homozygous 192-bp, homozygous 194-bp and 192-bp/194-bp alleles. Second group consisted of the remaining genotypes (192-bp/-, 194-bp/-, -/-). Data are presented as mean  $\pm$  SEM.

## DISCUSSION

In transfected cells, promoter elements in the 5' untranslated region of the IGF-I gene may influence basal gene transcription leading to a higher level of transcriptional activity, while truncation, deletions and mutations in the gene result in significantly lower transcriptional activity [24]. No studies so far have been performed on the transcriptional activity of alleles longer than the wildtype allele. Previous studies on the association of the different length of the alleles of the IGF-I gene promoter showed comparable frequencies in alleles, but suggested an inverse relation between the CA repeat and IGF-I levels [4, 9]. In this study we demonstrate that there seems to be an optimum in circulating IGF-I levels for the 192-bp and

the 194-bp allele, while both alleles shorter than 192-bp and longer than 194-bp seem to have lower circulating IGF-I levels.

A gender-specific association of the IGF-I alleles was found for body height. In males with homozygous short alleles, height was significantly less than in homozygous carriers of the 192-bp allele and the 194-bp allele. In women no such relation was found. In girls pubertal growth spurt stops earlier after the appearance of estradiol, whereas in boys IGF-I regulates for a longer period the effect of GH on body height. This may lead to genetically determined differences in body height between the sexes.

In males, a relation between IGF-I serum levels and body height for the different IGF-I genotypes was observed. This relation between IGF-I serum levels and body height was not observed in females. This suggests that IGF-I has less growth promoting effects in females than in males. Previously, it has been suggested that there is a prepubertal gender difference in GH sensitivity [25]. Our study suggests that the effect of IGF-I on linear growth is also gender-specific and opens the possibility that there is also (prepubertal) gender difference in IGF-I sensitivity. Unfortunately, in our cohort, we do not have information on prepubertal growth of these subjects to determine if the sex-specific effect of the alleles is indeed related to pubertal IGF-I gene expression. The number of males with the homozygous 194-bp genotype appears under-represented in the cohort selected to measure IGF-I levels. This is probably due to the selection on the basis of the IGF-I genotypes. Therefore, we did not investigate a population-based sample, but selection was made in order to maximize statistical power. We observed comparable effects of the 192-bp and the 194-bp alleles on IGF-I serum levels and body height, suggesting a broader optimum for IGF-I gene regulated transcriptional activity. This might shed new light on the conflicting data presented previously in several populations studied by Rosen [5], Frayling [3] and Allen [1]. By recalculating the association between the genotypes as suggested in our study, we anticipate that the conflicting reports so far might be harmonized, since frequencies of the IGF-I genotypes differed between the different populations studied.

The combination of the short (< 192-bp) and long alleles (> 194-bp) appear to have an IGF-I serum level and body height more comparable with homozygous carriers of the 192-bp and 194-bp alleles, than with homozygous carriers of shorter and longer alleles. This was unexpected and is probably due to a smaller number of subjects in this group.

As expected from previous studies, we observed a secular trend in body height over a 28-year period between 1917 and 1945. Mean body height increased approximately 5 cm over this period. The secular trend in the Netherlands has been observed since 1858 and is based on data from conscripts, with temporary interruptions or even decreases of the shift, during agrarian crises, during the world crisis around 1930 and during World War II [26]. From the middle of the 19<sup>th</sup> century onwards, a steep increase in body height has been reported which still continues [26, 27]. Anthropometry provides information on body composition in a non-invasive manner and reflects the interaction between genes, health and nutritional status [28,

29]. The magnitude of this shift is supposed to be influenced by genetic, environmental and lifestyle factors [16]. In all quartiles studied, the group of individuals with the 192-bp/192-bp, 192-bp/194-bp and 194-bp/194-bp alleles were approximately 1.5 cm taller compared to the group consisting of the remaining shorter and longer genotypes. The difference in body height reached only statistical significance in the period between 1938 and 1945 (time period after the recession and during World War II in the Netherlands). Since the difference in body height between the IGF-I genotype groups didn't increase over time, we believe that there is no specific effect of the IGF-I gene on the secular trend, nor in times of malnutrition. In conclusion, we observed an optimum in IGF-I levels for the 192-bp and 194-bp alleles of the promoter polymorphism in the IGF-I gene. For body height, the same optimum was only observed in males, suggesting that the effect of IGF-I on linear growth is gender-specific. A secular trend in body height was observed in our elderly Dutch population, which was similar for the different genotypes; carriers of the 192-bp and/or 194-bp allele remained significantly taller throughout time compared to the remaining IGF-I genotypes.

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# 4

**Insulin-like growth factor-I gene influences  
beta-cell function in normal glucose  
tolerance**



Insulin resistance and beta cell dysfunction are both prerequisites for the development of type 2 diabetes. Recent studies indicate that insulin-like growth factor-I (IGF-I) is an important regulator of pancreatic beta-cell growth and maturation. In this study we examined whether a genetically determined low expression of IGF-I has an effect on pancreatic beta cell function and insulin sensitivity.

We studied a polymorphism in the regulatory region of the IGF-I gene. Previously we found that variant carriers of this IGF-I gene promoter polymorphism have lower circulating IGF-I levels and lower body height than carriers of the wild type genotype. The relation between the IGF-I polymorphism, beta cell function and insulin sensitivity was assessed in a case-control study of 1069 subjects of the Rotterdam Study, selected on the basis of an oral glucose tolerance test. This selection comprised 595 persons with normal glucose tolerance, 254 individuals with pre-diabetes and 220 subjects with type 2 diabetes. Beta cell function was assessed by HOMA-B and first- and second phase insulin secretion. Furthermore we calculated HOMA-IR, the insulin sensitivity index and the disposition index (DI).

In the study population 787 subjects were carriers of the wild type IGF-I genotype (73.6%) and 282 subjects were variant carriers (26.4%). We observed in normal glucose tolerant individuals with genetically low IGF-I levels (variant carriers) decreased first- and second phase insulin secretion and HOMA-B but normal insulin sensitivity. In individuals with pre-diabetes and in persons with type 2 diabetes there were no differences in parameters of insulin sensitivity and beta cell function between variant carriers and carriers of the wild type of this IGF-I gene promoter polymorphism.

In conclusion, we observed in individuals with normal glucose tolerance, that genetically determined low IGF-I expression is associated with a decreased beta cell function and insulin secretion after an oral glucose load. This suggests that an IGF-I promoter polymorphism may in part determine a genetically programmed pancreatic beta cell dysfunction that leads to diabetes in persons with insulin resistance or obesity.

## INTRODUCTION

Insulin resistance is strongly related with obesity, which is growing in major proportions within the developed world [1]. Despite the fact that insulin resistance is one of the earliest features of type 2 diabetes mellitus, only 20 percent of persons with insulin resistance will ultimately develop diabetes mellitus [2]. This is largely explained by the ability of the beta cells to compensate insulin secretion in order to maintain normal glucose levels. The functional pancreatic beta cell mass is the major factor determining the amount of insulin that can be secreted. Beta cell mass is reduced in subjects with type 2 diabetes and in those with impaired glucose tolerance [3]. Changes in insulin sensitivity are compensated by inverse changes in beta cell responsiveness. Beta cell dysfunction in the presence of insulin resistance leads to type 2 diabetes.

Insulin-like growth factor-I (IGF-I) is a hormone that regulates proliferation and differentiation of numerous cell types. IGF-I has been shown to be an important regulator of the pancreatic beta cell mass [4-9]. Recent studies indicate that IGF-I is involved in pancreatic islet development during embryogenesis and also promotes compensatory beta cell proliferation and survival in situations of increasing insulin demands [4, 7, 8, 10, 11]. Patients with diabetes mellitus are characterized by low serum levels of IGF-I [12, 13]. In addition, the IGF-I/IGFBP3 complex protects NOD mice from developing type 1 diabetes [14].

We recently demonstrated that the absence of the wild type allele of a polymorphism in the promoter region of the IGF-I gene is associated with low circulating IGF-I concentrations, low body height, reduced birth weight and an increased risk for type 2 diabetes mellitus [15, 16]. Furthermore, non-carriers of the wild type allele of this polymorphism in the IGF-I gene had an increased intima media thickness when hypertensive [17] and did not show the age-related decline with circulating total IGF-I levels [18].

In this study we tested the hypothesis that a genetically determined reduction in IGF-I expression results in reduced pancreatic beta cell function and insulin sensitivity in normal glucose tolerant people and individuals with pre-diabetes. Furthermore, we investigated if pancreatic beta cell dysfunction and diminished insulin sensitivity is influenced by BMI and/or WHR as indicators of obesity.

## SUBJECTS AND METHODS

### *Study population*

All persons included in the present study were participants of the Rotterdam Study. The Rotterdam Study is a single-center prospective follow-up study in which all residents aged 55 years and over of the Rotterdam suburb Ommoord were invited to participate. The study was approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam and

written informed consent was obtained from all participants. The aim of the Rotterdam study is to investigate determinants of chronic and disabling cardiovascular, neurodegenerative, locomotor and ophthalmologic diseases. The design of the study has been described previously [19]. The baseline examination of the Rotterdam Study was conducted between 1990 and 1993. A total of 7983 participants (response rate 78 percent) were examined.

During the first follow-up examination in 1993-1994, 4830 participants aged 55 – 75 years participated. A sample was invited to participate in the present study [20]. Subjects with probable dementia were not invited. The subjects were divided into diabetics (using anti-diabetic medication or had a random or postload glucose level of 11.1 mmol/L or above), hyperinsulinemic (upper quintile of the sex-specific postload insulin distribution in subjects without impaired glucose tolerance or diabetes mellitus) or normal glucose tolerant (random glucose < 7.8, according to criteria used in 1994, when participants were selected). A random sample was taken from each glucose tolerance group, which resulted in 200 subjects with diabetes, 400 with hyperinsulinemia and 600 with normal glucose tolerance. In total, 1069 persons consented to participate. Based on a fasting OGTT a definitive classification of the glucose tolerance status was made. Normal glucose tolerance was defined as fasting glucose below 6.1 mmol/l and 2 hours post-load glucose below 7.8 mmol/l. Subjects with high glucose levels not meeting criteria for diabetes but too high to be considered normal are referred to as having pre-diabetes. Pre-diabetes includes persons with impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT). IFG was defined as fasting glucose between 6.1 and 7.0 mmol/l and IGT as a 2 hours postload glucose between 7.8 and 11.1 mmol/l. A diagnosis of diabetes mellitus was made if patients were treated for diabetes or had a fasting glucose level of 7.0 mmol/l or above and/or a 2 hours post-load glucose of 11.1 mmol/l or above [21].

### ***Data Collection***

Information concerning health status, smoking and drug use was obtained with a computerized questionnaire. Height and weight were measured and body mass index (BMI in kg/m<sup>2</sup>) was calculated. Body fat distribution was assessed by the ratio of waist and hip circumferences (WHR).

Blood sampling and storage have been described elsewhere [22]. Serum was separated by centrifugation and quickly frozen in liquid nitrogen. Glucose and insulin levels were measured in fasting serum and postload serum samples. Persons that were treated for diabetes did not undergo a glucose tolerance test. Therefore, postload glucose and insulin measurements, HOMA-B, HOMA-IR, first- and second phase insulin secretion, insulin sensitivity index and disposition index were only available for 990 persons not using anti-diabetic medication (595 subjects with normal glucose tolerance, 254 subjects with pre-diabetes and 141 newly diagnosed diabetic subjects). Glucose was measured by the glucose hexokinase method [23]. Insulin was measured by a commercially available assay (IRMA, Medgenix Diagnostics, Brussels, Belgium; intra-assay and inter-assay variation of 3-6 percent and 5-12 percent,

respectively). HbA1c was measured by HPLC (normal values: 5.0 – 6.3%) (Variant HPLC-Biorad, Veenendaal, The Netherlands).

To determine the relation between the IGF-I gene and beta cell function and insulin sensitivity we used the Homeostasis Model Assessment (HOMA), and the measurements proposed by Stumvoll et al., since they correlate well with parameters derived from the euglycemic clamp technique [24-29].

Beta cell function was assessed by HOMA-B [25] and first and second phase insulin secretion [29]:

First phase insulin secretion:  $1283 + 1.829 * \text{ins30} - 138.7 * \text{glu30} + 3.722 * \text{ins0}$

Second phase insulin secretion:  $286 + 0.416 * \text{ins30} - 25.94 * \text{glu30} + 0.926 * \text{ins0}$

As indicator of insulin sensitivity we used HOMA-IR [26] and the index (ISI) suggested by Stumvoll et al. [29]:  $0.213 - 0.00305 * \text{BMI} - 0.000308 * \text{ins0} - 0.000640 * \text{age}$

The disposition index (DI) was calculated as the product of first-phase insulin secretion and insulin sensitivity index (ISI) according to Bergman et al. [30].

### ***Genotyping of the IGF-I promoter polymorphism***

The human IGF-I gene contains a polymorphic cytosine-adenine repeat 1 Kb upstream of the promoter region [31]. IGF-I genotypes were determined as described previously [15]. The most common allele contains 19 CA-repeats, which equals a length of 192-bp as described in previous studies [15, 16]. Since carriers of the 194-bp-allele have similar IGF-I levels and body height as carriers of the 192-bp allele, two different IGF-I genotype groups were made as previously described [32]: 1) homozygous carriers of the 192-bp, homozygous carriers of the 194-bp and heterozygous carriers of 192-bp/194-bp alleles; “the wild type genotype” 2) All other combinations of alleles, i.e. heterozygous carriers of the 192-bp or 194-bp allele and non-carriers of the 192-bp and 194-bp alleles “the variant genotype”.

### ***Data analyses***

Differences in gender were compared using a chi-square test. Differences in age were compared using analysis of variance. BMI, WHR, fasting glucose and insulin concentration, HbA1c, postload glucose and insulin concentration, first and second phase insulin secretion, HOMA-B, HOMA-IR, ISI and DI were compared using analyses of variance and presented as mean values  $\pm$  standard error of mean (SEM) after adjustment for age and sex. First and second phase insulin secretion, HOMA-B, HOMA-IR, ISI and DI were additionally adjusted for BMI.

The relation between the IGF-I genotype and the risk to develop type 2 diabetes was re-examined with a new division of the IGF-I genotypes [32] using a logistic regression. Odds ratios are presented after adjustment for age and sex. The relation between the IGF-I gene and parameters of beta-cell function and insulin sensitivity was studied using a general linear model. Data are presented as mean values  $\pm$  SEM after adjustment for age and sex. A stratified analysis was conducted, based on a BMI lower (<) or equal or higher ( $\geq$ ) than

27 kg/m<sup>2</sup>, since it has been found that insulin sensitivity for glucose disposal is impaired in human subjects with normal glucose tolerance with a BMI of 27 kg/m<sup>2</sup> or above [33]. To evaluate the role of BMI as an intermediate factor in the relation between the IGF-I gene and beta cell function, we assessed the relation between the IGF-I gene and BMI using analyses of variance. All analyses were performed using the SPSS for Windows software package, version 11.0 (SPSS Inc., USA).

## RESULTS

Table 1 shows the characteristics of the study population. Diabetes patients and subjects with pre-diabetes were more frequently male ( $p < 0.05$ ). Mean age, BMI, WHR, fasting glucose and insulin levels, HbA<sub>1c</sub>, postload glucose and insulin concentrations, were significantly higher in persons with pre-diabetes and/or diabetes mellitus ( $p < 0.005$ ). First and second phase insulin secretion, ISI and DI were significantly lower in subjects with pre-diabetes or type 2 diabetes. HOMA-B and HOMA-IR were only significantly lower in subjects with diabetes.

**Table 1:** Characteristics of the study population

	Normal Glucose Tolerance	Pre-diabetes	Diabetes Mellitus
<b>Number of subjects</b>	595	254	220
Men (%)	45.9	51.2 <sup>†</sup>	56.4 <sup>†</sup>
Age - yr	65.9 ± 0.2	66.6 ± 0.3 <sup>*</sup>	67.9 ± 0.4 <sup>*</sup>
Body Mass Index - kg/m <sup>2</sup>	25.9 ± 0.1	27.3 ± 0.2 <sup>*</sup>	27.6 ± 0.2 <sup>*</sup>
Waist-to-hip ratio	0.90 ± 0.003	0.93 ± 0.005 <sup>*</sup>	0.93 ± 0.005 <sup>*</sup>
Fasting glucose - mmol/l	5.5 ± 0.1	6.2 ± 0.1 <sup>*</sup>	8.5 ± 0.1 <sup>*</sup>
Fasting insulin - mU/l	11.7 ± 0.5	15.5 ± 0.7 <sup>*</sup>	19.1 ± 0.8 <sup>*</sup>
Hemoglobin A <sub>1c</sub> - %	5.9 ± 0.03	6.0 ± 0.04 <sup>*</sup>	6.9 ± 0.05 <sup>*</sup>
<b>Number of subjects</b>	595	254	141 <sup>**</sup>
Postload glucose 30' - mmol/l	8.5 ± 0.1	9.9 ± 0.1 <sup>*</sup>	12.2 ± 0.1 <sup>*</sup>
Postload insulin 30' - mU/l	86.6 ± 2.5	87.1 ± 3.9	73.3 ± 5.2 <sup>†</sup>
First phase insulin secretion - pmol/l	1589.5 ± 37.7	1402.1 ± 59.0 <sup>†</sup>	930.1 ± 76.8 <sup>*</sup>
Second phase insulin secretion - pmol/l	410.8 ± 8.6	376.8 ± 13.5 <sup>†</sup>	283.9 ± 17.6 <sup>*</sup>
HOMA-B (%)	97.9 ± 1.5	96.6 ± 2.4	92.4 ± 2.5
HOMA-IR	1.95 ± 0.05	1.84 ± 0.08	1.71 ± 0.09 <sup>†</sup>
ISI - umol * kg <sup>-1</sup> * min <sup>-1</sup> * pmol/l	0.062 ± 0.001	0.056 ± 0.002 <sup>*</sup>	0.049 ± 0.002 <sup>*</sup>
DI - umol * kg <sup>-1</sup> * min <sup>-1</sup>	90.4 ± 2.0	63.7 ± 3.1 <sup>*</sup>	24.1 ± 4.1 <sup>*</sup>

Data are expressed as percentage or mean ± standard error of mean (SEM). Postload glucose and insulin concentration were measured at t = 30'. HOMA-B = homeostasis model assessment of steady state beta cell function; HOMA-IR = homeostasis model assessment of insulin resistance; ISI = insulin sensitivity index, DI = disposition index. Mean Body Mass Index, waist-to-hip ratio, fasting glucose and insulin concentration, hemoglobin A<sub>1c</sub>, postload glucose and insulin at t = 30' are corrected for differences in age and gender between the groups and presented after adjustment. First and second phase insulin secretion, HOMA-B, HOMA-IR, ISI and DI are additionally adjusted for BMI. Overall difference between groups <sup>†</sup>  $p < 0.05$  versus persons with normal glucose tolerance. <sup>\*</sup>  $p < 0.005$  versus persons with normal glucose tolerance. <sup>\*\*</sup> Only available for subjects not using anti-diabetic medication

In the whole study population 787 subjects were carriers of the wild type IGF-I genotype (73.6%) and 282 subjects were variant carriers (26.4%).

Table 2 shows the relation between the IGF-I genotypes and fasting glucose and insulin, HbA1c and postload glucose and insulin in subjects with normal glucose tolerance. No significant relation was observed between the IGF-I genotypes and fasting glucose and insulin, HbA1c and postload glucose and insulin. After stratification by BMI (< or  $\geq$  27 kg/m<sup>2</sup>) and WHR (< or  $\geq$  0.92), no significant differences were observed between carriers and non-carriers of the 192-bp and 194-bp alleles (data not shown).

**Table 2** Relation IGF-I genotype and fasting and postload glucose and insulin in normal glucose tolerance

	Wild type carriers	Variant type carriers
<b>Number (N = 582)</b>	<b>444</b>	<b>141</b>
Fasting glucose - mmol/l	5.5 $\pm$ 0.03	5.5 $\pm$ 0.02
Fasting insulin - mU/l	11.7 $\pm$ 0.3	11.2 $\pm$ 0.5
Postload glucose 30' - mmol/l	8.5 $\pm$ 0.1	8.6 $\pm$ 0.1
Postload insulin 30' - mU/l	88.0 $\pm$ 2.6	81.4 $\pm$ 4.5
Hemoglobin A <sub>1c</sub> - %	5.8 $\pm$ 0.02	5.9 $\pm$ 0.04

Data are expressed as mean  $\pm$  standard error of mean (SEM) adjusted for age and sex. Postload glucose and insulin concentration were measured at t = 30'. No significant differences were observed between the IGF-I genotypes

Total IGF-I levels were significantly lower in variant carriers than in carriers of the wild type of this IGF-I gene polymorphism (variant carriers: 15.3  $\pm$  1.2 nmol/L (N=30) versus carriers of the wild type: 19.0  $\pm$  0.8 nmol/L (N=60); p=0.03 after adjustment for age and sex). In subjects with normal glucose tolerance total IGF-I levels were lower in variant carriers than carriers of the wild type (14.4  $\pm$  1.8 nmol/L (N=15) versus 19.6 nmol/L (N=35), p=0.06). Risk of diabetes was 1.3 times higher in variant carriers compared to wild type carries. This was not significant (data not shown).

Table 3 shows the relation between IGF-I genotype and quantitative parameters of insulin sensitivity and beta cell function in subjects with normal glucose tolerance. Variant carriers of this IGF-I gene promoter polymorphism had a lower first and second phase insulin secretion and beta cell function (as assessed by HOMA) than wild type carriers. Insulin sensitivity (as

**Table 3** Relation between IGF-I genotype and quantitative parameters of insulin sensitivity, beta cell function and IGF-I levels in subjects with normal glucose tolerance

	Wild type carriers	Variant type carriers	p-value
<b>Number (N = 591)</b>	<b>441</b>	<b>150</b>	
Mean glucose OGTT (mmol/L)	6.4 $\pm$ 0.3	6.4 $\pm$ 0.06	0.73
First phase insulin secretion ( $\mu$ mol/L)	1573.7 $\pm$ 37.8	1452.0 $\pm$ 65.0	0.02
Second phase insulin secretion ( $\mu$ mol/L)	406.5 $\pm$ 8.7	379.2 $\pm$ 14.9	0.07
HOMA-B (%)	99.6 $\pm$ 1.8	92.5 $\pm$ 3.1	0.04
HOMA-IR	1.91 $\pm$ 0.07	2.05 $\pm$ 0.12	0.36

Data are expressed as percentages or mean  $\pm$  standard error of mean (SEM). First and second phase insulin secretion, HOMA-B and HOMA-IR are additionally adjusted for BMI

assessed by ISI and HOMA-IR) and DI did not differ between variant carriers and carriers of the wild type of this IGF-I gene promoter polymorphism.

The role of BMI as an intermediate factor in the relation between the IGF-I gene and beta cell function was assessed by determining the relation between the IGF-I gene and BMI. In addition to a lower first and second phase insulin secretion and beta cell function, we observed in non-obese variant carriers of this IGF-I gene promoter polymorphism a statistically significant lower DI than in carriers of the wild type (Table 4). In obese subjects no differences in parameters of insulin sensitivity and beta cell function were observed between variant carriers and carriers of the wild type of this IGF-I gene promoter polymorphism.

In individuals with pre-diabetes and in persons with type 2 diabetes, also no relation between the IGF-I genotype and parameters of insulin sensitivity and beta cell function was observed (data not shown).

**Table 4:** Relation between IGF-I genotype and some quantitative parameters of insulin sensitivity and beta cell function in subjects with normal glucose tolerance after stratification by BMI

	Wild type carriers	Variant type carriers	p-value
<b>Non-obese subjects (N = 400)</b>	<b>298</b>	<b>102</b>	
First phase insulin secretion	1472.9 ± 42.0	1293.4 ± 71.8	0.01
Second phase insulin secretion	382.4 ± 9.6	341.3 ± 16.5	0.02
HOMA-B	99.8 ± 2.3	91.1 ± 4.0	0.06
HOMA-IR	1.98 ± 0.10	2.19 ± 0.17	0.29
IS ( $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \cdot \mu\text{mol/L}$ )	0.074 ± 0.001	0.075 ± 0.001	0.67
DI ( $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ )	102.7 ± 2.4	92.7 ± 4.1	0.03
<b>Obese subjects (N = 179)</b>	<b>134</b>	<b>45</b>	
First phase insulin secretion	1197.9 ± 76.9	1784.8 ± 132.7	0.96
Second phase insulin secretion	459.8 ± 17.6	459.1 ± 30.4	0.93
HOMA-B (%)	99.5 ± 2.9	94.6 ± 5.0	0.40
HOMA-IR	1.80 ± 0.08	1.73 ± 0.13	0.62
IS ( $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \cdot \mu\text{mol/L}$ )	0.049 ± 0.002	0.049 ± 0.003	0.82
DI ( $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ )	78.5 ± 3.6	80.5 ± 6.1	0.74

Data are expressed as mean ± standard error of mean (SEM) after adjustment for age and sex. First and second phase insulin secretion are expressed in pmol/L

## DISCUSSION

IGF-I is an important regulator of the beta cell mass, possibly by regulating differentiation, proliferation and survival of pancreatic beta cells [7, 8, 11]. Diminished IGF-I expression may result in a physiologically relevant shortage of functional beta cells. Furthermore, it is reported that low plasma IGF-I levels predict a decline in whole body glucose uptake independent of body fat, free fatty acids, waist to hip ratio and basal respiratory quotient [34]. Within normal glucose tolerant people, a genetic predisposition for type 2 diabetes exists, which

may become evident with increasing BMI and decreasing insulin sensitivity. In our study, we observed in individuals with a normal glucose tolerance and who were variant carriers of an IGF-I gene polymorphism, a decreased beta cell function and a lower first and second phase insulin secretion and a normal insulin sensitivity. The relation between beta cell function and insulin sensitivity in healthy individuals is hyperbolic. Changes in insulin sensitivity are normally compensated by inverse changes in beta cell responsiveness, such that the product of insulin sensitivity and insulin secretion (the disposition index) remains constant. Hence the DI indicates the metabolic status of an individual, i.e. the relative contributions of insulin sensitivity and beta cell function to their degree of glucose tolerance [9].

Our study suggests, that in healthy subjects with genetically low IGF-I levels, beta cell responds insufficient to an oral glucose load. Our results are supported by a recent study showing a strong relation between low circulating IGF-I levels and subsequent development of glucose intolerance in an elderly population [35]. Furthermore, 't Hart et al observed the same trend in persons with normal glucose tolerance, but no significant association was observed, probably due to low numbers in their study population [36]. BMI could be a mediating factor in the causal chain of the IGF-I gene polymorphism leading to alterations in glucose metabolism. However, in our study no relation between the IGF-I gene and BMI was observed.

In patients with overt diabetes mellitus, it is not possible to distinguish defects in insulin secretion and sensitivity that are pathogenetically involved in the development of diabetes mellitus from defects that are secondary to hyperglycemia [37]. In addition, application of these OGTT based methods to individuals with an impaired beta cell insulin secretion capacity (for example those with IGT and frank diabetes) are confounded by differences in gastric emptying, beta cell function and glucose effectiveness and therefore might underestimate insulin resistance [38]. This may explain why we did not observe a relation between the IGF-I gene and HOMA parameters, ISI and DI in persons with pre-diabetes and diabetes.

In a previous publication we found that subjects with genetically determined low serum IGF-I levels had an increased risk to develop type 2 diabetes [15]. In the present study with a different approach this relation was again observed, although no statistical significance was reached. However, in contrast to our findings, Frayling et al. found that non-carriers of the wildtype alleles had higher IGF-I levels and reduced risk of type 2 diabetes [39]. It has been suggested that these inconsistent and opposite findings may have resulted from a variety of factors: e.g. linkage disequilibrium with another functional variant, differences in environmental factors and selection of participants [40]. In addition, the performed studies to date have been relatively small and it has been argued that all these studies have a reduced power to detect small genetic effects [41].

Obesity or weight gain plays a pivotal role in beta cell function and insulin sensitivity. Children with rapid postnatal weight gain had higher BMI and waist circumference and lower insulin sensitivity at 8 years of age [42]. IGF-I levels at 5 years of age predicted gain in height SDS

between 5 and 8 years of age and were also positively related to insulin secretion at 8 years of age [42]. Kloppel et al showed in 1985 that in diabetic as well as in non-diabetic patients, the beta-cell mass was about 40% higher in obese subjects compared with lean subjects, suggesting there is compensatory growth of beta-cell mass if insulin resistance increases in obesity [43]. In our study, we observed a significant association between the IGF-I gene and insulin secretion in the total study population and non-obese persons. This association was not found in obese individuals. An explanation for this latter observation is that our study lacked power to detect an effect of the IGF-I gene on insulin secretion in obese persons.

We estimated beta cell function from fasting and OGTT measurements using established, validated techniques [24-28]. Although estimates derived from these techniques correlate highly with more invasive and time-consuming gold-standard methods, i.e., the hyperglycemic and euglycemic clamp, some measurement error may have occurred. Since all clinical measurements were performed without knowledge of the genotype, such misclassification is most likely not related to the genotype and will therefore have resulted in an underestimation of the true effect.

In conclusion, we observed in individuals with normal glucose tolerance, that variant carriers of an IGF-I gene promoter polymorphism had a decreased beta cell function and insulin secretion after an oral glucose load. This finding supports the view that this polymorphism in the regulatory region of the IGF-I gene plays a role in the regulation of beta cell function and thus may contribute to the susceptibility for diabetes. When our findings could be reconfirmed in other studies, further studies could be started to investigate whether IGF-I increasing therapies may help to prevent the development of IGT and/or type 2 diabetes in normoglycaemic variant carriers of this IGF-I gene promoter polymorphism.

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# 5a

**An IGF-I gene polymorphism modifies the  
risk of diabetic retinopathy**



The role of IGF-I in the pathogenesis of diabetic retinopathy is unclear. We studied, prospectively, the relationship between an IGF-I gene polymorphism, retinal vessel diameters, and incident diabetic retinopathy in subjects with impaired glucose tolerance (IGT) or type 2 diabetes. In all 5505 participants of the population-based Rotterdam Study (775 with IGT, 394 with type 2 diabetes and 4336 control subjects), fundus color transparencies were taken at baseline (between 1990 and 1993) and at follow-up (from 1997 to 1999). The wild-type genotype (i.e. carriers of the 192- or 194-bp alleles) was present in 72.7% of the participants, while 27.3% were variant carriers. Variant carriers with IGT or diabetes appeared to have larger retinal arteriolar and venular diameters at baseline than individuals with the wild-type genotype, but these differences did not reach statistical significance. This trend was especially observed in subjects who developed retinopathy at follow-up. In variant carriers with IGT/diabetes, an increase (odds ratio 1.8 [95% CI 1.0 – 3.2];  $p = 0.04$ ) in the risk of retinopathy was observed compared with participants with the wild-type genotype. In conclusion, our findings suggest that this IGF-I gene polymorphism is associated with an increased risk of diabetic retinopathy.

## INTRODUCTION

The role of IGF-I in the pathogenesis of diabetic retinopathy (hereafter referred to simply as retinopathy) in both type 1 and type 2 diabetes mellitus is unclear and controversial. It has been suggested that hereditary factors are involved in the pathogenesis to develop retinopathy [1].

In humans, a CA<sub>n</sub> polymorphism in the promoter region of the IGF-I gene has been identified [2]. We previously observed that this CA<sub>n</sub> polymorphism indeed was associated with low normal serum total IGF-I levels, a lower body height, and a higher risk for developing type 2 diabetes and myocardial infarction [2]. Although these findings have not been consistently confirmed in other studies, they suggest that this IGF-I gene polymorphism may be used as a proxy for the genetically determined IGF-I expression in the body.

The purpose of our study was to examine the association between this CA<sub>n</sub> IGF-I gene promoter polymorphism and retinal vascular diameters, as well as diabetic retinopathy, in participants with type 2 diabetes and impaired glucose tolerance (IGT).

## RESEARCH DESIGN AND METHODS

The Rotterdam Study is a single-center prospective follow-up study in which all residents aged  $\geq 55$  years of the Rotterdam suburb Ommoord were invited to participate [3]. The appropriate medical ethics committee of the Erasmus University approved the study, and written informed consent was obtained from all participants. In total, 7,983 participants (response rate 78 percent) were examined. The ophthalmologic part of the study became operational after the screening of the participants had started, leading to ophthalmologic examinations of 6780 individuals. Of them, 6436 had transparencies taken. From these, 762 were excluded because they had ungradable fundus transparencies on either eye, resulting in 5674 eligible individuals [4]

Glucose was measured in 5505 of the 5674 individuals of the ophthalmologic cohort. Genotyping for the IGF-I gene was successful for 5393 of the 5505 individuals whose information on glucose tolerance was also available. The present study was based on these 5393 individuals (4247 control subjects, 759 subjects with IGT, and 387 type 2 diabetic subjects).

### *Ophthalmologic examination.*

Baseline examinations were conducted between 1990 and 1993, and for the prospective study, we used data from the follow-up period of 1997-1999 (n = 3296 participants [554 individuals with IGT/diabetes] and mean follow-up time 6.5 years [range 5.1 - 8.5]). At baseline and follow-up, 35° color fundus transparencies, centered on the fovea (field 2), were taken after pharmacological mydriasis [4]

To measure the sum of the retinal vessel diameters, 20° field color transparencies taken at baseline and centered on the optic disc were digitized [4]. Per person, the image with the best quality (left or right eye) was analyzed with a semiautomated system (Retinal Analysis; Optimate, Madison, WI) by four trained graders masked for any endpoint [5-7]. Summary retinal arteriolar and venular diameters (in micrometers) were calculated with the improved Parr-Hubbard formula, adjusted for magnification differences due to possible errors of the eye [5, 8]. In a random subsample of 100 participants, we found no differences between the right and left eyes for the arteriolar and venular diameters; therefore, only one eye of each individual was used.

For the prospective study, the level of retinopathy was graded in each eye according to the Early Treatment Diabetic Retinopathy Study scale [9, 10]. Grading was performed at baseline and at follow-up. Incident retinopathy was defined as absent retinopathy at baseline and presence of retinopathy in any eye at follow-up.

#### ***Other measurements.***

Information concerning health and smoking status was obtained during a home interview at baseline. Blood pressure was measured with a random-zero sphygmomanometer. Hypertension was defined as a diastolic blood pressure  $\geq 90$  mm Hg and/or a systolic blood pressure  $\geq 140$  mm Hg and/or the use of antihypertensive medication [11]. Blood sampling and storage have been described elsewhere. Glucose levels were measured by the glucose hexokinase method and were available due to logistic reasons in 97% of the ophthalmologic cohort. Diabetes mellitus was defined as a non-fasting glucose  $\geq 11.1$  mmol/L and/or use of anti-diabetic medication and, similarly, IGT as glucose between  $\geq 7.8$  and 11.1 mmol/L. Normal glucose tolerance was defined as a nonfasting glucose  $<7.8$  mmol/L without use of antidiabetic medication [12].

#### ***Genotyping of the IGF-I promoter polymorphism.***

IGF-I genotypes were determined as described previously [2]. In a previous study, we examined the role of the various lengths of the alleles of the IGF-I promoter polymorphism and observed for carriers of the 194-bp-allele similar IGF-I levels and body height as carriers of the 192-bp allele [13].

#### ***Statistical analyses.***

General and ophthalmologic characteristics were compared using ANOVA. Data are presented as means  $\pm$  SE after adjustment for age and sex. Hypertension, current smoking, and prevalent or incident retinopathies were compared between the groups using  $\chi^2$  statistics. Data are presented as numbers and percentages.

Logistic regression was used to calculate the odds ratios (ORs) and 95% CIs for incident retinopathy in diabetic and IGT case and control subjects stratified by the IGF-I genotypes,

after adjustment for age and sex. All analyses were performed using the SPSS for Windows software package, version 11.0.

## RESULTS

At baseline, participants with IGT or type 2 diabetes were younger, were more frequently male, had a higher systolic blood pressure, were more often diagnosed with hypertension and were more frequently current smokers than the control subjects (Table 1). They also had significantly higher mean retinal arteriolar and venular diameters than control subjects (Table 1).

**Table 1:** General characteristics and ophthalmologic parameters of the study population at baseline.

	<i>Control subjects</i>	<i>Participants with diabetes or IGT</i>	<i>p</i>
N	4247	1146	
Age (years)	67.5 ± 0.1	59.5 ± 0.2	<0.001
Males	1684 (39.7%)	549 (47.9%)	<0.001
Systolic blood pressure (mm Hg)	137.7 ± 0.3	141.8 ± 0.6	<0.001*
Diastolic blood pressure (mm Hg)	73.7 ± 0.2	73.8 ± 0.3	0.8*
Hypertension	1282 (30.2)	495 (43.2)	<0.001
Current smoker	961 (22.6)	298 (26.0)	<0.01
Arteriolar diameter (µm)	146.6 ± 0.2	147.8 ± 0.4	0.01*
Venular diameter (µm)	221.4 ± 0.3	223.7 ± 0.6	0.001*
Prevalent retinopathy		147 (13.0)	

Data are presented as means ± SE or n (%). \*Adjusted for age and sex.

Mean arteriolar and venular diameters at baseline were not different between participants with the wild type (n = 3922) and variant carriers (n = 1471) (arteriolar diameter [means ± SE] in participants with the wild type 146.7 ± 0.2 µm, p = 0.3; venular diameters in individuals with the wild type 221.7 ± 0.3 µm vs. variant carriers 222.5 ± 0.5 µm, p = 0.2).

Variant carriers with IGT or type 2 diabetes appeared to have larger retinal arteriolar and venular diameters at baseline than participants with the wild type, but these differences did not reach statistical significance (Table 2).

When further separated in those with and without incident retinopathy during follow-up, mean retinal arteriolar and venular diameters at baseline tended to be larger in variant carriers with incident retinopathy during follow-up than in those without (Table 3), but probably due to low numbers, these differences did not reach statistical significance (Table 3).

Retinal arteriolar and venular diameters at baseline tended to be largest in variant carriers with IGT or type 2 diabetes, who developed incident retinopathy during follow-up, compared with control subjects and variant carriers with IGT or type 2 diabetes, who did not develop

retinopathy (retinal arteriolar diameter  $p$  for trend = 0.03 and retinal venular diameter  $p$  for trend = 0.02; Figs 1 and 2).

In the group with IGT or type 2 diabetes, variant carriers had an increased risk (OR 1.8 [95% CI 1.0 – 3.2];  $p = 0.04$ ) (adjusted for age and sex) of incident retinopathy compared with participants with the wild type.

**Table 2:** Differences in baseline characteristics and ophthalmologic parameters in participants with IGT/diabetes comparing subjects with the wild type and variant carriers of an IGF-I gene polymorphism

IGT/DM	Wild type	Variant carriers	P value*
N	824	322	
Age (years)	69.4 ± 0.3	69.9 ± 0.5	0.3
Male	403 (48.9)	146 (45.3)	0.3
Systolic blood pressure (mm Hg)	142.9 ± 0.8	142.8 ± 1.2	0.9
Diastolic blood pressure (mm Hg)	73.6 ± 0.4	73.5 ± 0.7	0.9
Hypertension	353 (42.8)	142 (44.1)	0.4
Current smoker	219 (26.6)	79 (24.5)	0.7
Random glucose (mmol/L)	10.4 ± 0.2	10.5 ± 0.2	0.8
Arteriolar diameter (μm)	146.9 ± 0.5	148.5 ± 0.8	0.07
Venular diameter (μm)	222.6 ± 0.7	224.7 ± 1.2	0.1

Data are means ± SE or n (%). \*After adjustment for age and sex.

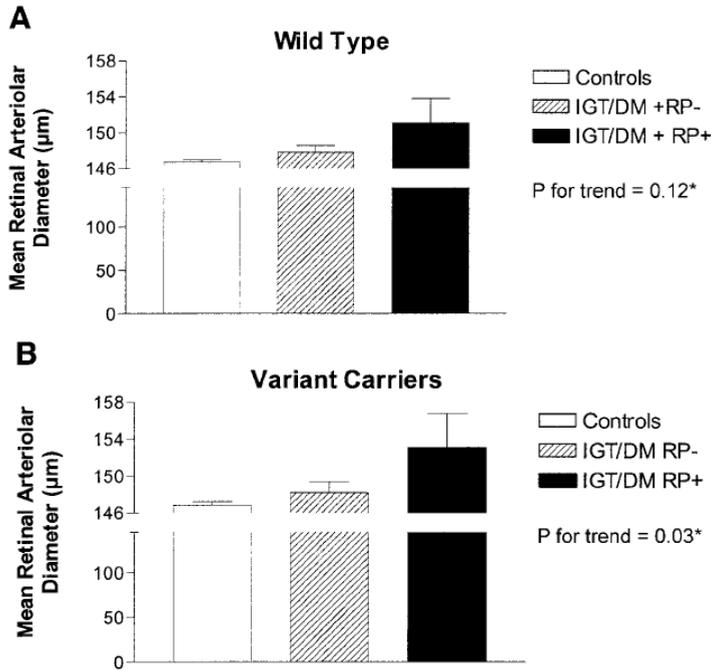
**Table 3:** Differences in baseline retinal diameters in participants with IGT/diabetes comparing subjects with the wild type and variant carriers of an IGF-I gene polymorphism

	Wild type	Variant carriers	P value*
Mean retinal arteriolar diameter (μm)			
Participants with IGT/diabetes			
Without incident retinopathy	147.8 ± 0.8 (361)	148.2 ± 1.2 (136)	0.45
With incident retinopathy	151.1 ± 2.7 (31)	153.1 ± 3.7 (18)	0.54
Mean retinal venular diameters (μm)			
Participants with IGT/diabetes			
Without retinopathy	224.6 ± 1.1 (361)	226.4 ± 1.8 (136)	0.39
With retinopathy	222.7 ± 3.8 (31)	230.8 ± 4.6 (18)	0.2

Data are means ± SE (n). For the analyses, the to genotypes were further separated in those with and without retinopathy. \*After adjustment for age and sex.

## DISCUSSION

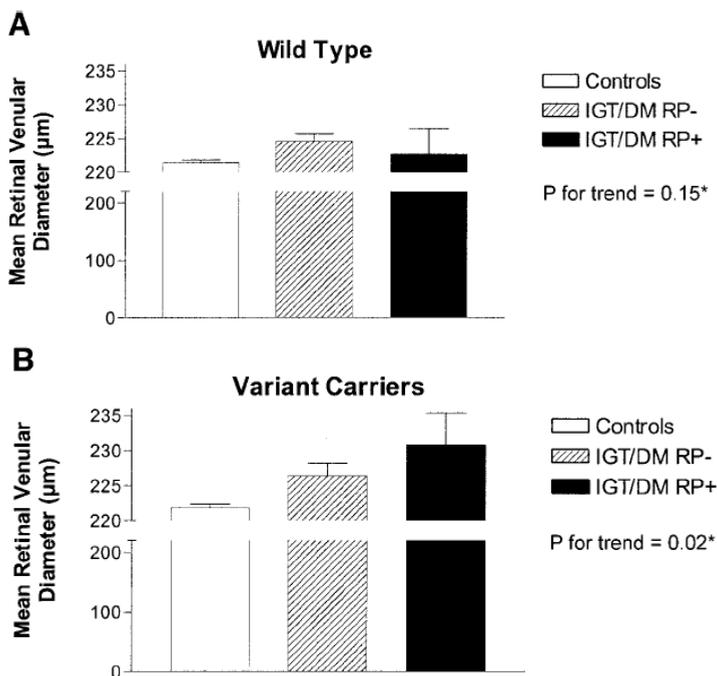
In participants with IGT or diabetes, the risk of retinopathy was significantly higher in variant carriers than in subjects with the wild-type genotype of this IGF-I gene promoter polymorphism. Thus, this suggests that variant carriers have an increased risk of retinopathy and opens the options that IGF-I gene variants are risk factors and/or markers for phenotypes related to overall IGF-I expression [14]. The IGF-I gene variant of an individual is fixed for life,



**Figure 1** Mean ( $\pm$  SE) baseline retinal arteriolar diameters comparing control subjects, participants with IGT/diabetes without retinopathy (IGT/DM RP -), and participants with IGF/diabetes with retinopathy (IGT/DM RP +) in subjects with the wildtype (A) and variant carriers (B). \*After adjustment for age and sex.

and the relationship between an IGF-I variant and a phenotype is not susceptible to potential confounding factors such as age, insulin deficiency, hyperglycemia and nutrition state. However, it is at present unknown whether this IGF-I gene polymorphism directly affects expression of IGF-I in vivo and mediates its effects through circulating IGF-I levels. No in vivo studies have been carried out demonstrating that this IGF-I gene promoter polymorphism is indeed associated with modified IGF-I gene expression. Variations in gene variants are traditionally difficult to study; subtle differences between gene variants may be present that are not detected by the presently available in vitro assays [15, 16]. Another unanswered question at the moment is whether this IGF-I gene promoter polymorphism is related to paracrine/autocrine IGF-I production in the body. This latter aspect could be very important because it is not only thought that the IGF-I system has important paracrine/autocrine actions but also that locally produced IGF-I may produce effects other than circulating IGF-I [17].

Although these differences did not reach statistical significance, variant carriers with IGT or diabetes who developed retinopathy at follow-up tended to have the largest retinal arteriolar and venular diameters (Figs 1B and 2B). In contrast, such a trend was absent in participants with the wild type with IGT or diabetes who developed retinopathy at follow-up (Figs 1A and 2A). Vascular dilatation of the retinal vessels, especially the retinal venules, is observed in the early stages of diabetic retinopathy [18]. Vascular dilatation is thought to indicate increased



**Figure 2.** Mean ( $\pm$  SE) baseline retinal venular diameters comparing control subjects, participants with IGT/diabetes without retinopathy (IGT/DM RP -), and participants with IGT/diabetes with retinopathy (IGT/DM RP +) in subjects with the wildtype (A) and variant carriers (B). \*After adjustment for age and sex.

retinal blood flow and is probably related to hyperglycemic hypoxia and reduced vascular tone [18]. In addition, larger arteriolar and venular diameters, independent of retinopathy severity level, have been associated with increased 4-year progression of retinopathy in type 1 diabetes [19, 20]. Moreover, larger venular diameters were positively associated with 4-year incidence of proliferative retinopathy [19]. Our study observed larger retinal diameters at baseline in variant carriers with IGT or diabetes, who developed incident retinopathy at follow-up and the absence of this trend in participants with the wild type may thus already point out at baseline an increased risk for progression of retinopathy in variant carriers than in participants with the wild type.

The low observed incidence of retinopathy in our study is probably related to the fact that we studied only field 2 of the standard diabetic fundus photographs. By studying only this field, we had no information on  $\sim$  50% of the retina and this will have contributed to an underestimation of the incidence of retinopathy (R. Klein, personal communication).

In conclusion, in individuals with IGT or diabetes, variant carriers of this IGF-I gene polymorphism had an increased risk of retinopathy compared with carriers of the wild-type genotype during a mean follow-up of 6.5 years. Variant carriers with IGT/diabetes, who developed retinopathy during follow-up, had a significant trend for larger retinal arteriolar and venular

diameters at baseline, while such trend was absent for subjects with the wild-type genotype with IGT or diabetes, who developed retinopathy at follow-up. Since arteriolar and venular diameters have been associated with an increased progression of retinopathy, our findings suggest that this IGF-I gene polymorphism may modulate the susceptibility and/or the progression of retinopathy.

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# 5b

**An insulin-like Growth Factor-I gene polymorphism modifies the risk of microalbuminuria in subjects with abnormal glucose tolerance**



Microalbuminuria (MA) is related to cardiovascular disease both in diabetic patients and non-diabetic subjects.

We investigated whether a polymorphism near the promoter region of the IGF-I gene was related to the development of MA.

For this study, 1069 participants of the Rotterdam study were selected (440 participants with an abnormal glucose tolerance (AGT); 220 participants with type 2 diabetes and 254 subjects with pre-diabetes, and 595 subjects with a normal glucose tolerance (NGT)).

787 subjects were carriers of the wild type IGF-I genotype (73.6%) and 282 subjects were variant carriers (26.4%) of this IGF-I gene polymorphism. Compared to subjects with NGT the risk for microalbuminuria was higher (Odds Ratio (OR): 3.1 (95% CI: 1.2–7.7);  $P = 0.02$ ) in variant carriers with AGT than in carriers of the wild type of this IGF-I gene polymorphism (OR: 2.2 (95% CI: 1.2–4.0);  $P = 0.009$ ). Compared with wild type carriers with AGT, the relative risk for MA was unadjusted and non-significantly increased in variant carriers with AGT (1.6; 95% CI: 0.8 –2.9). However, after adjustment for possible confounding factors (age, gender, mean blood pressure, fasting insulin, fasting glucose and smoking) this risk became significant (OR: RR 2.1; 95% CI: 1.1 – 4.4;  $P = 0.04$ ).

In subjects with AGT, a higher risk for MA was observed in variant carriers than in carriers of the wild type genotype of this IGF-I gene polymorphism. Since MA is primarily associated with cardiovascular disease in subjects with AGT, our study suggests that variant carriers have a higher risk for cardiovascular disease than carriers of the wild type when they develop an AGT.

## INTRODUCTION

The presence of microalbuminuria (MA) may be a marker of generalized vascular endothelial dysfunction reflecting generalized vascular disease [1, 2]. Development of MA is associated with glycaemic control, blood pressure, smoking, and male gender [3, 4]. MA may precede type 2 diabetes, occurring in parallel with the metabolic syndrome and its components, obesity and hypertension [1]. MA is also considered to be a marker for diabetic nephropathy in type 2 diabetes [5-7].

The insulin-like growth factor-I (IGF-I) system exerts multiple physiologic effects on the vasculature through both endocrine and autocrine/paracrine mechanisms [8]. Both macrovessel and microvessel endothelial cells express IGF-I receptor (IGF-IR; [9]). The expression of IGF-I in endothelial cells is low and might reflect mainly IGF-I sequestered from serum [10]. IGF-I has also been implicated in the development of cardiovascular disease [8, 11-13].

IGF-I has been further implicated in the development of diabetic nephropathy [14, 15]. IGF-I bioactivity is regulated by genetic and non-genetic factors like growth hormone, nutrition and insulin [16]. In humans, a cytosine-adenine (CA)<sub>n</sub> polymorphism in the promoter region of the IGF-I gene has been identified [17]. Studies of other genes have suggested that polymorphic CA repeats in the promoter region of a gene, affects transcription activity of a gene [18]. This polymorphism has thus the potential to influence directly the expression of IGF-I in the body [19].

Circulating IGF-I levels are often used as a substitute for tissue IGF-I bioactivity due to the lack of in vivo methods to measure the latter. However, determinations of circulating total IGF-I levels by radio-immunoassays may be especially problematic in pathological conditions, due to interferences of IGF-binding proteins. As a consequence, circulating IGF-I levels may not adequately reflect the IGF-I bioactivity. An alternative approach may be genetic association studies. In these studies, genetic variants can be treated as risk factors that cannot be influenced by secondary factors such as hypertension or hyperglycemia, unless these phenotypes are in the causal pathway. This opens the opportunity to characterize on a genetic basis, individuals who are at risk for MA. The aim of the present study was to investigate whether a polymorphism near the promoter region of the IGF-I gene was related to the development of MA.

## SUBJECTS AND METHODS

### *Study population*

All subjects included in the present study were participants of the Rotterdam Study. The Rotterdam Study is a single-center prospective follow-up study in which all residents aged 55 years and over of the Rotterdam suburb Ommoord were invited to participate. The Medical

Ethics Committee of Erasmus Medical Center Rotterdam approved the study and written informed consent was obtained from all participants. The aim of the Rotterdam study and the design of the study has been described previously [20]. The baseline examination of the Rotterdam Study was conducted between 1990 and 1993. A total of 7983 participants (response rate 78 percent) were examined.

Because of practical and financial reasons, only a proportion of the Rotterdam Study ( $n = 1069$ ) underwent a fasting glucose tolerance test. From this group we selected our cases and controls for a case-control study in which we assessed the relation between both IGF-I genotypes, glucose tolerance and albuminuria [21]. Criteria for the present study were age younger than 75 years, no use of anti-epileptics, corticosteroids, hormonal replacement therapy, cytostatics and people with dementia. After blood was drawn from the participants after an overnight fast, participants underwent an oral glucose tolerance test with 75 g glucose. Diabetic patients who were treated with anti-diabetic medication did not undergo a glucose tolerance test. Therefore, post load glucose measurements were only available for persons not using anti-diabetic medication. For the oral glucose tolerance test blood was drawn after 2 hours. Normal glucose tolerance (NGT) was defined as fasting glucose below 6.1 mmol/l and 2 hour post load below 7.8 mmol/l. Individuals with high glucose levels not meeting criteria for diabetes, but were too high to be considered normal were diagnosed as having prediabetes. Prediabetes includes persons with impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT). IFG was defined as fasting glucose between 6.1 and 7.0 mmol/l and IGT as a 2 hour post load glucose between 7.8 and 11.1 mmol/l. A diagnosis of diabetes mellitus was made if subjects were treated for diabetes or had a fasting glucose level of 7.0 mmol/l or above and/or a 2 hour post load glucose of 11.1 mmol/l or above [22]. All individuals meeting criteria for diabetes or prediabetes were to be considered as subjects with an abnormal glucose tolerance (AGT). To determine insulin sensitivity we used the homeostasis model assessment (HOMA) [23].

### **Measurements**

A questionnaire was used to collect data on glucose status, smoking status and use of anti-hypertensive medication. Blood pressure was measured in a sitting position at the right upper arm with a random-zero sphygmomanometer and the average obtained of two measurements at one occasion was used.

Blood sampling and storage have been described elsewhere [24]. Serum was separated by centrifugation and quickly frozen in liquid nitrogen. For the present study, we used blood measurements performed on fasting blood samples. Glucose levels were measured by the glucose hexokinase method (Medgenix Diagnostics, Brussels, Belgium) with an intra-assay variation of less than 2.5 and 6.0% respectively. Fasting insulin was measured by a commercially available assay (IRMA, Medgenix Diagnostics) with an intra- and inter-assay variation of 3 – 6 and 5 – 12 percent, respectively. Serum creatinine levels were assessed using an

automated enzymatic procedure (Roche, Mannheim, Germany). Creatinine clearance was determined by the formula of Cockcroft and Gault [25]. Serum cholesterol and triglycerides were determined by a standard laboratory method.

Participants collected timed overnight urine one day before the examination, albumin and creatinine were measured. Albumin and creatinine in urine were determined by a turbidimetric method and measured by a Hitachi 917 analyzer (Roche/Hitachi Diagnostics, Mannheim, Germany). Detection limit ranged from 2 to 400 mg/l and therefore all measurements below 2 mg/l were set at 1 mg/l. MA was defined as an albumin-to-creatinine ratio (ACR) of  $\geq 2.5$  mg/mmol in males and  $\geq 3.5$  mg/mmol in females [26]. From 34 subjects, no data of MA were available. Fourteen subjects (NGT: four subjects, AGT: ten subjects) met the criteria for macroalbuminuria, they were included in the analysis.

### ***Genotyping of the IGF-I promoter polymorphism***

The human IGF-I gene contains a polymorphic CA repeat 1 Kb upstream of the promoter region [27]. IGF-I genotypes were determined as described previously [17]. The most common allele contains 19 CA-repeats, which equals a length of 192-bp [28]. In a previous study, we examined the role of the various lengths of the alleles of the IGF-I promoter polymorphism and observed an optimum for IGF-I levels and body height [29]. Mean IGF-I levels and body height for homozygous carriers of the 192-bp allele, and homozygous carriers of the 194-bp allele of this IGF-I gene promoter polymorphism were equal and significantly higher than the values measured in subjects homozygous for alleles smaller than 192-bp and longer than 194-bp, respectively, suggesting an optimum for IGF-I expression by these two variants. Based on carrier ship of the 192-bp and 194-bp alleles, we therefore distinguished two different IGF-I genotypes in the present study: As wild type genotype we considered carriers homozygous for the 192-bp or for the 194-bp allele, and carriers heterozygote for these two alleles, participants with this genotype are further in the text denoted as carriers of the wild type. Participants with all other combinations of alleles were considered carriers of the variant genotype, which is further in the text denoted as variant carriers. 787 persons (73.6%) were carriers of the wild type and 282 subjects were variant carriers (26.4%).

### ***Carotid ultrasonography***

Carotid atherosclerosis was assessed by duplex scan ultrasonography of the carotid arteries, using a 7.5 MHz linear array transducer (ATL, Ultramark IV). Measurements of intima media thickness (IMT) were performed offline from the still images recorded on videotape. Details about this measurement have been published previously [30]. Briefly, the interfaces of the far and near walls of the distal common carotid artery are marked over a length of 10 mm. We used the average of the measurements of three still images of both the left and right arteries. Common carotid IMT was determined as the mean IMT of near and far wall measurements of both the left and right arteries. Results from a reproducibility study of IMT measurements

have been published elsewhere [31]. The mean differences $\pm$ S.D. in common carotid IMT between paired measurements of sonographers, readers, and visits were  $-0.04\pm 0.10$ ,  $0.066\pm 0.07$ , and  $-0.013\pm 0.13$  mm, respectively.

### Data analyses

Except when otherwise mentioned, data are presented as mean $\pm$ S.D. Diabetic and non-diabetic groups were compared with the Mann – Whitney test. Means of parameters were compared between genotypes after stratification for glucose tolerance using analyses of variance. Insulin, albuminuria and ACR did not meet the criteria for normality and were logarithmic transformed before analysis in order to obtain approximate normal distribution. Therefore results of these parameters are presented as geometric means with 95% confidence intervals (CI). Distribution of sex, use of antihypertensive drugs, lipid lowering drugs and frequency of the metabolic syndrome between genotypes was compared using a chi-square test.

Risk for MA was calculated by a binary logistic regression analysis and all results are presented unadjusted, and when explicitly mentioned, after adjustment for age, sex, mean blood pressure, fasting glucose and insulin levels and smoking. All analyses were performed using the SPSS for Windows software package, version 10.0.5 (SPSS Inc., Chicago, IL, USA).

**Table 1:** General characteristics of the study population, comparing subjects with normal glucose tolerance (NGT) and subjects with abnormal glucose tolerance (AGT) after stratification by IGF-I genotype

	NGT			AGT			P1
	Wild type	Variant type	P2	Wild type	Variant type	P2	
Number of subjects	444	151		343	131		
Age	66.2 $\pm$ 5.6	66.0 $\pm$ 4.8	0.67	67.7 $\pm$ 5.8	66.8 $\pm$ 5.8	<0.001	<0.001
Sex (M/F)	202/242	71/80	0.75	184/159	70/61	0.01	0.01
Creatinine ( $\mu$ mol/L)	89.3 $\pm$ 15.0	89.4 $\pm$ 13.9	0.93	92.2 $\pm$ 18.0	91.0 $\pm$ 17.9	0.61	0.27
Fasting glucose (mmol/L)	5.5 $\pm$ 0.4	5.4 $\pm$ 0.3	0.85	7.2 $\pm$ 2.0	7.3 $\pm$ 2.2	0.74	<0.001
2-h glucose post-load (mmol/L)	5.3 $\pm$ 1.2	5.3 $\pm$ 1.2	0.76	9.4 $\pm$ 3.9	9.2 $\pm$ 4.0	0.67	<0.001
Mean systolic bp (mmHg)	136 $\pm$ 21	135 $\pm$ 19	0.56	143 $\pm$ 20	142 $\pm$ 22	0.87	<0.001
Mean diastolic bp (mmHg)	77 $\pm$ 77	75 $\pm$ 11	0.52	78 $\pm$ 11	78 $\pm$ 12	0.67	0.002
Total cholesterol (mmol/L)	6.63 $\pm$ 1.06	6.54 $\pm$ 0.98	0.37	6.53 $\pm$ 1.13	6.46 $\pm$ 1.09	0.47	0.35
HDL-cholesterol (mmol/L)	1.33 $\pm$ 0.32	1.33 $\pm$ 0.32	0.88	1.22 $\pm$ 0.30	1.20 $\pm$ 0.30	0.48	<0.001
Triglycerides (mmol/L)	1.50 $\pm$ 0.66	1.44 $\pm$ 0.50	0.31	1.95 $\pm$ 1.10	1.96 $\pm$ 1.00	0.96	<0.001
BMI (kg/m <sup>2</sup> )	25.8 $\pm$ 3.1	26.0 $\pm$ 3.3	0.69	27.5 $\pm$ 3.6	27.4 $\pm$ 3.7	0.73	<0.001
Current smoking (%)	10.0	14.1	0.21	14.6	8.9	0.39	0.53
Antihypertensive drugs (%)	22.7	18.5	0.30	34.1	38.9	0.18	<0.001
Lipid lowering drugs (%)	5.0	2.6	0.24	7.3	6.1	0.67	0.08
IMT (mm)	0.75 $\pm$ 0.13	0.75 $\pm$ 0.12	0.69	0.78 $\pm$ 0.15	0.79 $\pm$ 0.11	0.51	<0.01
HOMA-IR	1.92 $\pm$ 0.06	2.05 $\pm$ 0.10	0.36	1.78 $\pm$ 0.07	1.78 $\pm$ 0.11	0.98	0.04

P1, p-value comparing subjects with NGT and subjects with AGT after adjustment for age and sex; P2, p-value comparing wild type with variant type adjusted for age and sex; HOMA-IR, homeostasis model assessment of insulin resistance.

## RESULTS

Table 1 presents clinical characteristics of 1069 subjects (595 subjects with NGT and 474 subjects with an AGT (254 subjects with pre-diabetes and 220 subjects with diabetes) stratified by IGF-genotype. Subjects with AGT were older and most of them were male in comparison to subjects with NGT (Table 1). Serum creatinine, blood glucose and cholesterol levels, blood pressure and body mass index (BMI) were higher in subjects with AGT than in subjects with NGT (Table 1). Subjects with AGT frequently used more antihypertensive drugs, had larger intima media thickness and were more insulin resistant than subjects with NGT. There were no differences in general characteristics between carriers of the wild type and variant carriers of this IGF-I gene promoter polymorphism in the group of NGT or AGT (Table 1).

**Table 2:** Relation between IGF-I genotype and parameters of renal function comparing subjects with NGT and subjects with AGT. Data are mean (95% CI) unless otherwise stated.

	NGT			AGT			P1
	Wild type	Variant type	P2	Wild type	Variant type	P2	
Number of subjects	441	151		343	131		
Albuminuria (mg/l)*	2.1 (1.9 – 2.4)	2.1 (1.8 – 2.5)	0.97	2.9 (2.5 – 3.3)	3.6 (2.9 – 4.7)	0.09	<0.001
Creatinuria (mmol/l)	7.7 (7.4 – 8.1)	7.5 (6.8 – 8.1)	0.45	8.1 (7.7 – 8.6)	8.2 (7.5 – 8.9)	0.86	0.20
Albumin-to-creatinine ratio (mg/mmol)*	0.32 (0.29 – 0.37)	0.34 (0.28 – 0.40)	0.70	0.41 (0.35 – 0.48)	0.52 (0.40 – 0.66)	0.12	0.002
Creatinine (μmol/l)	89 (88 – 91)	89 (87 – 91)	0.93	92 (90 – 94)	91 (89 – 94)	0.61	0.27
Clearance (ml/min)	68 (66 – 69)	67 (65 – 69)	0.71	69 (68 – 71)	70 (67 – 72)	0.75	<0.001

\* Geometric mean (95% CI); P1, p-value comparing subjects with NGT and subjects with AGT after adjustment for age and sex; P2, p-value comparing wild type with variant carriers adjusted for age and sex.

Table 2 shows the relation between the two IGF-I genotypes and parameters of renal function, comparing subjects with AGT to subjects with NGT. Subjects with AGT had significantly higher urinary albumin concentrations and ACRs than subjects with NGT. In addition, mean serum creatinine levels, creatinuria and clearance were higher in subjects with AGT than in subjects with NGT. In variant carriers with AGT, mean albuminuria and ACR were higher than in carriers of the wild type, but these differences did not reach statistical significance.

Subjects with an AGT had an increased risk of developing MA (Odds Ratio (OR): 2.5; 95% CI: 1.5 – 4.0; P = 0.001) in comparison to subjects with NGT. Compared to subjects with NGT, the risk for MA was higher (OR: 3.1; 95% CI: 1.2 – 7.7; P = 0.02) in variant carriers with AGT, than in carriers of the wild type of this IGF-I gene polymorphism (OR: 2.2; 95% CI: 1.2–4.0; P = 0.009; Fig. 1).

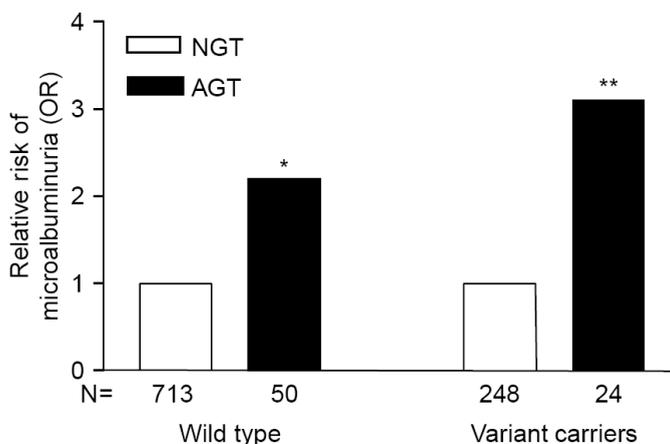
Table 3 shows the prevalence and relative risk of MA in subjects with AGT and subjects with NGT stratified for IGF-I genotype. Variant carriers with AGT had a higher prevalence of MA than carriers of the wild type of this IGF-I gene polymorphism, subsequently the risk of MA for variant carriers was investigated. In subjects with AGT, the relative risk for MA was non-sig-

nificantly increased in variant carriers when compared with carriers of the wild type: (Relative Risks (RR)): 1.5; 95% CI: 0.8 – 2.9; P = 0.19). When this analysis was repeated after adjustment for age, gender, fasting insulin, fasting glucose, mean blood pressure and smoking, the risk of MA in variant carriers with AGT became significant: (RR: 2.1; 95% CI: 1.1–4.4; P = 0.04). Compared with carriers of the wild type genotype, the relative risk for MA also increased in variant carriers with NGT after these adjustments, but it still remained not significant (RR: 1.6; 95% CI: 0.6 – 4.0; P = 0.35).

**Table 3:** The relative risk of microalbuminuria of subjects with AGT and subjects with NGT stratified by IGF-I genotype. Number of subjects for each category is given (% of cases and controls). Risks are given with a 95% confidence interval between brackets.

	Wild type	Variant type
Subjects with AGT		
Microalbuminuria	31 (9.3%)	17 (13.5%)
No microalbuminuria	303 (90.7%)	109 (86.5%)
Relative risk	Reference	1.5 (0.8 – 2.9)*
Relative risk	Reference	2.1 (1.0 – 4.4)**
Subjects with NGT		
Microalbuminuria	19 (4.4%)	7 (4.8%)
No microalbuminuria	410 (95.6%)	139 (95.2%)
Relative risk	Reference	1.1 (0.45 – 2.64)*
Relative risk	Reference	1.6 (0.61 – 4.03)**

\* Unadjusted; \*\* After adjustment for age, gender, mean blood pressure, fasting glucose, fasting insulin and smoking.



**Figure 1.** The relative risk of microalbuminuria comparing subjects with NGT and AGT per IGF-I genotype (wild type, left; variant carriers, right). Individuals with NGT were used as the reference group. \*p=0.009, \*\*p=0.02 vs the reference group.

## DISCUSSION

Compared to subjects with NGT, variant carriers with AGT had a higher risk to develop MA than carriers of the wild type genotype. In addition, variant carriers with AGT had a borderline and significantly higher prevalence of MA than carriers of the wild type genotype. These findings became significant after adjustment for a number of possible confounding factors, which have been previously found to be involved in the development of MA. Our findings suggest that this IGF-I gene polymorphism modulates the susceptibility and/or progression of MA as soon as a person develops AGT.

Compared with carriers of the wild type genotype, we observed that the increased risk for MA in variant carriers was relatively small. At first glance, this suggests that the role of this IGF-I gene polymorphism in the development of MA is not very important. However, the susceptibility to MA results probably from an interaction of multiple genetic and environmental factors. Consequently, the relationship between the prevalence of MA (phenotype) and any individual causal locus (genetic variant) will in general be fairly weak, even for major genes. MA will only develop when certain other risk factors are also present, such as hyperglycemia, hypertension and/or smoking. This may have obvious complications for both the number of subjects in the study and the power of analyses that aim to detect individual genetic signals among other genetic and environmental background. This may explain why we observed only a relative small and significant increase in the risk of MA in variant carriers with AGT, compared with carriers of the wild type genotype of this IGF-I gene polymorphism after adjustment for some possible confounding factors.

The number of subjects with MA was low in our study which might have further attributed to a reduced statistical power of our study. Although the prevalence of MA in our study was low, this prevalence does not differ from previous findings in another big Dutch population-based study [32]. In this latter study the prevalence of MA was 6.6% in non-diabetic subjects and 16.1% among diabetic patients. However, in this latter study, the diagnosis of MA was exclusively based on urinary albumin concentrations, while in our study this diagnosis was based on ACRs. A limitation of our study is that MA was assessed on one overnight sample. It is well known that there is considerable intra-individual variability in urinary albumin excretion in time [33]. As a consequence, the prevalence of MA in our study may have been significantly both underestimated as well as overestimated.

Another limitation of our study may be that there is not only an age-related penetrance of AGT, but also an age-related penetrance of MA. Subjects with a NGT at the time of the study may still develop both an altered glucose tolerance and MA in the near future. We previously observed that variant carriers of this IGF-I gene polymorphism have an increased risk of developing diabetes, in comparison to carriers of the wild type genotype. Thus, this suggests that especially in variant carriers with AGT, the risk for MA may have been underestimated. Moreover, the diagnostic umbrella MA probably gathers individuals who have developed MA

through a variety of pathological mechanisms (see below). This has obvious implications for the interpretation of our findings.

We did not find relationships between this IGF-I gene promoter polymorphism and renal function parameters, this was not unexpected. In subjects with type 2 diabetes MA is generally considered a stronger predictor of cardiovascular disease than it is of the risk of end-stage renal failure [34]. Type 2 diabetes patients with MA are at an increased risk of cardiovascular death compared with patients with normal albuminuria [35, 36]. In addition, in non-diabetic subjects, MA is even considered an independent risk factor to developing cardiovascular disease [3, 32]. The observed relationship between IGF-I genotype and MA in our study thus probably points mainly to an increased risk of variant carriers for cardiovascular disease. This is in accordance with our previous findings: we observed that variant carriers had a higher risk of developing myocardial infarction [17]. In addition, carotid IMT and aortic pulse wave velocity were significantly increased in variant carriers with hypertension [37].

MA may be the first sign that the vascular vessel wall, particularly the endothelium, is injured. It has been found that MA can be the specific consequence of a reduction in the fixed negative charges of the glomerular wall [38, 39]. Whether an IGF-I gene genotype of a subject may modulate the susceptibility and/or progression of MA by circulating IGF-I or rather via its effects at the tissue level is at present unknown. However, it is likely that a link should be found in alterations in the composition of the basal membranes of the capillaries and of the extracellular matrices, which has also been hypothesized for type 1 diabetes.

When our findings will have been replicated by others, these observations may suggest the existence of new etiological pathways for the development of MA, and together, provide some prediction of who will or will not get MA. In addition, it has been suggested that even if the risk is imparted by a certain genotype is low, or that only a subset of subjects carry the predisposing genotype, modulation of that gene by pharmacological intervention may be beneficial [40].

In conclusion, we observed in patients with AGT a higher risk for MA in variant carriers than in carriers of the wild type genotype of this IGF-I gene promoter polymorphism. Since MA is primarily associated with cardiovascular disease in subjects with type 2 diabetes, our study suggests that variant carriers with an AGT have an increased risk for cardiovascular disease.

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# 6

**An insulin-like Growth Factor-I promoter polymorphism is associated with increased mortality in subjects with myocardial infarction in an elderly Caucasian population**





We investigated whether an insulin-like growth factor I (IGF-I) promoter polymorphism is associated with excess mortality in elderly subjects with myocardial infarction (MI). This association was assessed in 7983 subjects of the Rotterdam Study during 14 years of follow-up. Among 345 subjects who developed a MI, the risk of mortality was 1.49 times higher in the variant carriers of the IGF-I promoter polymorphism than in the nonvariant carriers (95% confidence interval 1.10 to 2.10,  $p = 0.02$ ). The risk of death increased with the number of variant alleles. Our study suggests that genetically determined low IGF-I activity is an important determinant of mortality in subjects with MI.

Insulin-like growth factor I (IGF-I) plays an important role in regulating myocardial structure and function and promoting cardiac muscle growth, differentiation, and survival during myocardial ischemia [1, 2]. In addition to its anabolic properties, it has been suggested that IGF-I plays an important role in the development of cardiovascular diseases (CVDs) [1]. Subjects with low circulating IGF-I levels are at significantly higher risk of developing CVD [2]. Substantial genetic contributions to human blood IGF-I levels were first reported in 1996 [3]. A polymorphism in the IGF-I promoter region that may influence IGF-I production has been identified [4]. This polymorphism is also associated with circulating IGF-I levels [4]. We previously observed an increased risk of myocardial infarction (MI) in the absence of the 192-bp allele of the IGF-I promoter polymorphism [4]. We investigated whether this polymorphism is involved in the survival of subjects with incident MI and in the total population. The study was embedded in the Rotterdam Study, a population-based cohort study of 7983 subjects who were  $\geq 55$  years of age. Baseline data were collected between March 1990 and July 1993. The study was approved by the medical ethics committee of Erasmus University (Rotterdam, The Netherlands). Written informed consent was obtained from all participants. Data of the incident MI were verified by research physicians who collected information from patients' medical records. Information also included copies of discharge letters for hospital admissions. A MI was coded according to criteria of the *International Classification of Diseases, 10th Edition*. Follow-up data on overall mortality were available until January 1, 2004. CVD-specific mortality was based on data until January 1, 2000. In total, 7012 subjects were successfully genotyped for the IGF-I promoter polymorphism. We previously observed that circulating serum IGF-I levels are highest for subjects with 192- and 194-bp alleles, whereas IGF-I levels are lower in noncarriers of the 192- and 194-bp alleles [5]. For the present study, we used the same approach, which distinguishes 2 genotype groups: nonvariant carriers (homo- and heterozygous for the 2 common alleles) and variant carriers [5]. As nonvariant carriers, we included all subjects who were homozygous for the 192-bp genotype or homozygous for the 194-bp or the 192-bp/194-bp genotypes [5]. As variant carriers, we included subjects who were heterozygous for the 192-bp genotype, heterozygous for the 194-bp genotype, or noncarriers of these 2 alleles. We distinguished 2 subgroups within the variant carriers: heterozygous carriers (192-bp/- and 194-bp/- genotypes) and noncarriers of the 192- and

194-bp alleles (–/– genotypes). The effect of the IGF-I promoter polymorphism on survival was evaluated using Cox's proportional hazard analysis.

Mean age  $\pm$  SD of the study population was  $69.64 \pm 9.30$  years, and 60% were women. Mean body mass index was  $26.31 \pm 3.96$  kg/m<sup>2</sup>. At baseline, diabetes mellitus was present in 9% of the total population. Over a mean follow-up of 9.49 years, 345 subjects developed a MI, including 82 subjects with a previous MI. CVD-specific mortality accounted for 36.20% and 73.10% of total mortality in the total population and subjects with MI, respectively. Relative risk of mortality in subjects with incident MI and in the total population is presented in Table 1. In subjects with a MI, variant carriers had a 1.49 times higher risk of mortality compared with the nonvariant carriers (95% confidence interval 1.10 to 2.10,  $p = 0.02$ ). When we considered CVD-specific mortality, the result was similar to the overall mortality (hazard ratio 1.42, 95% confidence interval 0.92 to 2.19,  $p = 0.11$ ). No association was found between IGF-I promoter polymorphism and mortality in the total population (Table 1).

Figure 1 presents survival curves for subjects who developed a MI and in the total population. Noncarriers and heterozygous carriers of the 192-bp/194-bp alleles had higher mortality rates compared with nonvariant carriers after adjustment for gender and age. Mortality increased with the number of variant alleles ( $p$  for trend = 0.03; Figure 1). In subjects with a MI, risks of mortality were 1.42 in the heterozygous carriers (95% confidence interval 1.01 to 2.00,  $p = 0.04$ ) and 2.10 in the noncarriers (95% confidence interval 0.81 to 5.21,  $p = 0.13$ ) compared with nonvariant carriers after adjustment for age, gender, cardiovascular risk factors, and baseline history of CVD. Excluding 82 subjects with a previous MI did not change the results. In contrast, no significant association was found between IGF-I promoter polymorphism and survival in the total population ( $p$  for trend = 0.38; Figure 1).

The IGF-I promoter polymorphism has been associated with circulating IGF-I levels [4]. Our study suggests that subjects with a MI who are variant carriers of the IGF-I promoter polymorphism have an increased risk of mortality. We found no association between survival and IGF-I promoter polymorphism in the total population during the 14-year follow-up. We previously observed that noncarriers of the 192- and 194-bp alleles have lower circulating IGF-I levels [5] and a higher risk of incident heart failure [6]. Our findings suggest that absence of the 192-bp allele and/or 194-bp allele is particularly important in those who have developed vascular pathology. This is in line with previous studies that have suggested that IGF-I levels are markedly decreased in the early phases of a MI, which contributes to a poor outcome after a MI [2]. IGF-I may be a determinant of survival because it promotes survival of cardiomyocytes that are affected by ischemia [2].

Cardiomyocytes are highly differentiated cells that typically do not replicate after birth. Apoptosis in these cells results in myocardial function loss and contributes to the development of heart failure [1]. One of the most important effects of IGF-I is its ability to counteract apoptosis of cardiomyocytes. In various animal models of myocardial ischemia, IGF-I suppresses myocardial apoptosis and improves myocardial function. These studies suggest that

administration of IGF-I might decrease myocardial apoptosis and therefore the size of the infarction [7]. In contrast, IGF-I may also increase cardiac output and myocardial contractility [2], which may contribute to improved survival after

**Table 1:** Relative risk of mortality in insulin-like growth factor 1 variant carriers versus nonvariant carriers

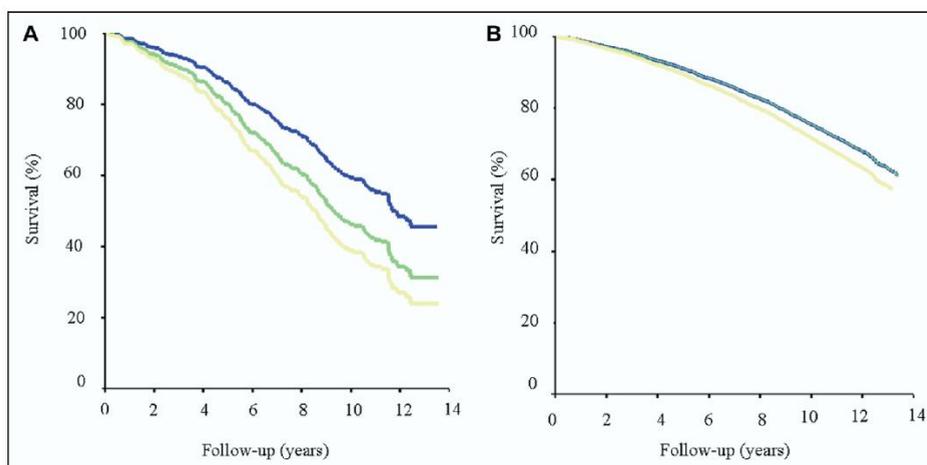
	Incident MI				Total Population			
	Total	Death	Hazard ratio (95% CI)		Total	Death	Hazard ratio (95% CI)	
			Model 1*	Model 2 <sup>†</sup>			Model 1*	Model 2 <sup>†</sup>
Variant carriers	101	64	1.50 (1.11-2.04) <sup>§</sup>	1.49 (1.10-2.10) <sup>§</sup>	1930	729	1.01 (0.93-1.11)	1.01 (0.92-1.10)
Nonvariant carriers	244	125	1.00 (reference)	1.00 (reference)	5082	1859	1.00 (reference)	1.00 (reference)

\* Adjusted for gender and age

<sup>†</sup> Adjusted for age, gender, body mass index, systolic and diastolic blood pressures, serum total cholesterol, serum high-density lipoprotein cholesterol, type 2 diabetes, smoking and baseline history of CVD.

<sup>§</sup> p = 0.01 versus nonvariant carriers; <sup>§</sup> p = 0.02 versus nonvariant carriers

CI = confidence interval



**Legend to figure 1:**

**Figure 1:** Survival curves for (A) subjects with incident MI and (B) the total population as stratified by IGF-I genotypes, i.e., nonvariant carriers (black line) and heterozygous carriers (dark grey line) and noncarriers (light gray line) of the 192- and 194-bp alleles, and adjusted for gender and age

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# 7

## General discussion



This thesis describes an investigation of the role of a (CA)<sub>n</sub> polymorphism near the promoter region of the IGF-I gene and associated (patho-)physiological conditions. The relationship between the IGF-I gene and serum IGF-I levels has been studied extensively in normal controls and also in relation with several disease states. Since IGF-I functions in an autocrine, paracrine and endocrine way, the local effect is difficult to examine. By studying the IGF-I genotype we tried to overcome this shortcoming. One of our aims was to investigate if the number of CA repeats was related to the total IGF-I level. This relationship could thereafter be used to study the relationship between IGF-I and physiological mechanisms, e.g. body height. Subsequently, this relationship was used to explore whether there was an association with beta cell function and macrovascular complications in patients with type 2 diabetes mellitus.

## CLINICAL IMPLICATIONS

In this thesis, IGF-I genotyping was used as a marker for total serum IGF-I levels to examine the relationship between functional properties like height and age-related decline of IGF-I as well as the development of diabetes and diabetic macrovascular complications. This was done in order to unravel a bit further the relationship between IGF-I and GH on the one hand, and the complex etiology of diabetes mellitus on the other. In our studies, IGF-I genotypes were associated with body height, age-related decline of IGF-I, and the development of diabetes and macrovascular complications, suggesting that IGF-I is indeed an important factor in these conditions.

As suggested before by Vaessen et al, the wild type alleles of the IGF-I gene are associated with the highest total IGF-I serum levels [1]. Instead of only one wild type allele, there seems to be an optimum for two wildtype alleles; the 192-bp allele ((CA)<sub>19</sub>), as well as the 194-bp allele ((CA)<sub>20</sub>). Persons with these "wildtype" IGF-I genotypes associated with the highest IGF-I levels were found to be taller and to show the well-known age-related decline in serum IGF-I levels. Our findings regarding IGF-I serum levels were replicated by only a few others [2]. Other investigators did not find the same relationship; homozygous carriers had equivalent [3-6] or lower [7-10] IGF-I serum levels. One study group found different relations in several ethnic groups [11]. An overview of study populations and their outcome is presented in table 1. In normal individuals, non-carrier state of the 192/194-bp alleles is associated with lower first and second phase insulin secretion, suggesting that people with low IGF-I serum levels are more susceptible to develop diabetes. A recent study demonstrated that persons with normal glucose tolerance (defined by glucose < 5.6 mmol/L) and lower IGF-I levels had lower HOMA-IR values [12]. In patients with diabetes, non-carriers of the 192/194-bp alleles have a higher risk to develop micro-albuminuria and retinopathy. In individuals with type 1 diabetes, the same relation between the IGF-I genotype and risk to develop micro-albuminuria (MA) was recently demonstrated in a Danish population [13]. In this study, variant carriers had an increased risk to develop persistent MA compared with subjects of the wild type, despite a similar metabolic control in the first 5 years of diabetes [13]. Protective effects of IGF-I on the kidney were demonstrated by a study of Zandbergen et al, where ACE-inhibitors raised IGF-I serum levels and were thought to protect diabetic kidney repair cells from ischemic injury and to accelerate tissue repair and recovery of renal function [14].

Also in the diabetic population, the risk of mortality after a myocardial infarction was significantly higher in variant carriers. Low circulating IGF-I levels can explain this relation, since they are involved in angina pectoris, atherosclerosis and the development of ischemic heart disease, even after correction for insulin sensitivity [15, 16]. Furthermore, in a prospective case-control study low levels of IGF-I were found in the acute phase of myocardial infarction [16]. A recent study demonstrated that low IGF-I serum levels on admission and in the acute

phase of myocardial infarction were related to a poor prognosis [17]. This suggests a potential beneficial effect of IGF-I therapy.

In GH deficient subjects, IGF-I therapy is currently more and more accepted. For children with severe primary GH insensitivity IGF-I therapy is already approved [18]. The effect of IGF-I therapy was reviewed in 4 large study groups, which all demonstrated an increase in 5-year growth rate from 3-5 cm/year pre-treatment to 8-9 cm/year during treatment in the first year. In subsequent years this effect attenuated [19]. Adverse effects observed included spontaneous hypoglycemia in 50% of the cases, which diminished when IGF-I therapy was given during meals, hyperstimulation of lymphoid tissue growth (e.g. tonsillar growth) and transient myalgia and arthralgia [19]. IGF-I therapy is not used in other disease states, but this is likely to happen in the future, e.g. in diabetics and after myocardial infarction. Recently, combination therapy with GH and IGF-I has been proposed as it achieves higher IGF-I serum levels and IGFBP-3 levels, it alters IGF-I clearance, counteracts disadvantageous effects on glucose metabolism, optimizes effect of IGF-I on bone tissue and improves tissue IGF-I levels [20]. Further studies are needed to examine the above mentioned possible mechanisms, but also to investigate side effects and long-term effects [20]. In addition, benefits of IGF-I therapy should be tested in diabetics with low IGF-I levels.

## **LIMITATIONS**

The studies performed were carried out in a population-based investigation because large numbers of individuals increase the generability of the results. Population based studies are in general mostly used in the discovery of susceptibility loci and can be subsequently followed by genetic association studies in cases. Before performing an association study, it is important to have evidence that genetic variation plays some role in determining the phenotype based on heritability [21]. In complex diseases, prior odds associated with any given gene can rarely be exactly calculated [22]. Association studies test whether a genetic marker occurs more frequently in cases than in controls [23]. If a significant association emerges and the possible bias of population stratification is excluded, the polymorphism itself is either in the susceptibility locus or in linkage disequilibrium with the susceptibility locus [23].

Positive results of genetic association studies have frequently not been replicated in subsequent studies. Several factors may contribute to these problems: heterogeneity in the phenotype, the methodology used and chance finding. The relation we observed between IGF-I genotype and IGF-I levels could not be replicated by others, due to the above mentioned factors. We will comment on these confounders below.

### ***A Heterogeneity***

Confounding factors in this type of study are phenotypic differences in the cases due to variable definitions for cases and controls, difference in skills of the investigators and phenotypic heterogeneity of the disease [23, 24]. In the case of type 2 diabetes, many apparently healthy people have diabetes without knowing they are affected. Calculating risk ratios based on the frequency of IGF-I genotypes may underestimate the relation. Development of glucose intolerance is caused by alterations in beta cell balance. Insulin sensitivity index (ISI) and disposition index (DI) are sensitive measures to detect disturbances. In a study of normal glucose tolerance (NGT) subjects who were relatives of type 2 diabetics and IGT subjects in a cohort in the Netherlands, non-carriers of the 192-bp alleles were associated with lower ISI and DI, conforming the relation between the IGF-I gene and risk to develop diabetes [25]. In the recently published Danish study population, no relation was observed between the IGF-I polymorphism and IGF-I levels, possibly due to loss of endogenous insulin production and/or the subcutaneous administration of insulin which may have masked genetically mediated differences in circulating IGF-I levels [13].

### ***B Methodology***

Failure of replication may be due to heterogeneous study populations based on ethnicity, selection of cases and controls and age difference.

Differences in the genetic background of cases and controls may lead to difference in outcome between a polymorphism and disease [21, 23, 24]. Regarding the IGF-I gene, the most frequent alleles in older ethnic groups are shorter compared to the Caucasian population [5, 26]. In the mentioned studies, some study populations had included at least 50% of subjects of a different ethnic background compared to our study [4, 5, 10, 11]. It is still unknown what the effect is of different IGF-I alleles across different ethnic groups. Mean IGF-I levels in longer existing ethnic groups are lower, but the reason why remains unclear [11, 27]. One might argue that this is the result of coding for different mRNA classes [28-30]. Since they all give rise to the same mature IGF-I protein, IGF-I is synthesized as a precursor molecule. Several studies have shown that there are multiple mRNAs due to transcription from tandem promoters, alternative RNA splicing and differential RNA polyadenylation [28, 29, 31]. It is still unknown what the effect is of different mRNA classes on function and synthesis.

Selection of the study population determines outcome of IGF-I levels, since levels are influenced by many factors like age, nutrition, liver disease and certain drugs e.g. estrogen replacement therapy [32-36]. Many of the studies regarding IGF-I genotype and levels were performed in controls of incident breast- or prostate cancer cases [5, 10]. Compared with our study population, these controls were selected in a hospital setting and may have complaints related to prostate or breast cancer. This type of confounding is called confounding by indication and may have blurred the true relationship between genotypes and levels.

IGF-I levels are known to decrease with age [35]. In our observation, this was only seen in homozygous carriers of the (CA)<sub>19</sub> repeat allele, suggesting that IGF-I serum levels become less GH dependent with age. The relationship between IGF-I genotypes and serum levels are therefore strongly dependent on age. A few studies examined the relation in younger cohorts [6, 8, 13], where age might interact with this relation. Since the mean age in these studies is approximately 30 years lower compared to the Rotterdam study, it is possible that the effect of this polymorphism on clinical endpoints is abolished by environmental factors. Later in life, the effect of the polymorphism becomes more and more important.

Methodological differences e.g. methodological artefacts, lack of specificity and insufficient number of cases also influence results [23]. One of the most reported methodological problems is multiple testing [21]. Another methodological deficit arises from technical problems in genotyping. Genotyping error or missing genotypes can over- or underestimate frequency of alleles, which will lead to a problem when concerning rare alleles [21, 22]. Statistical programs assume normality, but if the trait is not normal, outliers with extreme values can influence the result [21]. In every study population, missing IGF-I genotypes exist depending on method used and interpretation of the investigators. In two studies, either no homozygous carriers of the 19 CA repeat allele were observed [5] or only a few [4]. This can be another reason for the differences observed between the IGF-I gene and IGF-I serum levels.

There are several assays for the measurement of total IGF-I, which are used in the studies discussed. Radioimmunoassay (RIA) has been used for screening for the presence of growth hormone deficiency and for establishing a diagnosis of GH excess states, but in large clinical populations the observed results for patients with well established diagnoses were less than optimal [37]. Another problem regarding analytical problems is the separation of IGF-I from its binding proteins, as well as the relatively large inter- and intra-assay variation [37, 38]. Several assays focussed on eliminating binding-protein interference. One of the assays, immunoradiometric (IRMA), can be divided into non-extraction methods and methods involving extraction f.e. acid-ethanol extraction [37]. Disadvantage of this method is that the supernatant contains residual concentrations of IGFBP-1 and IGFBP-4 with variation in amount which in some cases may lead to a serious confounding problem [37]. Another extraction method utilized is hydrophobic interaction chromatography, which extracts almost 100% of IGFBP's, although some of the IGF-I is lost in the sample [37]. After extraction, IGF-I is measured with a detection antibody, which binds to a binding site of IGF-I. Chemiluminescence has been introduced which enhanced sensitivity [37]. Besides problems with different assays, interpretation of IGF-I levels can be difficult since there is a biological variability within a given patient varying from 5 to 37%, mostly due to nutritional intake [37].

Directly comparing above mentioned assays revealed in general higher IGF-I levels with RIA [38]. Other assays include ligand immunofunctional assay, KIRA and measurement of free IGF-I [38]. Immunofunctional assay was developed as means of detecting the ratio of IGF-I to intact IGFBP-3 and an index of the fraction of IGF-I that was exchangeable but was also

bound to IGFBP-3 [38]. The amount of radiolabeled IGF-I that binds intact IGFBP-3 is detected [38]. With this method, in diabetics a high amount of bioactive IGF-I can be detected, but this was not the case in subjects with GHD [38].

In 1999, a kinase receptor activation assay was introduced, utilizing purified IGF-I receptor to determine the ability of IGF-I to activate receptor protein kinase [39]. Recently, a study was performed which compared the KIRA assay with 2 RIA assays, IRMA, and two chemiluminescence immunometric assays [40]. Only the KIRA assay showed normal distribution of the IGF-I levels and lower variation [40]. Since free IGF-I is IGF-I in the bioactive form, measurements of free IGF-I were performed [38, 41]. However, measurement of total IGF-I levels remained superior, since assays for free IGF-I were sensitive to room temperature and incubation time [42]. When using free IGF-I in studies, one must be sure to have stored immediately serum samples, because of rapid proteolysis and therefore unreliable results already when there is delay of 2 hours [43].

Difference in storage of IGF-I leads to differences in outcome; samples stored at  $-80^{\circ}$  gave similar results compared to freshly collected samples, while stored at  $-20^{\circ}$  gave different results in different assays [44]. Especially the IGF-I IRMA assay didn't correlate compared to levels measured in fresh plasma samples [44].

Presence of non-19 CA allele is rare, so finding a true association will need a study with sufficient subjects. In the study of DeLellis, only in a subset the relation between the IGF-I gene and IGF-I levels was tested [11]. Also the study of Kato was performed in a population with a minimal number of subjects [5]. Small sized studies deviate from population characteristics since a wider range in genotype frequencies is observed.

### ***C Chance finding***

Spurious chance finding is the most likely explanation for difficulty in replication [24]. Chance finding is an issue in subgroup analyses, since the number of possible subgroup analyses that can be undertaken is large [24]. Sufficient sample size is needed to obtain statistical power to detect a given association and depends on relative risk and frequency of the allele [22]. Type 2 diabetes is highly prevalent and therefore population-based studies are able to enroll a sufficient number of diabetic cases for association studies.

## **FUTURE PERSPECTIVES**

The relation we observed between IGF-I genotypes and IGF-I serum levels was not found by other investigators, most likely due to differences in the study population and the methods used. Also it is possible that our observation was a false positive result due to selection in survival, since our cohort consisted of subjects of 55 years and over, who were physically able to visit our research Center. In order to define the true relationship, confirmation studies

should be performed in similar study populations. Since we observed that the age-related decline in IGF-I levels was only present in homozygous carriers of the 19CA repeat allele, it is likely that this relation is different in various age groups. Therefore, the associations should be studied (and confirmed) in more age categories. Finally, the classification of the IGF-I genotypes should be done in a more physiological way; namely based on the biologic effects. Based on our study this means variant carriers vs. wildtype alleles.

In the past years, it has become clear that developing complex diseases like diabetes mellitus is influenced by a combination of environmental and genetic factors. Currently more emphasis is put on the role of genetic factors, to understand the pathophysiology of the diseases. To determine which genes play a role in pathophysiology two genetic approaches can be used: a candidate gene approach and genome wide screen. For the candidate gene approach, a relation with the disease is already expected based on the protein the gene is coding for. In case of IGF-I, it's easy to imagine that because of functional and physiological homology with insulin, a role in development of diabetes is suspected. Genome wide association studies are being performed, because they can detect a subtle effect of common genetic variants. In this type of genetic study, no pre-selection of genes is made, which allows investigators to detect the effect of multiple gene variations on several diseases [45]. Genome wide association studies already detected several genes which are related to the development of type 2 diabetes [46]. The biological importance of these genes needs to be established, before we can determine the relevance of the IGF-I gene between and in interaction with other genes. It would be clarifying if we could perform IGF-I serum measurement in all diabetics in early stage of the disease and later on to determine the effect or consequence of IGF- serum levels in development or progression of the disease.

## **CONCLUSION**

The studies described in this thesis demonstrated an allele specific effect on IGF-I levels. IGF-I genotypes related to the lowest IGF-I levels in our study population, were more susceptible to develop diabetes, had a higher risk to develop diabetic retinopathy and micro-albuminuria. Also after myocardial infarction, survival was worse. The relation between the IGF-I gene and total serum IGF-I levels was not replicated by others, due to various differences in the study population, which made it hardly possible to compare. Similar studies should be conducted to see if our results could be replicated. Furthermore, this will give information on the relation of the polymorphism and levels during aging. Eventually, relation with disease states can be established. Finally, effect size of risk alleles should be compared with other genes involved in type 2 diabetes.

**Table 1:** Relation of IGF-I gene, based on carrierhip of 192-bp allele/19 CA repeat allele, and circulating total serum IGF-I levels in several studies

	<b>Selection of study population and ethnic background</b>	<b>N</b>	<b>Years of data collection</b>	<b>Mean age (range)</b>	<b>IGF-I levels in IGF-I genotypes</b>	<b>% IGF-I genotypes<sup>1</sup></b>	<b>IGF-I assay</b>
Vaessen, 2001 [1]	Caucasian population, selected on IGF-I genotype	150	'90 - '93	65.9 (5.9)*	X/X highest	44.5-43.3-12.2	RIA
Jernstrom, 2001 [6]	Caucasian. University of Toronto, Center for Birth control, community of Toronto	311		25.4 (1.7 - 3.5)	Similar	Data not given	ELISA
Kim, 2002 [4]	Postmenopausal Korean Menopause clinic for BMD measurement	220			Similar	0.2 - 24.5-73.6	RIA after bio-spin Separated from BP's
Frayling, 2002 [8]	Children from subjects of BCG cohort: milk during pregnancy vs no supplements. Caucasian	640	'97 - '99	25	X/X <sup>1</sup> lowest;	40 - 46 - 14	RIA after acid-acetone extraction
Allen, 2002 [3]	Men from EPIC cohort (European Investigation into Cancer and Nutrition) Relation between nutrients and proteins	660	'94 - '97	47 (20 - 78)	Similar	41 - 47 - 12	Not mentioned
Misner, 2002 [2]	Controls matched to incident breast cancer cases, prospectively followed from Nurses' Health Study	622	'89 - '90	58 (43 - 69)	X/X Highest	39 - 42 - 19	ELISA
Kato, 2003 [5]	Controls prostate cancer Controls breast cancer Controls were healthy employees or patients with regular check-up. African-American, postmenopausal	60 53	'98 - '99	51 (48 - 86) 52 (22 - 73)	Similar	0 - 53 - 47 0 - 43 - 57	ELISA
DeLellis, 2003 [11]	Multi-ethnic cohort: diet and lifestyle in aetiology of cancer. Postmenopausal women African-American Japanese American Non-latino white Latino american	65 50 47 68	'93 - '96	45 - 75			RIA
Hovind, 2007 [13]	Newly diagnosed type 1 diabetes	233	'96 - '98	26.9 (13.8)*	Similar	73-72 <sup>3</sup>	IRMA
Hernandez, 2007 [10]	Controls of incident prostate cancer in urology department African-Americans	401	'01 - '04	65.8 (9.3)*	X/X lowest	14 - 7 - 79	Immunochemi-luminometric
Fehringer, 2008 [9]	Premenopausal women, Caucasian Recruited from mammographic units	162	'94 - '97	44.8 (4.6)*	X/X lowest	41 - 43 - 16	Competitive binding RIA

<sup>1</sup> IGF-I genotypes are presented as % in homozygous - heterozygous - non carriers of 19 CA repeat

<sup>2</sup> X/X represents homozygous carriers of 19 CA repeats

<sup>3</sup> IGF-I genotypes are presented as % wild type - variant type carriers. Wild type carriers: homozygous carriers; homozygous for the 19 CA repeat allele or for the 20 CA repeat allele, and carriers heterozygote for these two alleles.

Variant carriers: all other combinations of alleles

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# Summary

Insulin like growth factor-1 (IGF-I) is a polypeptide which most important function is mediating physiological growth. It is also involved in fat, carbohydrate and protein metabolism and influences cell proliferation, differentiation and survival in many tissues. IGF-I is synthesized by most organs and may act as an endocrine, paracrine and/or autocrine growth factor. Its production is dependent on several factors. The most important regulators are growth hormone (GH), insulin, IGF-I binding proteins and the IGF-I gene. A CA repeat polymorphism in the promoter region of the IGF-I gene has been identified. This CAn polymorphism has a CA repeat range varying between 10 and 24 CA repeats. In the Caucasian population, the most common allele comprises 19 CA repeats (also called the 192-bp allele), suggesting that this is the wild type allele. Homozygous carriers of this allele have been found to have the highest total IGF-I serum levels, highest body height and an increased risk for diabetes mellitus compared to non-carriers of the 192-bp allele.

In this thesis, we investigated the role of this CAn polymorphism on physiologic endpoints. We also examined the role of this polymorphism and beta cell function, diabetic retinopathy, micro-albuminuria and mortality. For all the investigations, we used the data of the Rotterdam Study, a population-based cohort study of diseases in the elderly. Baseline examination of the Rotterdam Study was conducted between 1990 and 1993 and a total of 7983 participants were examined.

In the **introduction** part of this thesis, the functions and regulation of IGF-I and its role in the development of diabetes mellitus and development of micro- and macrovascular complications of diabetes are described.

Serum IGF-I levels are known to decline with age as the influence of GH on IGF-I seems to become less important. Most of the IGF-I is bound in a trimeric complex with IGFBP3 and an acid lable subunit (ALS). The production of all of the components of this complex is under the influence of GH. In **chapter 2** the role of the CAn promoter polymorphism in the age related decline of IGF-I and IGFBP-3 levels was studied in a subgroup of the Rotterdam Study. This group was partly selected on genotype, to maximize power to detect a relation between the IGF-I genotype and serum levels. In the total study group the well known inverse relation between age and total IGF-I levels was observed. After stratification according to the IGF-I genotypes, this relationship disappeared in heterozygous and non-carriers of the 192-bp allele and only remained highly significant in homozygous carriers of the 192-bp allele. Also IGFBP-3 levels decreased with age and stratification per genotype showed again only a significant correlation in homozygous carriers of the 192-bp allele. This suggests that the age related decline of IGF-I is more GH dependent in homozygous carriers of the 192-bp allele compared to heterozygous and non-carriers of the 192-bp allele. In heterozygous and non-carriers of the 192-bp alleles, IGF-I concentration is likely to become more dependent of other factors like nutrition, sex steroids and insulin levels.

Our observation that homozygous carriers of the most frequent allele have the highest total serum IGF-I levels, was not consistently found by other investigators. Other study groups have found lower, equal and higher IGF-I levels in homozygous carriers of the wild type allele compared to non-carriers.

IGF-I is not only a candidate for influencing body height, but determines also the response to famine in animals. Secular trends in body composition seem primarily related to lifestyle, environmental and socio-economic factors. Genetic factors might also play a role, but so far no major genes have been found to be related to secular trends in body composition.

In **chapter 3** we studied the relationship between the number of CA repeats in the IGF-I gene and total IGF-I serum levels and body height. We also studied the influence of this CAn polymorphism on the secular trend in body height. We observed that total IGF-I serum levels of homozygous carriers of the 194-bp alleles were similar compared to the levels of homozygous carriers of the wild type allele. Homozygous carriers of 192-bp and 194-bp alleles had higher levels compared to homozygous carriers of alleles longer than 194-bp alleles and homozygous carriers of alleles shorter than 192 bp alleles. Also no difference was observed between homozygous carriers of the 192-bp alleles and 194-bp alleles when regarding body height. When we examined all genotypes, a clear optimum in IGF-I serum levels and body height was observed for the group comprising homozygous carriers of the 192-bp and 194-bp alleles, as well as the combination of the 192 and 194-bp alleles. In addition, no relation between the CAn polymorphism and the secular trend in body height was observed. Since the difference in body height between the IGF-I genotype groups didn't increase over time, we believe that there is no specific effect of the IGF-I gene on the secular trend, nor in times of malnutrition.

IGF-I is an important regulator of pancreatic beta cell growth and maturation. In a previous study an increased risk for diabetes mellitus was observed in non-carriers of the 192-bp allele. Insulin resistance and beta cell dysfunction are both prerequisites for the development of type 2 diabetes. The relation between beta cell function and insulin sensitivity in healthy individuals is hyperbolic. Changes in insulin sensitivity are compensated by inverse changes in beta cell responsiveness, such that the product of insulin sensitivity and insulin secretion (the disposition index) remains constant. The disposition index indicates the metabolic status of an individual, i.e. the relative contributions of insulin sensitivity and beta cell function to their degree of glucose tolerance. A higher disposition index means a higher ability of the beta cells to compensate for the decrease in insulin sensitivity. In **chapter 4** we investigated the relation between the IGF-I gene and beta cell function and insulin sensitivity in a case-control study, comprising persons with normal glucose tolerance, pre-diabetes and type 2 diabetes. Pre-diabetes was defined as subjects with impaired glucose tolerance and impaired fasting glucose. We used the genotypes based on the observation that carriers of 194-bp alleles had similar IGF-I serum levels as carriers of 192-bp alleles. Two groups were made: wild type and variant carriers. Wild type carriers are carriers homozygous for the 192-bp or for

the 194-bp allele, and carriers heterozygote for these two alleles. Participants with all other combinations of alleles were considered variant carriers. In subjects with normal glucose tolerance, variant carriers had lower first and second phase insulin secretion than wild type carriers. We also performed a stratified analyses based on a BMI lower (<) or equal or higher ( $\geq$ ) 27 kg/m<sup>2</sup>, since it has been found that insulin sensitivity for glucose disposal is impaired in humans with normal glucose tolerance with a BMI  $\geq$  27 kg/m<sup>2</sup>. The parameters for beta cell function as well as the disposition index were significantly lower for variant carriers when looking in non-obese persons only. In obese subjects no differences in parameters of insulin sensitivity and beta cell function were observed between the IGF-I genotypes. Our study suggests that in individuals with a normal glucose tolerance, beta cells respond insufficiently to this glucose load in variant-carriers. This polymorphism in the IGF-I gene may thus contribute to diabetes susceptibility.

The existing literature on the role of IGF-I in the development and progression of diabetic retinopathy is conflicting. Intravitreal levels have been found to be higher in diabetic patients compared to controls. On the other hand, GH deficient dwarfs with glucose intolerance rarely develop severe proliferative retinopathy. Proliferative retinopathy has been associated with low, normal and high total IGF-I serum levels. In **chapter 5a**, we studied the association of the IGF-I gene as a marker of IGF-I production and retinal vascular diameters as well as diabetic retinopathy. Vascular dilatation of the retinal vessels, especially the retinal venules, has been observed in the early stages of diabetic retinopathy. Furthermore, especially larger venular diameter has been associated with a 4-year incidence of proliferative retinopathy. At baseline, variant carriers with impaired glucose tolerance (IGT) or diabetes appeared to have a larger retinal arteriolar and venular diameter than wild type carriers with IGT/diabetes, but the differences were not statistically significant. Variant carriers with IGT/diabetes who developed retinopathy during follow-up already tended to have even larger retinal vascular diameter at baseline when compared with IGT/diabetes subjects who did not develop retinopathy. Finally, variant carriers with IGT/diabetes had a 1.8 increased risk of incident retinopathy compared with participants of the wild type. Our study suggests that the CAN IGF-I promoter polymorphism may modulate the susceptibility and/or the progression of diabetic retinopathy.

Micro-albuminuria (MA) is an early marker for renal disease in diabetics. It is also related to cardiovascular disease in diabetic and non-diabetic persons. IGF-I has been implicated in the development of diabetic nephropathy and cardiovascular disease. IGF-I enhances renal plasma flow and creatinine clearance and has been associated with renal hypertrophy and compensatory renal growth in amongst others diabetes. Raised renal concentrations are thought to protect diabetic kidney cells from ischemic damage and to accelerate tissue repair and recovery of renal function. In **chapter 5b**, we investigated if the promoter polymorphism in the IGF-I gene was related to the development of micro-albuminuria. In persons with an abnormal glucose tolerance (AGT), comprising persons with impaired glucose tolerance,

impaired fasting glucose and diabetes, variant carriers had almost significantly more albuminuria and a higher albumin-to-creatinine ratio (ACR) than wild type carriers. Risk of development of MA was higher in AGT compared to normal glucose tolerance (NGT) and variant carriers had a higher risk compared to carriers of the wild type alleles in both glucose tolerance conditions. After stratification for glucose tolerance, variant carriers had a higher risk of development of MA in individuals with AGT, which was significant after adjustment for potential confounders. This suggests that the IGF-I gene polymorphism modifies the susceptibility and/or progression of MA as soon as a person develops MA. Susceptibility to MA probably results from an interaction of multiple genetic and environmental factors, meaning that MA only will develop in with a certain genetic background when other risk factors are also present.

In **chapter 6**, the relation between the polymorphism and mortality was examined. In a population of 7983 elderly individuals, 345 of them developed a myocardial infarction during follow-up. The risk of mortality following myocardial infarction was 1.5 times higher in variant carriers compared to non-carriers, where as no relation between the polymorphism and mortality in the overall population was observed. IGF-I may be a determinant of survival because it promotes survival of cardiomyocytes that are affected by ischemia

In **chapter 7** possible explanations for the differences in outcome between IGF-I genotype and circulating IGF-I levels are discussed as well as the clinical implications of our results. Furthermore, we outlined limitations of the candidate gene approach and the study design used in our study. Finally, perspectives for future research are described.

# Samenvatting (summary in Dutch)

Het insuline-achtige groeifactor-I (IGF-I) is een polypeptide en heeft als belangrijkste functie het bevorderen van de lichaamslengte. Daarnaast speelt het een rol in het vet-, koolhydraat- en eiwitmetabolisme en bevordert het de groei, uitrijping en de overleving van verschillende weefsels. IGF-I wordt in diverse weefsels geproduceerd en functioneert als een endocriene, paracriene en/of autocriene groeifactor. De productie van IGF-I hangt af van diverse factoren. De belangrijkste zijn groei hormoon (GH), insuline, IGF-I bindende eiwitten en het IGF-I gen. Recent werd een CA repeat polymorfisme in de promotor regio van het IGF-I gen ontdekt. Dit polymorfisme heeft een variatie van CA repeats, variërend tussen de 10 en 24 CA repeats. In de Caucasische populatie is het meest voorkomende allel het allel bestaande uit 19CA repeats (ook wel het 192-bp allel genoemd), suggererend dat dit het wild type allel is. Homozygote dragers van dit allel hebben de hoogste serum IGF-I spiegels, zijn het langst en hebben een hoger risico op het ontwikkelen van diabetes mellitus vergeleken met niet dragers van dit allel.

In dit proefschrift onderzochten we de rol van dit CA polymorfisme en fysiologische eindpunten. Ook bestudeerden we de rol van dit polymorfisme met de beta cel functie, diabetische retinopathie, micro-albuminurie en mortaliteit. Voor alle studies maakten we gebruik van de data van de Rotterdam Studie, een populatie studie naar aandoeningen bij ouderen. Het baseline onderzoek werd uitgevoerd tussen 1990 en 1993 en in totaal deden 7983 mensen mee.

In de **inleiding** van dit proefschrift, worden de diverse functies en de regulatie van IGF-I beschreven evenals zijn rol in de ontwikkeling van diabetes mellitus en de ontwikkeling van zowel de micro- als macrovasculaire complicaties hiervan.

De hoogte van de serum IGF-I spiegels nemen af met de leeftijd doordat de invloed van GH op IGF-I minder belangrijk lijkt te worden. Het merendeel van het IGF-I wordt in een trimeer complex gebonden met IGFBP-3 en een acid labile subunit (ALS). De synthese van alle componenten van dit complex is onder invloed van GH. In **hoofdstuk 2** bestudeerden we de rol van het CA promotor polymorfisme op de leeftijdsafhankelijke daling van zowel de IGF-I als de IGFBP-3 spiegels in een subgroep van de Rotterdam Studie. Deze groep is deels geselecteerd op IGF-I genotype om de relatie tussen het IGF-I genotype en spiegels te kunnen bestuderen. Bij bestudering van de gehele studie populatie zagen we de welbekende inverse relatie tussen de IGF-I spiegels en leeftijd. Na stratificatie op IGF-I genotype was deze relatie niet zichtbaar bij heterozygoten en niet-dragers van het 192-bp allel, maar was alleen aantoonbaar bij homozygote dragers van het 192-bp allel. Daarnaast zagen we ook de IGFBP-3 spiegels dalen met de leeftijd en na stratificatie op IGF-I genotype was deze relatie wederom alleen aanwezig bij homozygote dragers van het 192-bp allel. Deze bevindingen suggereren dat de leeftijdsafhankelijke daling van IGF-I meer GH afhankelijk is bij homozygote dragers van het 192-bp allel dan heterozygote en niet dragers van het 192-bp allel. De IGF-I concentratie in

heterozygote en niet-dragers van het 192-bp allel lijkt dus meer afhankelijk te worden van andere factoren zoals voeding, geslachtshormonen en insuline spiegels.

Onze bevinding dat homozygote dragers van het meest voorkomende allel de hoogste totale IGF-I serum spiegel hebben, wordt niet gevonden door andere studie groepen. In de diverse studies worden lagere, dezelfde en hogere IGF-I spiegels gevonden bij homozygote dragers van het wildtype allel ten opzichte van niet-dragers. IGF-I is niet alleen een belangrijke factor voor lichaamslengte, het bepaalt bij dieren ook hoe er wordt gereageerd in tijden van honger. Seculaire trends in lichaamssomvang lijken primair gerelateerd te zijn aan levensstijl, omgevings- en socio-economische factoren. Genetische factoren lijken ook een rol te spelen, maar tot dusver zijn er nog geen genen gevonden die een belangrijke invloed uitoefenen in de seculair trends van lichaamssamenstelling. In **hoofdstuk 3** onderzochten we de relatie tussen het aantal CA repeats van het IGF-I gen met IGF-I spiegels en lichaamslengte. We onderzochten eveneens de relatie van dit CAn polymorfisme met de seculaire trend in lichaamslengte. We vonden dat IGF-I spiegels van homozygote dragers van het 194-bp allel gelijk waren aan de spiegels van homozygote dragers van het wild type-allel. Homozygote dragers van het 192-bp allel en het 194-bp allel hadden hogere spiegels dan homozygote dragers van allelen langer dan 194-bp en homozygote dragers van allelen korter dan 192-bp. Ook de lichaamslengte was hetzelfde bij homozygote dragers van het 192-bp en het 194-bp allel. Als we alle IGF-I genotypen bestudeerden, vonden we duidelijk een optimum in IGF-I spiegels en lichaamslengte bij homozygote dragers van het 192-bp allel, homozygote dragers van het 194-bp allel en dragers van de combinatie van het 192-bp en het 194-bp allel. Er werd geen relatie gevonden tussen het CAn polymorfisme en de seculaire trend van lichaamslengte. Aangezien het verschil in lichaamslengte tussen de IGF-I genotypen niet met de tijd toenam, denken we dat er geen effect is van het IGF-I gen op de seculaire trend, ook niet in tijden van ondervoeding.

IGF-I is een belangrijke regulator van groei en uitrijping van de beta cellen van de pancreas. In een eerdere studie werd een hoger risico op diabetes mellitus gevonden voor niet dragers van het 192-bp allel. Insuline resistentie en disfunctie van de beta cellen zijn voorwaarden voor het ontwikkelen van diabetes mellitus. Veranderingen in insuline gevoeligheid gaan samen met inverse veranderingen in respons van de beta cel zodat het product van insuline gevoeligheid en insuline secretie (de dispositie index) constant blijft. De dispositie index geeft de metabole status van een individu weer, met andere woorden, de relatieve contributie van insuline gevoeligheid en de beta cel functie op basis van de glucose tolerantie. In **hoofdstuk 4** onderzochten we de relatie van het IGF-I gen met de beta cel functie en insuline gevoeligheid in een case-control studie, bestaande uit mensen met een normale glucose tolerantie, een gestoorde glucose tolerantie, afwijkend nuchter glucose en personen met diabetes mellitus. De IGF-I genotypen werden samengesteld op basis van de observatie dat dragers van 194-bp allelen even hoge IGF-I serum spiegels hebben als dragers van 192-bp allelen. Er werden 2 groepen gemaakt: wild type dragers (homozygote dragers van het 192-bp

allel, homozygote dragers van het 194-bp allel en dragers van de combinatie 192 met 194-bp allel) en variante dragers (alle overige genotypen). Van personen met een normale glucose tolerantie hadden variante dragers een lagere eerste en tweede fase van de insuline secretie dan wild type dragers. Vervolgens deden we een analyse gestratificeerd op een BMI onder of  $\geq 27 \text{ kg/m}^2$ , aangezien bij mensen met een normale glucose tolerantie de mogelijkheid van insuline om glucose op te nemen gestoord is indien de BMI  $27 \text{ kg/m}^2$  of meer bedraagt. Bij variante dragers waren de beta cel functie en de dispositie index significant lager ten opzichte van wild type dragers indien ze niet-obees waren. In obese mensen werden geen verschillen gevonden in de insuline gevoeligheid en de beta cel functie tussen de IGF-I genotypen. Onze studie suggereert dat bij variante dragers met een normale glucose tolerantie, de beta cellen onvoldoende reageren op een glucose belasting. Het onderzochte polymorfisme in het IGF-I gen lijkt dus mogelijk bij te dragen tot de ontwikkeling van diabetes mellitus.

De bestaande literatuur over de rol van IGF-I in de ontwikkeling en progressie van diabetische retinopathie is niet eenduidig. Intravitreale IGF-I spiegels zijn hoger in diabeten tov controles. Daarentegen ontwikkelen GH deficiënte dwergen met glucose intolerantie zelden ernstige proliferatieve retinopathie. Proliferatieve retinopathie zijn geassocieerd met lage, normale en hoge IGF-I serum spiegels. In **hoofdstuk 5a** bestudeerden we de associatie van het IGF-I gen als marker voor de IGF-I productie met retinale vaatdiameters en diabetische retinopathie. Dilatatie van de retinale diameter, met name van de venulen, wordt gezien in de vroege fase in de ontwikkeling van diabetische retinopathie. Daarnaast is een grotere diameter van de veneuze vaten gerelateerd aan een hogere 4 jaars incidentie van proliferatieve retinopathie. Bij aanvang van onze studie bleken variante dragers met IGT/diabetes een grotere retinale vasculaire diameter te hebben dan wild type carriers met IGT/diabetes, maar deze verschillen zijn niet statistisch significant. Variante dragers met IGT/diabetes die retinopathie ontwikkelden tijdens het verloop van de studie bleken zelfs bij aanvang van de studie al de grootste retinale vaatdiameter te hebben ten opzichte van personen met IGT/diabetes die geen retinopathie ontwikkelden. Tot slot hebben variante dragers met IGT/diabetes een 1.8 keer hoger risico op het krijgen van retinopathie ten opzichte van deelnemers met het wild type genotype. Onze studie suggereert dat het CAn promotor polymorfisme in het IGF-I gen mogelijk een rol speelt bij de ontwikkeling en/of de progressie van diabetische retinopathie. Micro-albuminurie is een vroege marker voor nierziekte bij diabeten. Daarnaast is het een voorbode voor cardiovasculaire ziekte bij zowel diabeten als niet-diabeten. IGF-I speelt een rol bij de ontwikkeling van diabetische nefropathie en cardiovasculaire ziekten. IGF-I verhoogt de glomerulaire plasma flow en kreatinine klaring en is geassocieerd met renale hypertrofie en compensatoire renale groei in onder andere diabeten. Men veronderstelt dat toegenomen concentraties van IGF-I diabetische niercellen beschermen tegen ischemische schade, weefselherstel versnellen en herstel bevorderen van de renale functie. In **hoofdstuk 5b** onderzochten we of het promotor polymorfisme in het IGF-I gen was gerelateerd aan het ontstaan van micro-albuminurie.

Bij personen met een gestoorde glucose tolerantie (AGT), hebben variante dragers significant meer albuminurie en een hogere albumine-kreatinine ratio dan wild type dragers. Personen met een gestoorde glucose tolerantie hebben een hoger risico op het ontwikkelen van micro-albuminurie (MA) dan individuen met een normale glucose tolerantie. In beide glucose tolerantie groepen is het risico hoger bij variante dragers dan bij wild type dragers. Na stratificatie voor glucose tolerantie lopen variante dragers met een gestoorde tolerantie meer kans op het krijgen van MA. Deze relatie blijft significant na correctie voor confounders. Dit suggereert dat het polymorfisme in de promoter regio van het IGF-I gen de gevoeligheid en/of de progressie van MA beïnvloedt zodra men MA ontwikkelt. De mogelijkheid MA te ontwikkelen hangt af van de interactie tussen multi-pele genetische en omgevings factoren, wat inhoudt dat men alleen MA ontwikkelt bij een bepaalde genetische predispositie bij het tevens aanwezig zijn van andere risico factoren.

In **hoofdstuk 6** werd de relatie van het IGF-I polymorfisme en mortaliteit onderzocht. In een populatie van 7983 oudere individuen kregen 345 van hen een myocard infarct. Het risico op overlijden was 1.5 keer hoger bij variante dragers en er werd geen relatie gevonden tussen het polymorfisme en mortaliteit in de totale populatie. IGF-I kan een determinant van overleving zijn aangezien het de overleving van cardiomyocyten beïnvloedt bij ischemische schade.

In **hoofdstuk 7** worden mogelijke verklaringen voor de verschillende studie uitkomsten van het IGF-I genotype en IGF-I spiegels besproken alsmede de klinische implicaties van onze studie resultaten. Daarnaast worden de beperkingen van kandidaatgen studies en de door ons gebruikte studie opzet besproken. Tot slot worden mogelijkheden voor vervolgonderzoek besproken.

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## About the author

Ingrid Rietveld was born on January 16, 1975 in Udenhout, the Netherlands. She graduated in 1993 at the “Thomas More College” in Oudenbosch. In 2000, she obtained her medical degree at the Erasmus University in Rotterdam. Subsequently, she worked 6 months as a resident in Neurology at the Amphia Hospital in Breda. In June 2001, she started her work described in this thesis at the Department of Internal Medicine and the Genetic Epidemiology Unit of the Department of Epidemiology & Biostatistics of the Erasmus MC in Rotterdam. During this period she also participated in the research for Creutzfeldt Jakob disease. In 2003, she obtained a Master of Science degree in Genetic Epidemiology at the Netherlands Institute for Health Sciences. She started her specialist training in Internal Medicine at the Amphia Hospital, Molengracht, in Breda in January 2005 under supervision of Dr. G.J. Wenting and Dr. C. van Guldener. From January 2008 the specialist training was continued at the Erasmus Medical Center in Rotterdam under supervision of Prof. Dr. J. van Saasse. Since July 2009, she is working as a fellow in Endocrinology under supervision of Prof. Dr. A.J. van der Lelij. She lives together with Sander van Roodselaar in Breda.



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6. 't Hart LM, Hansen T, **Rietveld I**, Dekker JM, Nijpels G, Janssen GM, Arp PA, Uitterlinden AG, Jorgensen T, Borch-Johnsen K, Pols HA, Padersen O, van Duijn CM, Maassen JA. *Evidence that the mitochondrial leucyl tRNA synthetase (LARS2) gene represents a novel type 2 diabetes susceptibility gene*. Diabetes 2005 June;54(6):1892-5
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# PhD Portfolio

Summary of PhD training and teaching activities

## **General and Research courses**

Master of Science in Genetic Epidemiology 2001-2003. Courses:

- Principles of Research in Medicine and Epidemiology. 2001
- Methods of genetic Epidemiology. 2001
- Searching Genes of Complex Disorders. 2001
- Genetic Epidemiology of Complex Diseases. 2001
- Bioinformatics in medicine. 2001
- Current concepts in epidemiologic study design. 2003
- Bayesian Analysis. 2003
- Analysis of Repeated Measurements. 2003
- Study Design. 2002
- Classical Methods for Data-Analysis. 2001
- Genetic-Epidemiologic Research Methods. 2001
- Modern Statistical Methods. 2001
- Discussion Meeting Research Proposal. 2003
- Principles of Epidemiologic Data Analysis. 2004
- Advances in population-based Studies of Complex Genetic Disorders. 2002
- Genetic Linkage Analysis I: Model Based Analysis. 2002
- Genetic Linkage Analysis II: Sib-pair Analysis. 2002
- S.A.G.E. 2003

Communication course: Het drama van de arts. Slachtoffergedrag en de dramadriehoek. 2007

## **Specific courses**

Internal Medicine, residency, Erasmus MC, Rotterdam, NL. 2005-current

Endocrinology, residency, Erasmus MC, Rotterdam, NL. 2009-current

## **(Inter)national conference presentations**

Endocrine society 2003: Poster presentations:

- A polymorphism in the promoter region of the IGF-I gene is associated with albuminurie
- Functional aspects of a polymorphism in the promoter region of the IGF-1 gene

ESPE 2003, posterpresentation:

Relation between IGF-I gene polymorphism and bone mineral density and lean body mass in healthy children and young adults

A.M. Boot, I.M. van der Sluis, S.M.P.F. de Muinck Keizer-Schrama, I. Rietveld, H.A.P. Pols, A.G. Uitterlinden

NDESG 2003. Oral presentation: A polymorphism in the promoter region of the IGF-I gene is associated with albuminuria

ADDG 2003. Oral presentation: A polymorphism in the promoter region of the IGF-I gene is associated with albuminuria

HOVO onderwijs 2004. Oral presentation: "IGF-I van geboorte tot ouderdom"

### ***Teaching***

Supervising and teaching medical students, Department of Internal Medicine and Endocrinology. 2001-present

Residents of Internal medicine Erasmus MC: presentation about "aliskiren"



