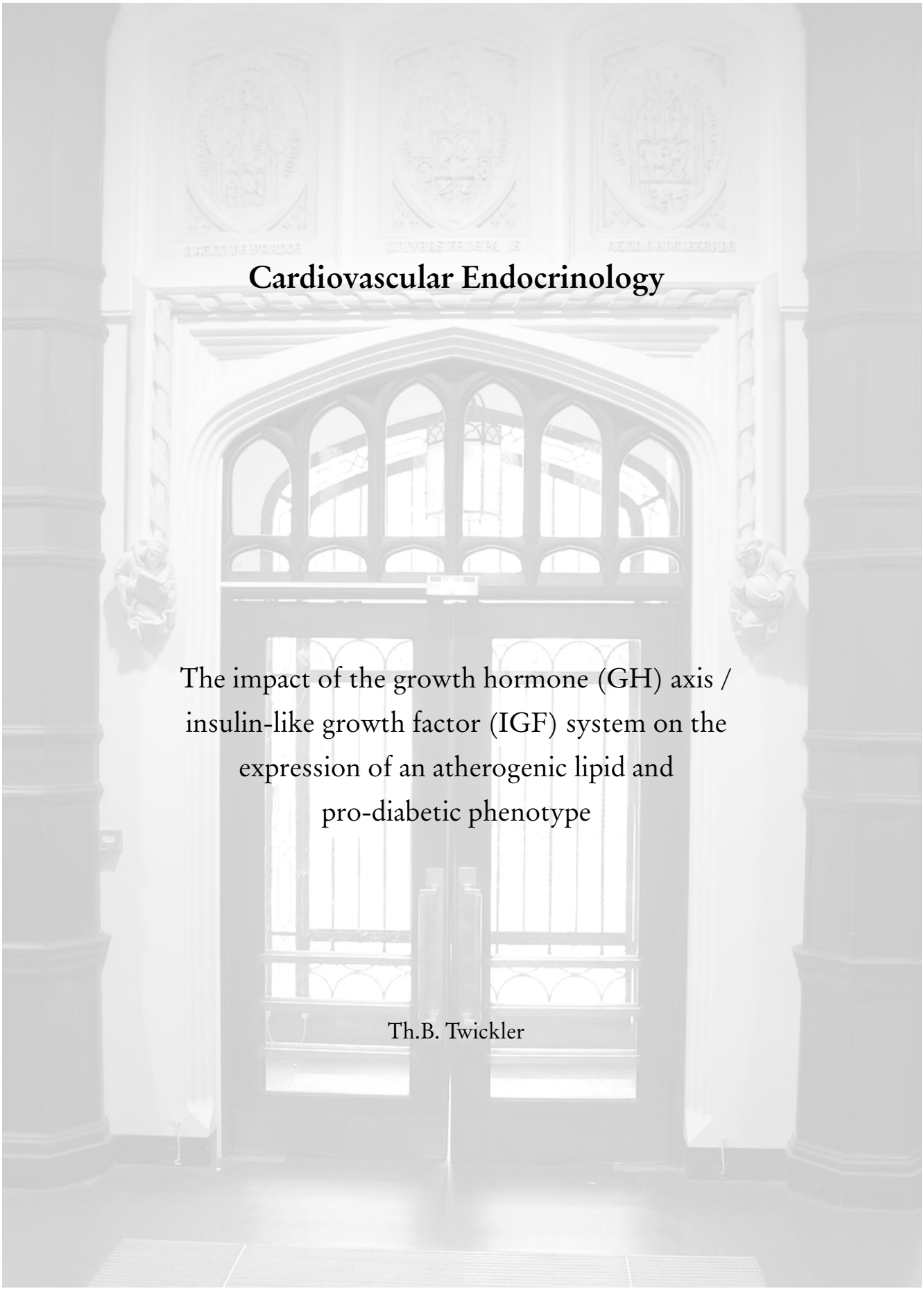

Cardiovascular Endocrinology

The impact of the growth hormone (GH) axis / insulin-like growth factor (IGF) system on the expression of an atherogenic lipid and pro-diabetic phenotype

Th.B. Twickler

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Cardiovascular Endocrinology

The impact of the growth hormone (GH) axis / insulin-like growth factor (IGF) system on the expression of an atherogenic lipid and a pro-diabetic phenotype (including an English summary)

Cardiovasculaire Endocrinologie

Het belang van de GH as/ IGF systeem voor de expressie van atherogene lipiden en pro-diabetische fenotype (met een samenvatting in het Nederlands).

Endocrinologie Cardiovasculaire

L'importance de l'axe GH et de la système d'IGF sur l'expression de la phénotype athérogène lipidique et pro-diabétique (avec une résumé en Francais)

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht
Op gezag van de rector magnificus, professor doctor W.H. Gispen
Ingevolge het besluit van het college voor promoties
In het openbaar te verdedigen
op dinsdag 29 april 2003 des namiddags te 2.30 uur precies

Door

Theodorus Bartholomeus Twickler

Geboren op 13 april 1970 te Musselkanaal

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"De mens, die belooft, wordt zelf toekomst, in plaats van er slechts slachtoffer van te zijn. Hij drukt zijn eigen stempel op de toekomst, in plaats van die te laten bepalen, of zelfs wegnemen door het verleden".

« L'homme qui promet, devient du futur soi-même, au lieu d'en être seulement la victime. I l s'en construit son futur, au lieu de le faire déterminer, ou même de le faire prendre par le passé »

Professor dr. P van Tongeren (department of Philosophy, Catholic University Nijmegen) in the Thym Essay 2003 called "Over het verstrijken van de tijd", of the Thijm Foundation

This thesis is dedicated to
Alexandra and Lieska.

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Abbreviations

AGHD	adult-onset growth hormone deficiency
ALS	acid label subunit
Apo	apolipoprotein
AUC	area under the curve
BMI	body mass index
BIA	bio-impedance assessment
CAD	coronary artery disease
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
Chol	cholesterol
CVD	cardiovascular disease
DM	diabetes mellitus
FC	free cholesterol
FFA	free fatty acids
FH	familiar hypercholesterolaemia
FMD	flow mediated dilation
rhGH	(recombinant) growth hormone
GNG	gluconeogenesis
GL	glucogenolysis
HOMA(-IR)	homeostasis model assessment (of insulin resistance)
HDL	high-density lipoprotein
HL	hepatic lipase
IL-6	interleukin-6
IL-10	interleukin-10
IGF	insulin-like growth factor
IGF-BP	insulin-like growth factor binding protein
IMT	intima media thickness
kDa	kilo Dalton
LDL	low-density lipoprotein
LDL-ox	oxidised LDL
LDL-r	LDL receptor
LPL	lipoprotein lipase
NCEP ATP III	National Cholesterol Education Program Adult Treatment Panel III
NO	nitric oxide
OD	once a day
7-OH ase	7-alpha hydroxylase
PP	postprandial
RLPc	remnant-like particle cholesterol
RE	retinyl ester
TG	triglyceride
TNF	tumour necrosis factor
TRL	triglyceride rich lipoproteins
TSH	thyroid stimulating hormone
VLDL	very low density lipoprotein
WHR	waist-hip ratio

Prologue

"Cardiovascular Endocrinology, a new dimension in Medicine"

Sir-From daily clinical practice we are aware that only about 40% of all cardiovascular events can be explained by the classical cardiovascular risk factors (such as hypertension, dyslipidaemia, and obesity). In line with James Parle and colleagues' report [1] additional pathophysiological mechanisms need to be investigated, especially in relation to hormonal disturbances.

We have noted increased concentrations of highly atherogenic lipoprotein remnants in active acromegaly [2]. Moreover, premature atherosclerosis is a clinical feature in adult-onset growth hormone (GH) deficiency syndrome. We have reported an improved postprandial atherogenic lipoprotein remnant profile and endothelial function after GH substitution [3].

Bengtsson and Johansson [4] summarised the beneficial effects of GH therapy on early atherosclerotic changes in GH-deficient adults. The cardiovascular importance of the GH/insulin-like growth factor (IGF) axis and the efforts to treat disturbed activity is a further example of endocrinological intervention in cardiovascular disease. Scientific progress has been made in this area, in which left-ventricle dysfunction improved after chronic subcutaneous Ghrelin administration in a rat model [5]. Ghrelin in treatment of chronic heart failure in men may, therefore, become an option in the future.

Concordant with effects of disturbed activity of the GH/IGF axis in cardiovascular function, we postulate that other disturbed hormonal systems will have an effect on cardiovascular disease. Of course, this hormonal-cardiovascular interaction needs to be studied more thoroughly. We believe these developments support the importance of a new multidisciplinary approach, which may create a new dimension in medicine-*cardiovascular endocrinology*.

Lancet 2002; 359:799

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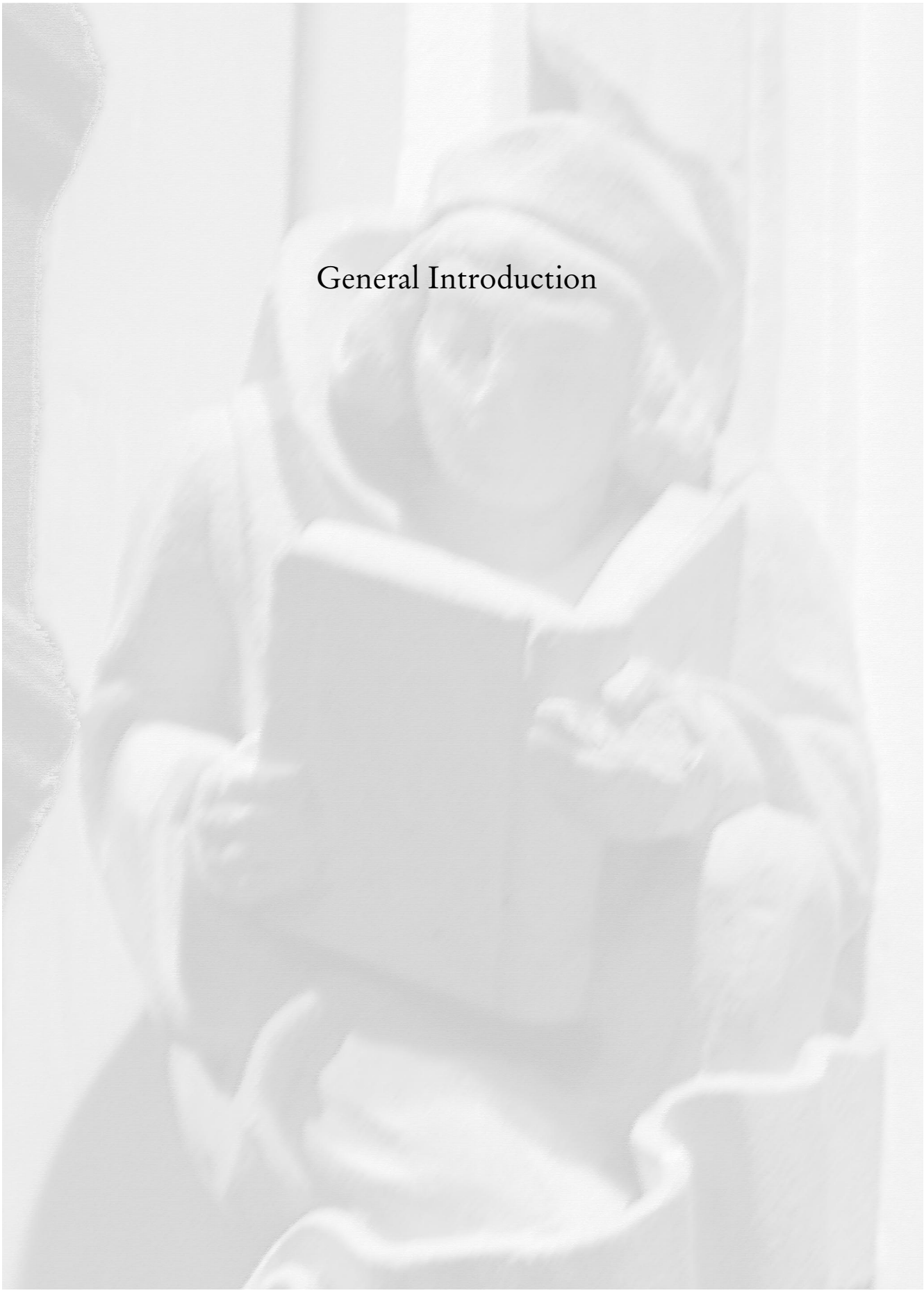


General Introduction

A complex relationship exists between disease of the cardiovascular system and a spectrum of neural and humoral factors. Recently, the modulating role of hormones, such as thyroid hormone (6-9), in the atherosclerotic process has been emphasized. However, several other hormones, in addition to thyroid hormone, may contribute to atherogenesis, thereby constituting a key element in the concept of cardiovascular endocrinology (10). Recently, evidence has been provided to suggest that disturbances of the pituitary growth hormone (GH) axis and its mitogenic partners, including insulin-like growth factor-1 (IGF-1) and IGF-binding proteins (IGFBP), are critical actors in the initiation of atherosclerotic processes (4; 11; 12).

GH axis/IGF system

The GH axis originates in the cerebrum with the brain structures, hypothalamus and pituitary, as regulation centers (figure 1). Growth hormone releasing hormone (GHRH) releases, whereas insulin-like growth factor-I (IGF-I) inhibits secretion of GH from the somatotrope cells in the anterior lobe of the pituitary. Recent evidence supports the notion that GH release from the pituitary is controlled not only by GH-RH and Somatostatin from the hypothalamus, but also by GHrelin from the stomach and hypothalamus. GH is secreted from the anterior pituitary in an individual diurnal pattern, with the highest serum peak levels early in the night. In the circulation, GH is mostly bound to GH binding protein (GHBP). Only unbound GH has biological activity. The GH axis is superimposed on the IGF system. The insuline-like growth factors (IGF-I and IGF-II) are important factors in the regulation of somatic growth, cellular proliferation and metabolism. This regulation is modulated further by at least six distinctive insulin-like growth factor binding proteins (IGF BPs) and IGF BP proteases (13). Both IGF-I and IGF-II are synthesized and secreted from the liver and they are mainly bound to the IGF

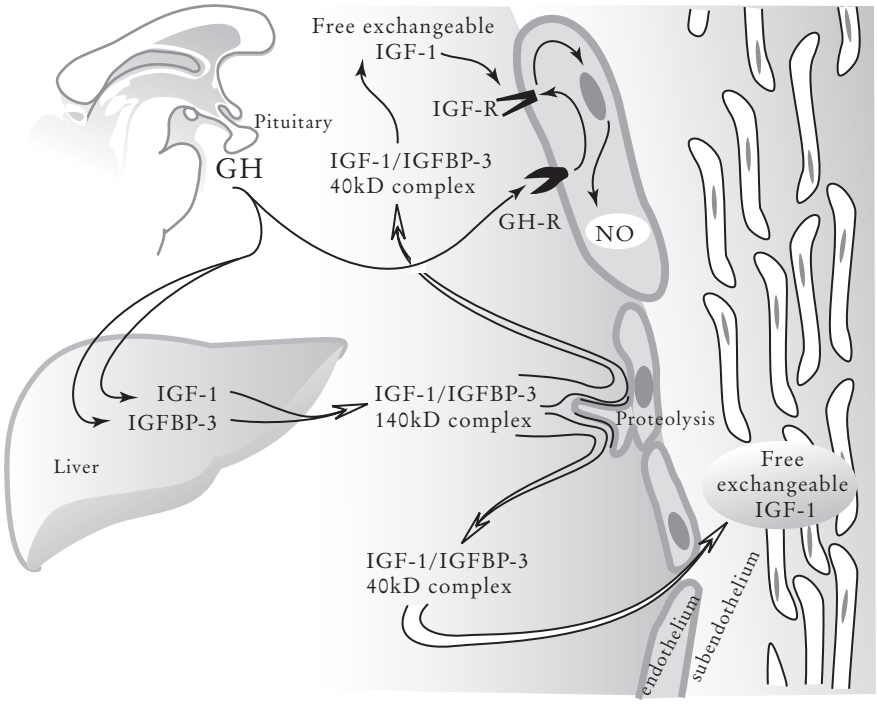


Figure 1.
The G Haxis/ IGF system with its principal components.

BP-3. The total plasma pool of IGF-II is twice that of IGF-I. The synthesis of IGF-I and IGF BP-3, but not IGF-II, is under regulation by GH and environmental factors (e.g. nutritional status)(14). Both IGF-I and IGF-II are products of a single gene, located on the arm of chromosome 12 (IGF-I), and on the short arm of chromosome 11 (IGF-II)(15) In addition, the proportion of variance attributable to genetic effects for the concentration of IGF-I was 38%, IGF-II: 66% and IGF BP-3: 60%. Therefore a substantial genetic contribution is responsible for the interindividual variation of circulating IGF-I, IGF-II, and IGF BP-3 (16).

The availability of biological active (free circulating) IGF-I is determined by its binding on the IGFBP complex, which consist of a 140 kDa, or a smaller 40 kDa IGF-IGF BP complex. Most circulating IGF-1 and IGF-II (in an equimolar ratio) is sequestered by IGF BP-3 (38 kDa to 43 kDa, depending upon the number of sites that are glycolsylated), associated with the 80 kDa acid labile subunit (ALS) in a GH-dependent large complex, leading to an increased residence time in plasma (17). The proteolytic enzymes that are bound to the apical side of tissue capillaries break down the large fraction into smaller GH-independent 40 kDa fractions (consisting only of IGF-I and IGF BP-3), that are capable to transfer into extra capillary tissues. This extravasation results in dissociation of IGF-I, and probably of IGF-II, from the 40 kDa complex, thereby enabling its biological activities in local tissues (18). The local level of free exchangeable IGF-1 is of biological importance for its paracrine/autocrine effects, such as cell proliferation, prevention from apoptosis and synthesis of nitric oxide (NO). In addition to the synthesis of IGF-1 in the liver, GH also stimulates IGF-1 expression in other tissues; which has local autocrine and paracrine actions (vide infra).

GH deficiency

The GH axis is one of the first hormonal axis that is defective in pituitary disease. Nowadays, the effects of a relative deficiency in GH axis/IGF system on metabolic processes are recognized. A decreased GH secretion, and subsequently, a decreased plasma level of total IGF-1 is observed in ageing and in patients with type 2 diabetes mellitus and/or premature atherosclerosis. In the general population, a low serum total IGF-1 level (without the analysis of GH secretion) gives rise to an increased risk on ischemic heart disease (IHD)(19). In addition, disturbances in the signalling of the GH receptor lead to a GH resistant state (defined as: inappropriately high serum GH with low serum total IGF-1 and IGFBP-3 levels) that is mostly associated with catabolic conditions, such as

Table 1: Cardiovascular mortality in patients with adult-onset GH deficiency, due to panhypopituitarism

Authors	Study design	Cardiovascular mortality	Remarks
Bulow, et al (26)	retrospective	increased; SMR 1.4	F>M; cerebrovascular > cardiovascular
Nilsson, et al (23)	retrospective	increased; SMR 1.6	F>M; cerebrovascular > cardiovascular
Bates, et al (27)	retrospective	not increased; SMR 1.2	
Tomlinson, et al (28)	prospective	increased; SMR 1.8	associated with androgen deficiency

SMR: standard mortality rate; F: female; Male:male

chronic heart failure, progressive cancer or end-stage renal failure (20; 21). Taken together, the term of GHD includes nowadays a broader range of separate insufficiencies or deficiencies in the GH axis IGF system than a decade ago.

Acromegaly
GH-secreting pituitary adenomas are the most frequent cause of acromegaly. Chronic high circulating GH levels (with plasma GH levels in the range from 5 to 500 ng/mL) result in an increased plasma IGF-I level, which results in several metabolic disturbances, one of which is insulin resistance.

Accelerated atherosclerotic disease
AGHD
In several retrospective studies, cardiovascular mortality in AGHD is increased in comparison with a matched healthy population. The first report by Rosèn and Bengsston showed an increased cardiovascular mortality in subjects with panhypopituitarism substituted with adrenal, gonadal and thyroid hormones, as compared to an age- and gender-matched control population (standard mortality rate, 1.8) (22). Subsequent reports have confirmed this observation (23-28) (Table 1).

No long term effects of GH therapy on the vascular mortality are known at present, although short term analysis of GH intervention in AGHD patients shows a decrease in the increased cardiovascular mortality. However, long term randomised follow-up GH intervention trials will definitively answer whether GH treatment in AGHD patients will result in reduction of cardiovascular mortality.

Acromegaly
Intriguingly, increased mortality from cardiovascular disease is observed in acromegaly. Although cardiomyopathy is presented as a major cause of death, atherosclerotic disease is equally reported as an underlying cause in acromegaly.

In general, the relationship between mortality due to cardiovascular diseases and disturbances in the GH axis/IGF system is best represented by a U-curve: revealing an increased mortality in both GH deficiency and in GH excess.

Lipoprotein metabolism
Disturbances in the GH axis/IGF system coincides with abnormalities in lipoprotein metabolism. Lipoproteins which originates from the intestine or liver, are major carriers for lipids in the circulation. Dietary fatty acids are absorbed in the small intestine, packaged into chylomicrons and secreted into the circulation via the lympatic system (exogenous lipid pathway). Chylomicrons display a size of 0.1 to 1.0 μM, and a chemical composition in weight percentage of TG 87%, cholesterol 3%, phospholipids 9% and proteins 2%. The major structural protein of chylomicrons is apolipoprotein B-48. In the circulation, chylomicrons are enriched with apo E which facilitate receptor-mediated uptake in the liver.

The liver secretes very low density lipoproteins (VLDL) that possess apo B-100 as their major structural protein. This pathway is called the endogenous lipid pathway. Major functional apolipoproteins are apo C-I, apo C-II, apo C-III and apo E. VLDL particles have a size between 300 and 800 Å, and chemical composition expressed as weight percentage consists of: TG 50-60%, cholesterol 17%, phospholipids 19% and proteins 10%. This pathway is called endogenous lipid pathway.

In the circulation, both chylomicrons- and VLDL-triglycerides are hydrolysed by lipoprotein lipase (LPL) via a so-called common saturable pathway (29). This enzyme is attached to the luminal side of the endothelium. Lipolysis is catalysed by apo C-II, and inhibited by apo C-III. The lipid particle which remains after lipolysis and intravascular remodelling by hepatic lipase and CETP,

is called a remnant particle. Apo B-48 containing remnant particles are preferentially taken up by apo B-48 receptors at the hepatic surface (30), whereas apo B-100 containing particles may either be taken up via the LDL-receptor by hepatocytes or be further processed into smaller lipid particles, such as intermediate density lipoprotein (IDL) and low density lipoproteins (LDL) that are more enriched in cholesteryl-esters by cholesteryl-ester transfer protein (CETP) (31).

High density lipoprotein (HDL) with apo AI as principal protein, removes cholesterol from the peripheral cells. HDL cholesterol is esterified by lecithin: cholesterol acyltransferase (LCAT), forming cholesteryl esters (CE). This HDL-CE is returned to the liver by: 1. transfer of CE from HDL to triglyceride-rich particles by CETP (as apo B-48 and apo B-100) or 2. by selective uptake by scavenger receptor B1(32).

Catabolic lipid pathways are mediated through receptors that are expressed at the hepatic surface. Apo B-48 lipid particles are taken up by LDL-receptors, apo B-48 receptors and LRP (LDL-receptor related protein) receptors (33). The LDL-receptor expression is dependent upon intracellular cholesterol content; the more cholesterol in the hepatocyte, the less expression of LDL-receptors. No relation is found between intracellular cholesterol content and hepatic LRP expression. Apo E facilitates particle uptake by the LDL receptor.

Glucose homeostasis

Glucose is a major substrate for metabolic fuel. Some tissues and cells are completely dependent on glucose (e.g. erythrocytes and brain) for their energy metabolism. Plasma glucose levels are therefore strictly controlled by several hormones. Dietary glucose is absorbed by enterocytes and delivered to insulin-sensitive tissues, such as liver and skeletal muscles for storage of glucose in the form of glycogen (glycogenesis). In the fasting and postabsorptive period, glucose is released by degradation of stored glycogen (glycogenolysis), and by gluconeogenesis. Glycogen stores are limited (150 g in the liver, and 300 g in skeletal muscles). The glycogene stores in skeletal muscles are direct sources for energy substrate (e.g. during exercise). Regulation of the glycogenolysis metabolism is reciprocal and depends upon two key enzymes: glycogen phosphorylase (glycogenolysis) and glycogene synthase (glycogenogenesis). These enzymes are activated by phosphorylation that is itself regulated by hormones (such as adrenalin, insulin and glucagon). Due to limited stores of glycogen, gluconeogenesis (GNG) will contribute most to the circulating glucose after 24 to 36 hours of fasting. The process of GNG takes place for 80% in the liver, and 20 % in the kidney. After a 10 day period of fasting, both kidney and liver contribute equally through GNG to the amount of circulating glucose. For GNG, the precursors are pyruvate and lactate (a total contribution of 35%) that are derived from the red blood cell and skeletal muscle, alanine (a total contribution of 35%) that is derived from skeletal muscle, and glycerol (a total contribution of 8 %) that is derived from adipose tissue. Entry in the GNG pathway is at three levels: 1. through pyruvate (lactate and alanine), 2. through phosphoenolpyruvate (glutamate) and 3. through dihydroxyaceton phosphate (glycerol). Its regulation occurs at different levels in the GNG pathway, that depends mostly upon glucagon, the substrate availability (alanine), the NADH/NAD+ balance and the level of available ATP. Fatty acids degradation provides additional components, such as acetyl-Co A and NADH/NAD+, that facilitates GNG.

Under physiological conditions, catabolism of amino acids from the skeletal muscle to supply substrate for GNG is quantitatively not important. However, in pathological conditions, the release of alanine by skeletal muscle, as a precursor for GNG, results in an increase in GNG (through pyruvate), but also of an increase of urea synthesis through glutamate (Felig Cyclus). Consequently,

increased plasma glutamate levels are found. All steps that limit the availability of glucose for degradation (glycolysis) are related to the biological action of insulin. Insulin resistance may be a manifestation of a defect in glucose transport (GLUT4; in muscle and adipose tissue), in decreased expression of enzymes required in the glycolytic cascade (ie hexokinase, glucokinase, fosfokinase I, pyruvate dehydrogenase) or further downstream in the glycolytic pathway. In general, progressive hyperglycemia, or finally type 2 diabetes mellitus, is due to combination of peripheral insulin resistance and impairment in insulin secretion by the insulin-secreting β-cells in the pancreas (34). The function of insulin secreting beta cells is under influence of the different receptors that are involved in the action of insulin and the IGF system. The capacity to compensate for hyperglycemia is related to the maximal insulin secretion. Indeed, in type 2 DM patients who are unable to compensate for hyperglycemia, a decrease in β-cell mass, due to increased apoptosis of insulin secreting cells, was detected (35). Local growth factors, such as IGF-I and IGF-II, control apoptosis (in case of IGF-I), and increase β-cell growth (in case of IGF-I and IGF-II). Receptors for IGF-I and IGF-II are present in the pancreas. Knock-out mice for the IGF-I receptor in the pancreas cell showed an absent first phase and a blunted second phase insulin secretion response (36). IGF-II is a part of the glucose sensitising mechanism in the pancreas that forms an autoregulatory loop to control the definite insulin secretion; insulin secretion is known to adapt to systemic needs for insulin, and in systemic insulin profiles mostly reflect peripheral sensitivity to insulin action in man (37).

GH/IGF and myocardial adaptation

Both GH and IGF-I have trophic effects on cardiac muscle. Receptors for GH and IGF-I are found on the surface of cardiomyocytes, but also in the endothelium of the coronary artery. The expression of the IGF-I receptor in cardiomyocytes is facilitated by GH. An increase of systemic GH, due to hormonal substitution in AGHD or in excess in acromegaly, give rise to vasodilation of arteries, with a decrease in cardiac afterload. The ejection fraction of the left ventricle increases after start of GH therapy in AGHD. In excessive amounts of systemic GH, a decreased after load increases heart frequency to maintain constant heart minute volumes, and subsequently the work load of the heart increase. Such long-term periods definitely result in diastolic dysfunction, with a decrease in left ventricular function.

In situations of an increased work or stress load, the heart is in adaptation. During adaptation, cardiomyocytes express more local tissue IGF-I. The expression of local IGF-I is under influence of the GH axis that determine the level of IGF-I receptors on the cardiomyocyte. The exact pathway that regulates the local expression of IGF-I in tissue is not elucidated yet. Downregulation of the expression of IGF-I with a subsequent rise in angiotensin-II gives maladaptation with heart failure. Therefore, IGF-I has trophic effects, controls apoptosis and hypertrophy of cardiomyocytes. Hypertrophic adaptations of the heart muscle are also found in cardiomyopathy, that most frequently results from ischemic heart disease (38). Indeed, although results are not yet conclusive, intervention with GH in patients with ischemic heart disease gives rise to an increase in ejection force of the left ventricle, and GH substitution may therefore be beneficial in this kind of patients (39).

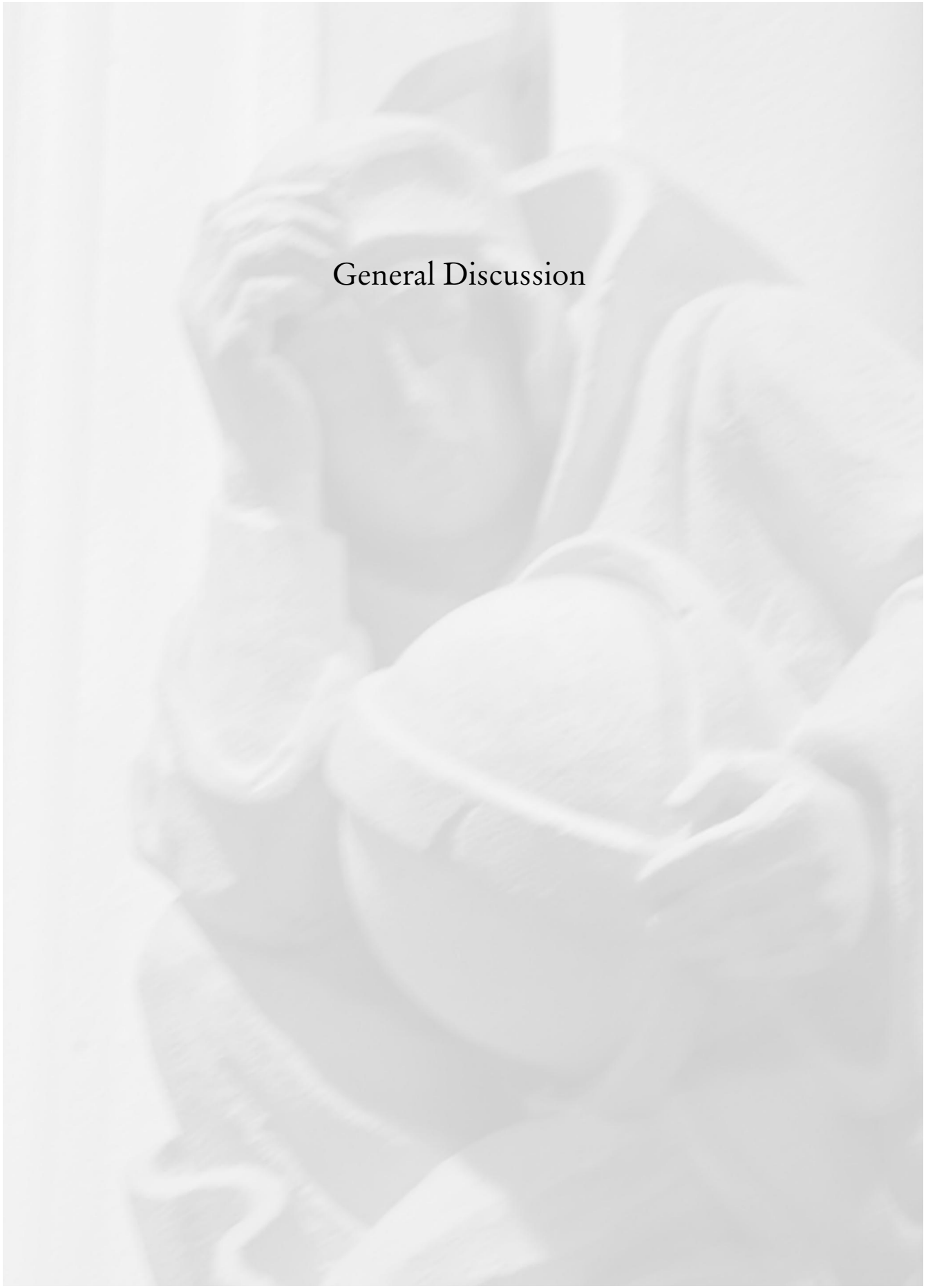
Outline of the thesis

To study metabolic disturbances in lipoprotein metabolism in the fasting and postprandial state, that may explain the increased cardiovascular risk, observed in patients with disturbances in the GH axis/IGF system (adult-onset growth hormone deficiency (AGHD) and acromegaly).

To study the suitability of a recently introduced immuno-separation method (remnant-lipoprotein particle; RLP) to further characterize the atherogenic lipoprotein phenotype in patients with disturbances in the GH axis/IGF system, and in patients with heterozygous familial hypercholesterolemia (FH).

To study disturbances in glucose homeostasis occurring during dysfunction in the GH axis/IGF system.

To study the relationship between cardiac function and acromegaly.



General Discussion

Endothelial dysfunction, as a surrogate marker for atherosclerotic disease Previous studies in adult-onset GH deficiency have revealed a significant association with GH deficiency and the progression atherosclerotic disease (4; 4; 22). Pfeifer et al (40) showed an increased carotid intima-media thickness in AGHD, which was decreased by growth hormone treatment. In addition, the function of the endothelium is impaired (**chapter 1.6**). GH and IGF-1 both have an inductive effect on the endothelial nitric oxide (NO) system. In GH deficiency, a deficit in NO production occurs in the endothelium. This depletion in endothelial NO is associated with endothelial dysfunction, and thus early atherogenic disease (41;42). In **chapter 1.6**, the flow mediated dilation (FMD) in AGHD patients was decreased before the start of GH therapy (5.9 ± 3.3%), and improved after substitution with GH to 10.2 ± 4.0%. An improvement of endothelial function, arterial stiffness and IMT in AGHD patients after the start with GH therapy is supported by several recent studies (43; 44). Evans et al (45) indicate that an increased oxidative stress, measured as the concentration of lipid-derived free radicals by paramagnetic resonance, may influence endothelial function in AGHD patients. Treatment with GH results in improvement of these parameters. Baseline FMD values (**chapter 1.11**), were impaired in active acromegalic patients (5.4 ± 3.1 %, Twickler et al, personal communication). Although treatment of active acromegalic patients will improve the FMD, the values are still not in the range that are observed in healthy subjects, matched for BMI, age and sex (46). These observations confirm the importance of the GH axis/IGF system in endothelial dysfunction.

Atherogenic Lipoprotein Phenotype in AGHD

The origin of the "progressive", but also to some extent rapidly reversible, atherosclerotic disease in AGHD patients, as shown by distinctive methods (such as IMT, FMD and arterial impedance), remain a subject of discussion. It has been reported that the elevated plasma LDL-cholesterol levels in AGHD patients (47) are the most prominent risk factor. However, plasma LDL-cholesterol is only marginally elevated (**chapter 1.5, 1.6, 1.7**) (3.37 - 4.12 mmol/L)(48). The clinical impact of small elevations in LDL-cholesterol levels is still under debate, and moreover, no point is obtained for a borderline high plasma LDL-cholesterol in the risk assessment in the NCEP score sheet that estimates the 10-year risk. During GH therapy, plasma LDL-cholesterol level in AGHD patients decrease by 16% (**chapter 1.6**). According to ATP III of the NCEP guidelines, in **chapters 1.4, 1.5, 1.6 and 1.7** plasma TG levels in AGHD patients are within the borderline high range from 1.71 to 2.27 mmol/l. After starting GH therapy, plasma TG levels in AGHD patients tend to increase. Plasma HDL levels are in the normal range, and decrease only slightly during GH treatment. LDL-cholesterol, TG, and HDL-cholesterol do not completely explain the progressive atherosclerotic disease and increased cardiovascular mortality in disturbances in the GH axis/IGF system.

Triglyceride-rich remnant particles (TRP) are of special interest in the assessment of an atherogenic lipid phenotype. Several studies have shown that the importance of smaller, more atherogenic, TRPs (such as intermediate density lipoprotein; IDL), are related to carotid artery IMT (MARS study (49)). The calculated non-HDL cholesterol was a better predictor for cardiovascular disease than plasma levels of LDL-cholesterol. The mortality after a first cardiovascular ischemic event is dependent upon plasma RLP-C levels at entry of the study (50). In an evaluation of fasting and postprandial plasma RLP-C levels in healthy subjects, the fasting plasma RLP-C levels were <0.20 mmol/L, and tended to be higher in male than in female subjects (**chapter 1.2**). Postprandially the RLP-C peak level was at 3 h, while the peak level of retinyl ester, a marker for

dietary fatty acid uptake and metabolism, was 2 h later, thereby illustrating that both markers represent different features of the intravascular metabolism of the TG-rich particle fraction. Probably, RE that is isolated from the Sf<1000 fraction, represent the fate of larger-sized TG-rich chylomicron particles (Sf<1000). Postprandial peak in RE Sf<1000 was higher in AGHD patients than in normolipidemic control subjects. In line with recent literature, RLP-C is considered to be more closely related to the atherogenic process in vivo, such as endothelial dysfunction, IMT and induction of pro-inflammation (**chapter 1.8, 1.11**). This recent focus on lipoprotein remnants could be an important development in defining more closely the atherogenic lipid phenotype, in general and in patients with disturbances in the GH axis/IGF system in particular.

Plasma RLP-C levels in AGHD patients are 1.5 fold increased, as compared to control subjects (table 1). In acromegaly, plasma RLP-C levels were even higher (0.40 ±(0.13 mmol/l). In Japanese patients, in whom normal plasma RLP-C levels are twofold lower than in Caucasians, plasma RLP-C > 0.14 mmol/l are a strong predictor of subsequent cardiovascular events (OR 6.38, 95% CL 2.3-17.6; p<0.01), even after the inclusion of high LDL-cholesterol (> 3.4 mmol/l) in the Cox analysis (50). In a subanalysis of the Framingham study in which the relationship between plasma RLP-C levels and cardiovascular events in postmenopausal women was investigated, plasma RLP-C of more than 0.14 mmol/L were associated with an increased cardiovascular mortality, independent from plasma levels of TG, HDL- and LDL-cholesterol (51). In conclusion, plasma RLP-C levels are part of the atherogenic profile in AGHD patients.

Knowledge of plasma RLP-C levels in primary dyslipidemic diseases, such as heterozygous familiar hypercholesterolemia, is rare. Familiar hypercholesterolemia is characterized by a defect in the function of the LDL-receptor, leading to accumulation of LDL-cholesterol. This defective removal of plasma LDL-cholesterol may give rise to a disturbed removal of lipoprotein remnants. Notably, the LDL-receptor is also part of the lipopro-

Table 2: Plasma fasting and postprandial RLP-C levels in populations at elevated cardiovascular risk.

	Plasma RLP-C (mmol/L) levels	Area under the incremental postprandial RLP-C curve (mmol/l/h)
Healthy control subjects	0.18 ± 0.06	1.14 ± 0.61
Patients		
Familial Hypercholesterolemia (FH)		
Before Statin therapy	1.09 ± 0.50[a]	3.14 ± 1.8[a]
During Statin therapy	0.26 ± 0.12[b]	0.34 ± 0.6[b]
Adult-onset GH deficiency (AGHD)		
Before rhGH therapy	0.29 ± 0.14	2.13 ± 1.60[a,b]
During rhGH therapy	0.32 ± 0.09	0.73 ± 0.34
Acromegaly	0.40 ± 0.13[a]	2.14 ± 1.19[a]

All values are expressed as mean ± SD. [a]: P<0.05, compared to control subjects, [b]:P<0.05, patients treated vs. untreated.

tein remnant removal pathway. Ongoing discussion exists regarding the presence of lipoprotein remnant accumulation in FH in the postprandial period. In **chapter 1.10 and 1.11**, we showed that plasma RLP-C levels in heterozygous FH patients are increased in both baseline as well as in the postprandial state (Table 2). Treatment with high dose simvastatin (80 mg once a day) decreased, but did not completely normalise plasma RLP-C levels. Therefore, focussing on LDL-cholesterol profile only in these patients may limit the assessment of the atherogenic phenotype and the interpretation of treatment goals. To confirm the hypothesis that plasma RLP-C is related to atherosclerotic disease, IMT of the carotid artery was analysed together with the RLP-C profile, in a subset of FH patients (**chapter 1.11**). Indeed, the carotid artery IMT was positively associated with plasma RLP-C levels, but LDL-cholesterol levels remain the major predictor for IMT in a multivariate analysis. These observations strengthen the hypothesis that plasma RLP-C levels complete the atherogenic profile, even in the classic dyslipidemia FH.

Previous studies have confirmed that increase in postprandial RLP-C is related to abnormality in endothelial function. Reduction in postprandial plasma levels of RLP-C by statin treatment attenuates endothelial function (52;53). As stated previously, endothelial dysfunction is one of the first key steps in athrosclerotic diease. Postprandial plasma RLP-C levels are increased in both AGHD (**chapter 1.6**), and in active acromegalic patients (**chapter 1.12**). In addition to disturbances in the GH axis/IGF system, these elevated postprandial RLP-C levels increase the atherogenic burden. This conclusion is confirmed by Doi et al who found that incubation of RLP in endothelial models shows a dose-dependent effect of RLP on the endothelial function (54). Further evidence was obtained by Dichtl et al (55) showed that an increase of arterial expression of NF-$\kappa\beta$, VCAM-I, ICAM-I, and TNF-α was found in rats, but that this phenomenon occurred after a lag time of 12 hours after infusion of VLDL. A pro-inflammatory reaction is recognized as one of the first steps in the initiation of early atherosclerosis (56-58). In **chapter 1.8**, an induction of the inflammatory response with an increase in TNF-α and IL-6 in AGHD patients was found in the postprandial period. The peak level of both TNF-α and IL-6 was found between 10 and 12 hours after intake of the fat, and 6 to 8 hours after the peak level of RLP-C with a positive association between the postprandial IL-6 and RLP-C profile ($r^2=0.44$, $P<0.05$).

In the classic representation of initiation of the atherosclerotic process, oxidation of LDL is an important step. After retention of LDL in the subendothelial space, oxidative processes and oxidized LDL give rise to inflammation (59). Huff et al (60) previously showed that lipolysed triglyceride-rich particles (remnants) may be oxidized and taken up in macrophages. In line with these results, we found that RLP-C of type II-B dyslipidemic patients can be easily oxidized (**chapter 1.3**). Moreover from FPLC analysis we learned that 85 % of the RLP in type II-B dyslipidemic patients is in the VLDL-1 range, and 15 % in the VLDL-2 and IDL size range. A recent report noted that VLDL-1 is a favourable substrate for lipid accumulation in human monocyte-macrophages (61). The in vitro experiments indicate that RLP is a fraction that induces early atherogenic components, such as oxidation and foam cell formation (not shown in the present results), and that induction of a postprandial inflammatory response in AGHD patients may be caused by RLP related interactions with the endothelium and components in the subendothelial space.

In GH deficiency, the expression of the hepatic LDL-receptors is decreased in both human and in animal models (62). Decreased removal of lipoprotein particles by the LDL-receptor result in higher plasma levels of LDL-cholesterol and RLP-C. (figure 2) Additionally, a second receptor, LRP, is

involved in the removal of lipoprotein remnants so that the removal of RLP-C is only partly inhibited. An increase in synthesis and secretion of apo B-100 VLDL (enriched in TG) in AGHD patients is found (63; 64). However, the intravascular remodelling of TG-rich lipoproteins by lipolysis in AGHD patients remained unchanged because no decrease in postheparin activity of both lipoprotein lipase and hepatic lipase was found (in contrast to rodents) (65). During GH therapy, cholesterol synthesis, reflected by plasma concentrations of cholesterol intermediates as mevalonic acid, is still increased, compared to control subjects (**chapter 1.7**). However, plasma LDL-cholesterol is decreased in AGHD patients due to the up-regulation of the hepatic LDL receptor (66). GH substitution in a LDL-receptor knock-out model showed an increase in the 7-alpha hydroxylase pathway (67). This leads to the hypothesis that a larger part of the intrahepatic cholesterol pool will be released into the bile acid pool, resulting in an increased removal of cholesterol from the circulation via LDL uptake. In humans, depletion of intracellular cholesterol also reduces synthesis of the VLDL-2 sub-fraction (68). Thus, although plasma LDL-cholesterol levels in AGHD patients decrease during GH therapy, plasma RLP-C and VLDL-1 remain elevated, due to the fact that TG secretion is still elevated.

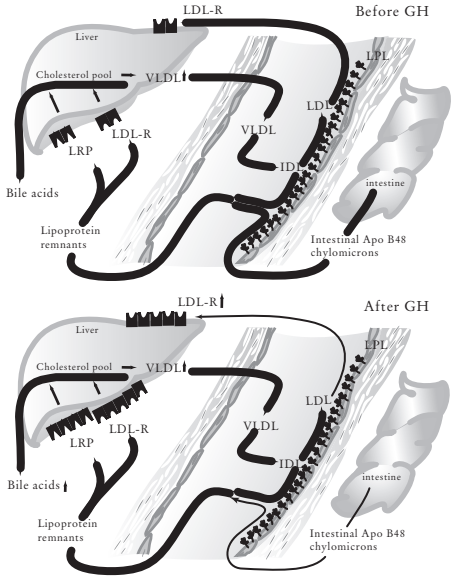


Figure 2 The relastionship between the GH axis/IGF system and the lipoprotein metabolism; the effect of GH therapy

In acromegaly, plasma RLP-C in both the fasting and postprandial state was elevated. The origin of this disturbed RLP-C profile is related to a lower postheparin LPL activity in active acromegalics (**chapter 1.12**). This reduced LPL activity give rise to postprandial accumulation of both intestinal and liver derived TRP. Fasting plasma levels of TG was positively related to the postprandial RLP-C response. Besides the deficient intravascular remodelling pathway, the increased insulin resistance, that is reflected by an elevated HOMA index, will give rise to a disturbed postprandial lipoprotein metabolism, with an increase in especially the VLDL-1 pool and dense LDL. As previously stated, RLP fraction is mostly within the size range of VLDL-1, and this physical relation may explain its increased baseline levels in active acromegaly.

The baseline plasma RLP-C levels are closely associated with postprandial RLP response, when studying postprandial mtabolism. Postprandial studies are time consuming and laborious for both patient and clinician. Consequently, Schaeffer et al (69) therefore questioned whether a postprandial approach is necessary to define the atherogenic lipid phenotype, with a final negative conclusion From our results, the RLP-C response was associated with baseline RLP-C in both control subjects, as in studied patient groups (AGHD, FH, and acromegaly).

GH/IGF and pro-diabetic phenotype

Glucose homeostasis in GH deficient patients is similar to that in normal subjects. Several reports have shown a decreased glycogen store in skeletal muscle, and a decrease in insulin sensitivity. In contrast, we and others were not able to find any difference in the insulin sensitivity. GH therapy in

AGHD patients is the cause of a shift in glucose homeostasis with a decrease in insulin sensitivity. The amount of rhGH substitution is directly associated with the insulin sensitivity. Therefore, lower start dosages of daily rhGH are not associated with major changes in insulin sensitivity. Indeed, we have observed (**chapters 1.5, 1.6, 2.4, 2.5**) a decrease in insulin sensitivity (as reflected by HOMA index) in AGHD patients during GH therapy. Although, fasting plasma insulin increase as a consequence of GH therapy, no hyperglycaemia occurs. The decrease in insulin sensitivity during GH therapy is therefore compensated by an increased insulin secretion. In male AGHD patients, the increase of HOMA index during GH therapy was not significantly increased. In our studies, the dosage of GH was low (0.5 IU/day) at the start, and was titrated to age and sex adjusted normal IGF-I levels. In excessive increased GH levels, such as in active acromegaly, insulin resistance (reflected by HOMA) is higher and the HOMA index is associated to the plasma IGF-I levels. The insulin resistance in AGHD patients, that is reached during GH therapy (with equal amounts of GH administered daily), is also related to pre-treatment IGF-I levels (**chapter 2.4**).

The major substrate for the energy supply in humans is glucose, but glucose may be partly replaced by fatty acids during decreased insulin sensitivity. Glucose remains the principal substrate for energy supply during GH therapy, despite the increase in plasma FFA levels. In our study, these changes from glucose to fat as a preferable substrate for energy supply in AGHD male patients did not reach a significant difference after a 12 months' GH therapy. Glucose oxidation contributes for 55 % of the total oxidation in mild obese control subjects

In addition to glucose that is derived from exogenous sources (diet), glucose is actively formed in several tissues (muscle and liver) through GNG and GL (Figure 3). Both processes occur simultaneously. Several steps in GL are pertubated in children with a GH deficiency, and therefore a state of hypoglycemia develop during a fasting period. In GHD in adulthood, GL contributed most to total glucose turnover. During GH therapy, the contribution of GNG increases. The major precursor for the increased GNG is pyruvate, that is derived from acetyl Co-A. This last molecule is part of the fatty acid oxidation pathway (β-oxidation), which is induced by GH.

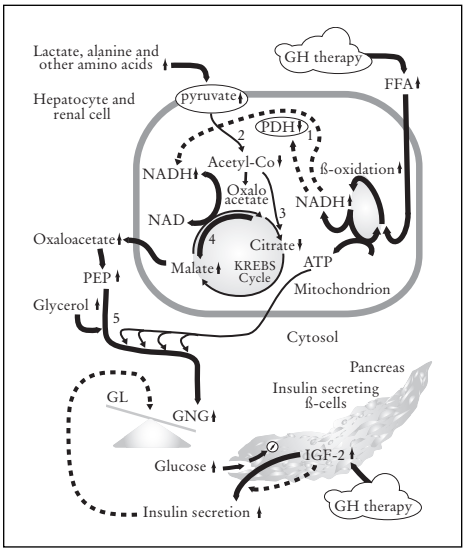


Figure 3. Adipose tissue lipolysis increases due to GH therapy, with consequently higher plasma free fatty acid (FFA) levels. In the liver, oxidation of FFA gives rise to an increase of β-oxidation and formation of NADH. Increased mitochondrial levels of NADH lead to a decrease in the activity of pyruvate dehydrogenase (1) with consequently less production of acetyl Co-A, and therefore less substrate for the Krebs cyclus (3). Equally, elevated NADH levels increase pyruvate carboxylase with an increase in the production of oxaloacetate (2); in addition, increased availability of NADH favours conversion of oxaloacetate to malate (4). Hereafter, cytosolic oxaloacetate is converted to phosphoenol pyruvate (PEP), that results in increase of gluconeogenesis (GNG). In the fasting state, glycogenolysis (GL) is the principal glucose provider. A regulated balance exists between GNG and GL, that is slightly in favour of GNG during GH therapy (5). The increase in circulating glucose is detected by pancreatic β-cells. An increase in intrapancreatic glucose level increases de-novo insulin synthesis and secretion. This process is facilitated by IGF-2 (6). Within this background, prenatal development of the capacity of the insulin secreting cell may be determined by plasma levels of IGF-2, that are highly genetically determined. The increase in insulin secretion, which is dependent on the capacity of the β-cell, normalises hyperglycaemia.

Substitution of GH increases the efflux of glutamate from the liver, that is part of a nitrogen (N) sparing pathway. Indeed, in **chapter 2.5**, plasma levels of glutamate in AGHD patients were higher (with a decrease in 24 h urine content) during GH therapy, than in control subjects. The plasma glutamate levels are increased in pre-treatment AGHD patients, as compared to control subjects.

To overcome insulin resistance, insulin secretion increases. The reserve capacity of insulin secreting β-cells in the pancreas is therefore of importance. In healthy non-diabetic humans, a positive association was found between insulin secretion and IGFBP-3 (**chapter 2.2**), and IGF-II (**chapter 2.3**). The relationship with IGF BP-3 was decreased, after correction for BMI. IGF BP-3 is the principal transporters of both IGF-I and IGF-II in circulation. The IGF-II pool is larger that IGF-I pool in human adults. Plasma levels of IGF-II are an important factor for the development of the individual capacity to secrete insulin. In foetal life, IGF-II is an essential component in the development of the pancreas, and in tissues of mesodermal origin. In line with Barkers' hypothesis, it may be argued that pancreatic insulin secretion capacity is predetermined, because 66% of the IGF-II levels are genetically determined as has been reported in human twin studies. In adult life, the paracrine and autocrine effect of IGF-II prevents apoptosis of pancreatic beta cells. An increased apoptosis of beta cells with a decrease in mass was found in insulin dependent type II diabetes. The biological effect of IGF-II is mediated by IRS-2 pathway. The limited insulin secretion capacity may therefore be mostly determined by the amount of circulating IGF-II. Lower IGF-II levels may provoke a faster expression of a prodiabetic phenotype. In dorsal pancreas agenesis, a decrease in beta cell mass results in a modest increase in GNG, also a feature in the syndrome of insulin resistance. Insufficiencies in beta cell function accounts therefore for several key symptoms in insulin resistance. Higher plasma levels of IGF-II, therefore, improve the capacity of the insulin secreting beta cells to compensate for hyperglycaemic events. In line with this conclusion, the negative association that was found in a GH intervention in AGHD male patients, compared to mild obese matched control subjects, between GNG and IGF-II may be a consequence of an adequate insulin response. Type II diabetes has an increased GNG due to less inhibition of the GNG process by insulin.

GH/IGF and heart adaptation

The GH axis/IGF system, through an autocrine and systemic effect, influence the capacity of cardiomyocytes to adapt on both volume and pressure stress. In a case report (**chapter 3.2**), we described that a decrease in plasma GH levels (and not IGF-I) was associated with a decrease of chronic hypertrophic cardiomyopathy in a patient with active acromegaly. Simultaneously, the ejection fraction improved. GH receptors are abundantly present on the heart, and activation of these local GH receptors result in trophic changes and a decrease in apoptosis. If GH is chronically present in excess, the expression of local GH receptors is disturbed that lead to a GH resistant state. Less stimulation of the GH receptor results in less induction of local IGF-I. Consequently, the heart will enter in a maladaptation process, which leads to overt heart failure (**chapter 3.1**).

To conclude, the data that are presented in this thesis indicate that:

1. The assessment of RLP-C is essential in assessing the atherogenic lipoprotein phenotype.
2. RLP possess atherogenic properties, such as oxidizability and foam cell formation.
3. The metabolism of fasting and postprandial RLP-C in AGHD is disturbed and postprandial RLP-C profile is improved during GH therapy. These disturbances in RLP metabolism may be partly responsible for the high susceptibility to premature atherosclerosis in AGHD.
4. Endothelial dysfunction in AGHD patients improves during GH therapy.
5. The postprandial RLP-C profile is related to a pro-inflammatory response.
6. In heterozygous FH patients, increased fasting plasma RLP-C levels are part of the atherogenic lipid phenotype (independent from plasma LDL-cholesterol levels). Simvastatin (80 mg) reduces RLP-C levels to within the normal range in a quarter of FH patients.
7. Postprandial RLP-C response in heterozygous FH patients decreases during Simvastatin therapy.
8. Plasma RLP-C levels in heterozygous FH patients are associated with an increased carotid IMT, but not independent of plasma LDL-cholesterol.
9. The fasting and postprandial RLP-C profile is disturbed, not only in GH deficiency, but also in acromegaly, due to a decreased insulin sensitivity and decreased LPL activity.
10. Insulin secretion in adult healthy subjects is related to the IGF system through plasma IGF-II.
11. Baseline plasma IGF-I levels in AGHD patients determine the decline in insulin sensitivity during GH therapy.
12. The contribution of GNG in AGHD patients increase with pyruvate as major GNG precursor
13. In addition to insulin secretion capacity of adult pancreatic b-cells, plasma IGF-II levels are inversely associated with the amount of GNG. The adult plasma levels of IGF-II, which is mostly genetically determined, therefore link two major features of the insulin resistance syndrome.
14. Severe dysfunction of the left ventricle in acromegaly is reversible, and is closely related to plasma GH levels.

Prospectives

Clinical features that are related to disturbances in the GH axis/IGF system (such as disturbed lipoprotein remnant metabolism, endothelial dysfunction, increased visceral fat with obesity, hypertension, relation with insulin secretion and gluconeogenesis) resemble closely to the entity syndrome X or the plurimetabolic syndrome. Analysis of the GH axis/IGF system will therefore teach us more about insulin resistance, unravel the plurimetabolic syndrome, and may give rise to novel therapeutic approaches.

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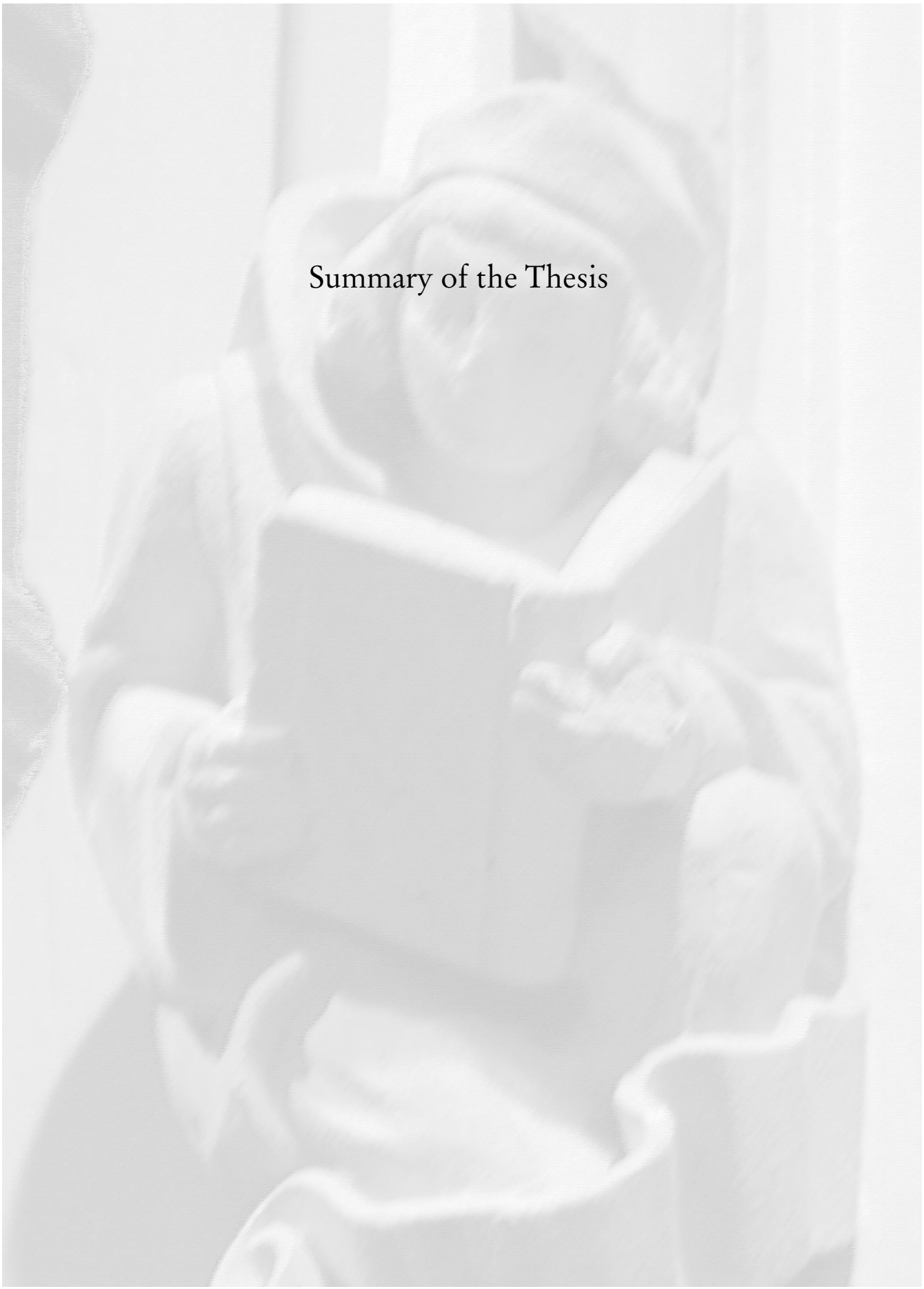


Summary of the Thesis

Atherosclerosis is a major cause of premature cardiovascular morbidity and mortality in the Western World. One of the principal risk factors in atherosclerotic disease is dyslipidemia with a major focus on elevated plasma LDL-cholesterol levels. However, triglyceride-rich particles also possess atherogenic properties and elevated fasting (and postprandial) plasma levels of these particles are therefore associated with an increased cardiovascular morbidity and mortality (***Chapter* 1.1**). Postprandial levels of lipoprotein remnants reflect the composition and quantity of the diet and lipoprotein metabolism itself. Because Western individuals eat at least three times a day, and intrinsic defects in lipoprotein metabolism are frequently present, most of these people are in an accentuated postprandial state during the day. In the recent NCEP ATP III guidelines, the importance to treat high plasma triglyceride-rich particle levels in order to reduce cardiovascular disease was emphasized. It is relevant that cardiovascular mortality is increased in patients with disturbances in the growth hormone (GH) axis/insulin-like growth factor (IGF) system (such as in GH deficiency and acromegaly). GH is secreted from a small gland just down the brain; the pituitary. GH induces IGF and IGF-related proteins principally in the liver, and other functional tissues. Probably, disturbances in the complex GH IGF system are involved in atherogenesis, via changes in lipoprotein metabolism and in endothelial nitric oxide synthesis (***Chapter 1.4***). The synthesis of nitric oxide by endothelial cells leads to vasodilatation and influence structures in the subendothelial matrix (such as smooth muscle cells). Such an interaction between hormones and/or hormone-like growth factors and the pathophysiology of the cardiovascular system is encapsulated in a recently introduced term, cardiovascular endocrinology.

The presence of increased plasma triglyceride-rich particle levels, and especially of lipoprotein remnants (RLP), has been assessed in several patient groups with an increased risk of cardiovascular disease. In ***Chapters 1.9 and 1.10***, we showed that both fasting and postprandial plasma RLP-C levels were elevated in patients with a primary atherogenic dyslipidemia: heterozygous familial hypercholesterolemia [FH]. In addition, in ***Chapters 1.5, 1.6, 1.7 and 1.12*** in patients with a secondary dyslipidemia: overt acromegaly, and growth hormone deficiency [GHD]) displayed similar increased fasting and postprandial levels, as compared to healthy BMI, age and gender-matched control subjects (***Chapter 1.2***).

Although plasma RLP levels were elevated in all these patient groups, the origin of these disturbances was detected at different levels;

1. in the postheparin lipoprotein lipase activity (lower in overt acromegaly; ***Chapter 1.12***),
2. in the production of very low-density lipoproteins (increased in GHD and FH; ***Chapters 1.6, 1.7, and 1.9***),
3. in the activity of cholesteryl ester transfer protein (lower in GHD),
4. and in the expression of hepatic LDL-receptors (decreased in FH and GHD; ***Chapters 1.7 and 1.9***).

The atherogenic process consists of several initial steps of which a key feature is endothelial dysfunction. Treatment with recombinant growth hormone (GH) decreases the rise of plasma RLP levels after a fatty meal, and improves endothelial dysfunction in adult-onset GHD (***Chapter 1.6***). Moreover, such increased postprandial plasma RLP levels induced a pro-inflammatory state, a condition that is associated with premature atherosclerosis (***Chapter 1.8***). In addition, fasting plasma RLP-C in FH patients are associated with elevated carotid artery intima media thickness, a surrogate marker of atherosclerotic disease (***Chapter 1.11***). In in-vitro studies, RLP consist mostly of cholesteryl ester and TG, have mostly the size of VLDL-1 (85%), and could be easily oxidized and give rise to formation of macrophage foam cells (***Chapter***). With these

results, we showed that RLP particles are closely associated to atherogenic processes. In line with this remark, elevated plasma RLP levels make an individual more susceptible to develop premature atherosclerotic disease.

Type 2 diabetes mellitus is generally due to a combination of insulin resistance and impairment in insulin secretion by the pancreatic β-cell. Progression towards type II DM is related to failure of insulin secreting cells to compensate hyperglycaemia. Deterioration of glucose homeostasis, although mostly limited and temporarily, is noted after the start of GH therapy in AGHD patients (as presented in ***Chapters 1.5, 1.6, 1.7, and 2.4***) and more profoundly, in overt acromegaly (***Chapters 1.12 and 3.2***). Besides a relationship between the GH axis/ IGF system and plasma RLP-C levels, a similar relationship (but hitherto only observed in animal studies) is found with glucose homeostasis, and insulin secretion (***Chapter 2.1***). A central component of the IGF system, IGF-2, was positively related to insulin secretion in healthy adults, independent of IGF-I, IGF BP-3 and BMI (***Chapters 2.2 and 2.3***). Receptors of IGF-2 are found on the insulin secreting cells in the pancreas, and IGF-2 is a principal factor in the development of mesodermal tissue, such as the pancreas, muscle, liver and the heart. The variability of GH independent plasma IGF-2 levels is mostly genetically determined (66%), and therefore the total capacity of insulin secretion is partly determined in the prenatal period. A decline in insulin secretion is a key symptom in type II diabetes mellitus, as is an increase in glucose synthesis through the gluconeogenic (GNG) pathway. Indeed, we observed a negative correlation between plasma IGF-2 levels and GNG in GHD patients before and during GH therapy, in comparison to age and BMI matched control subjects (***Chapter 2.5***). Moreover, the degree of decrease in insulin sensitivity (as determined by estimated HOMA index) due to GH therapy is related to baseline plasma IGF-1 level (***Chapter 2.4***). The increase of GNG during GH therapy has its origin in increased oxidation of the higher circulating free fatty acid levels, that supply energy to the GNG pathway (with pyruvate as a dominant substrate) (***Chapter 2.5***). The increase in plasma glucose levels is almost completely compensated by higher circulating insulin levels.

In addition to relationships between the IGF system and insulin secretion, GNG and lipoprotein metabolism, cardiomyocytes are highly influenced by the local and systemic GH/IGF system (Chapter 3.1). High plasma levels of IGF-1, but more special GH, were responsible for heart failure due to cardiomyopathy. After strict control of such increased GH levels, the impaired heart function could be progressively restored to normal (***Chapter 3.2***).

In conclusion, plasma levels of lipoprotein remnants, which are analysed with the immuno-isolation method, are suitable in testing the susceptibility to premature atherosclerotic disease. Moreover, several metabolic (such as lipoprotein remnants and glucose homeostasis) and endothelium-related components that are considered as key factors in atherogenesis are related to disturbances in the GH axis/IGF system.

Résumé de la Thèse

L'athérosclérose est la cause principale de la morbidité et de la mortalité cardio-vasculaire précoce dans le monde occidental. Un des facteurs de risque cardiovasculaire majeurs est la hyperlipoprotéinémie, comportant une augmentation des taux plasmatique de cholestérol-LDL. De plus, les particules riches en triglycérides possèdent aussi des actions pro-atherogènes et des taux élevés (à'jeuns et postprandiaux) sont associés à une morbidité et mortalité cardiovasculaire augmentée (***Chapitre 1.1***). Les taux postprandiaux des lipoprotéines remnants dans le sang sont la résultante conjointe de l'alimentation, et du métabolisme lipidique soi-même, tous les deux. Parce que l'homme occidental mange au moins trois fois par jour, et que les anomalies du métabolisme lipidique sont fréquents, il se trouve la majeure partie la journée en condition postprandiale. Dans les recommandations de NCEP ATP III l'importance du traitement des taux plasmatique élevés des lipoprotéines riches en triglycérides est soulignée pour la réduction de la maladie cardiovasculaire. Une observation supplémentaire est l'augmentation des maladies cardiovasculaires chez les patients avec une pathologie de l'axe somatotrope (comme en cas de déficit de GH et d'acromégalie). Les anomalies de l'axe GH/system d'IGF peuvent démarrer l'athérogénèse, par les modifications du métabolisme lipidique et par une modification de la synthèse d'oxyde nitrogène (NO) d'origine endothéliale (***Chapitre 1.4***). L'endocrinologie cardiovasculaire est un terme récent qui rassemble l'interaction entre le système endocrine et cardiovasculaire.

La présence de taux plasmatiques élevés de particules riches en triglycérides, et principalement des remnants de lipoprotéines (RLP), est déterminée dans plusieurs groupes de patients dont le risque cardiovasculaire est augmenté. Aux ***Chapitres 1.9 et 1.10***, nous avons montré que les taux plasmatiques RLP (à'jeun et postprandiaux) sont élevés chez des patients avec une hyperlipoprotéinémie primaire: hypercholestérolémie familial hétérozygote [FH]. De plus, aux ***Chapitres 1.5, 1.6, 1.7 et 1.12*** chez des patients avec une hyperlipoprotéinémie secondaire: acromégalie, et déficit en GH [GHD]) ont des taux élevés, en comparaison avec des sujets en bon santé (***Chapitre 1.2***).

Bien que les taux plasmatiques RLP étaient élevés dans ces groupes de patients, l'origine de ces anomalies est reliée à plusieurs niveaux différents;

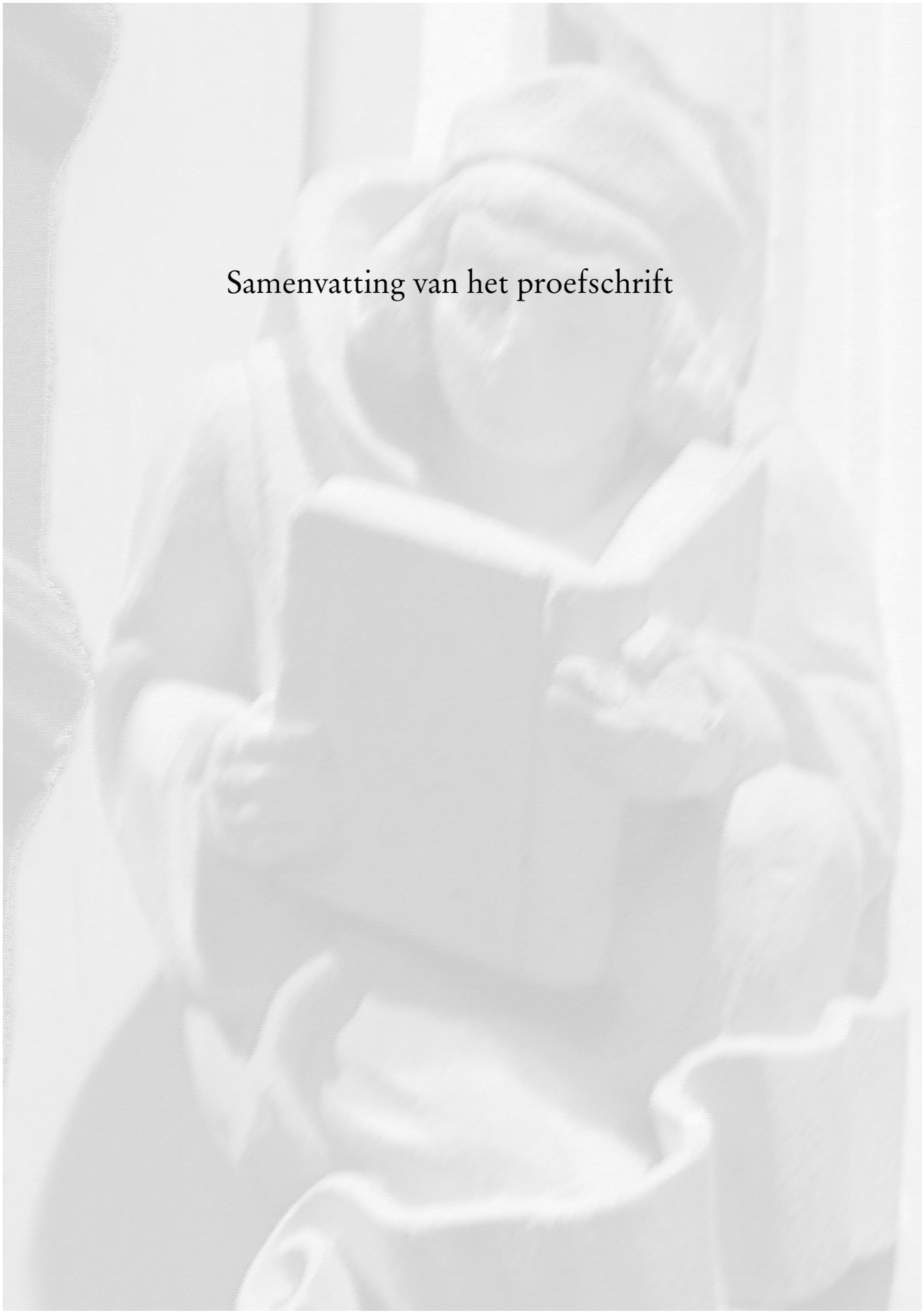
1. à l'activité posthéparine de lipoprotéine lipase (diminuée dans l'acromégalie; ***Chapitre 1.12***),
2. à la sécrétion de very low-density lipoproteins (élevée dans GHD et dans FH; ***Chapitres 1.6, 1.7, et 1.9***),
3. à l'activité de cholesteryl ester transfer protein (CETP ; diminuée dans GHD),
4. et à l'expression des récepteurs de LDL dans le foie (diminué dans FH et dans GHD; ***Chapitres 1.7 et 1.9***).

La processus d'athérogénèse comprends des plusieurs étapes initiales dont l'une entre elles est une dysfonction de l'endothélium. Le traitement avec hormone de croissance recombinante diminue l'augmentation des taux plasmatiques RLP-C dans la période postprandiale, et améliore la fonction d'endothéliale dans GHD (***Chapitre 1.6***). De plus, ces taux élèves induisent une réponse inflammatoire, qui est une des caractéristiques de l'athérosclérose (***Chapitre 1.8***). Les taux plasmatiques RLP-C à'jeun chez les patients FH sont associés à l'épaisseur de la paroi artérielle (IMT, en anglais) des carotides, qui est un marquer de l'athérosclérose (***Chapitre 1.11***). Dans des études in-vitro, ces RLP qui sont composées principalement de cholestérol ester et de TG, ont une taille de VLDL-1 (à 85%). Elles peuvent être oxydés facilement et peuvent initier la formation des cellules spumeuses (***Chapitre 1.3***). En compilant ces résultants, nous avons montré que l'isolement de RLP est utile pour une définition complète du phénotype lipidique proathérogène. Ainsi, les taux plasmatiques élevés de RLP sont associé à l'athérosclérose et qu'en déroulant aux maladies cardiovasculaires.

Le diabète de type 2, ou la résistance de l'insuline, est le résultat de la combinaison d'insulinorésistance et la diminution de la sécrétion d'insuline par les cellules β pancréatique. Le passage à l'insuline résistance dans les DM type II est associé à un déficit total de la sécrétion d'insuline par les cellules. Détérioration, dans la plupart des cas temporaire et modérée, de la homéostasie glucidique est notée après le début de traitement par l'hormone de croissance chez les patients avec un déficit de GH (comme présenté aux ***Chapitres 1.5, 1.6, 1.7 et 2.4***) et plus profondément en cas d'acromégalie active (***Chapitres 1.12*** **et** ***3.2***). Parallèlement, cette relation entre de l'axe de GH/ système d'IGF et du taux plasmatique de RLP-C, une relation similaire (mais surtout décrite dans les études animales) est retrouvée entre l'homéostasie glucidique, et la sécrétion d'insuline (***Chapitre 2.1***). Parmi les composants du système d'IGF, le taux d'IGF-2 est associé positivement avec la sécrétion d'insuline chez les sujets en bon santé, indépendamment d'IGF-I, d'IGF BP-3 et du BMI (***Chapitres 2.2 et 2.3***). Des récepteurs d' IGF-2 sont présents au niveau des cellules pancréatiques qui secrètent l'insuline. L'IGF-2 est un des facteurs principaux dans le développement des tissus de mésodermes, comme le pancréas, le foie, et le cœur. La variation des taux plasmatiques d'IGF-2, non dépendant de l'hormone de croissance, est déterminée principalement par des facteurs génétiques (66%), et par conséquent la capacité totale de la sécrétion d'insuline est déterminée pendant la période prénatale. La diminution de la sécrétion d'insuline est un symptôme clé dans les diabètes de type II, simultanément avec l'augmentation de la synthèse glucidique par les voies métaboliques de la néoglucogenèse (GNG). Nous avons trouvé une corrélation négative entre le taux plasmatiques d'IGF-2 et la GNG chez des patients avec GHD, avant et après leur traitement avec GH, en comparaison avec des sujets contrôlés pour l'âge et le BMI (***Chapitre 2.5***). De plus, lors de traitement avec GH la baisse de la sensibilité d'insuline (déterminé par l'index d'HOMA) est relié aux taux plasmatiques d'IGF-1 avant le traitement (***Chapitre 2.4***). L'augmentation de la GNG est due à une augmentation de la bêta oxydation mitochondriome des acides gras circulant du faut de la lipolyse induit par la GH et qu'offrent l'énergie pour dérouler des étapes successives dans la GNG (avec la pyruvate comme substrat préféré) (***Chapitre 2.5***). Les élévations des taux plasmatiques de glucides sont compensées par l'augmentation des taux plasmatiques d'insuline.

Enfin à coté de la relation entre métabolisme gluco-lipidique et système d'IGF, il existe une autre et bien entre des cardiomyocytes et le système d'IGF (***Chapitre 3.1***). Des taux plasmatiques élevés d'IGF-1, mais plus principalement de GH, sont associés avec une insuffisance cardiaque dans la cardiomyopathie des acromégalies. Apres traitement de l'acromégalie et surtout de taux de GH, la récupération de la fonction cardiaque peut être rétabli (***Chapitre 3.2***).

En bref, les taux plasmatiques des lipoprotéines remnants, mesurés grâce aux anticorps anti-Apo B100 et anti-Apo AI, est prêts à être introduit en clinique pour la détermination du phénotype lipidique proathérogène, supplémentairement au taux de cholestérol-LDL. Les affections du métabolisme glucolipidique, lesquelles sont constatées dans la pathologie de l'axe somatotrope sont aussi associées avec une susceptibilité à l'athérogénèse.



Samenvatting van het proefschrift

Aderverkalking is een veelvoorkomende oorzaak van chronische hart- en vaatziekten. Zelfs vroegtijdig overlijden aan aderverkalking in samenlevingen met een Westerse georiënteerde leef- en voedingsgewoonten hebben. Eén van de meest belangrijke risicofactoren voor aderverkalking is een afwijkend vetspectrum in het bloed (dyslipidemie). Een verhoogd LDL-cholesterol, dat een van de vetten in het bloed is, werd tot voor kort als belangrijkste oorzaak van aderverkalking aangemerkt. Uit recent onderzoek wordt steeds duidelijker dat de lipoproteinen remnants, andere vetdeeltjes dan het LDL-cholesterol in het bloed, ook eigenschappen bezitten die tot vervroegde aderverkalking kunnen leiden. Lipoproteinen remnants zijn die triglyceride-rijke deeltjes, die in het bloed deels afgebroken en daardoor kleiner van formaat zijn. Tevens wisselen de triglyceride-rijke en remnant deeltjes, met de andere vetdeeltjes in het bloed componenten uit. Door deze processen blijven de lipoproteinen remnant deeltjes langer in het bloed. Door hun geringe formaat zijn ze in staat om door de vaatwand heen te dringen en onder de vaatwand schade aan te richten. Dit geheel van factoren kan een eerste stap richting aderverkalking zijn. In patiënten die verhoogde nuchtere als postprandiale (na de maaltijd) waarden van lipoproteinen remnant deeltjes hebben, is verhoogde sterfte aan hart- en vaatziekten vastgesteld. (**Hoofdstuk 1.1**).

De postprandiale waarden van de lipoproteinen remnants in het bloed zijn een resultaat van de dagelijks genoten voeding, en van de stoornissen die in vetstofwisseling zelf voorkomen. De Westerse mens bevindt men zich voor het grootste deel van de dag in een postprandiale staat vanwege zijn gewoonte om minimaal driemaal per dag te eten en omdat verstoringen van de vetstofwisseling met regelmaat voorkomen. In de recent uitgekomen NCEP ATP III richtlijn werd het belang van de behandeling van triglyceriden en triglyceriden rijke deeltjes ter voorkoming van hart- en vaatziekten benadrukt. Een interessante observatie is dat de sterfte aan hart- en vaatziekten verhoogd is bij patiënten met een verstoring in de groeihormoon (GH) as/ "insulin-like growth factor" (IGF) systeem (zoals in GH deficiëntie en in GH overschot; acromegalie). Het GH wordt gemaakt in een kleine klier die zich aan de schedelbasis bevindt. Het in het bloed circulerende GH stimuleert in de lever de productie van IGF-, en aan IGF-, verbonden eiwitten. Waarschijnlijk hebben deze verstoringen een stimulerend effect op processen die tot aderverkalking leiden, zoals afwijkingen in de vetstofwisseling en in de aanmaak van stikstofoxide (NO) in de vaatwand (**Hoofdstuk 1.4**). Dit NO is belangrijk voor een optimale functie van de vaatwand, maar ook voor de relatie met de directe omgeving, de subendotheliale matrix. Dit samenspel tussen hormonen of hormoonachtige groeifactoren en de pathofysiologie van het hart- en vaatstelsel wordt samengevat onder de recent geïntroduceerde term, cardiovasculaire endocrinologie.

De aanwezigheid van verhoogde waarden van de lipoproteinen remnant deeltjes (RLP), werd bepaald in het bloed van verscheidene groepen patiënten met een verhoogd risico op hart- vaatziekten. In de **hoofdstukken 1.9 en 1.10** werd aangetoond dat nuchtere en postprandiale RLP-C waarden verhoogd zijn in het bloed van patiënten met een primaire dyslipidemie: heterozygote familiaire hypercholesterolemie [FH]. In de **hoofdstukken 1.5, 1.6, 1.7 en 1.12**, vonden we tevens verhoogde nuchtere en postprandiale plasma RLP-C waarden in patiënten met een secundaire dyslipidemie (onbehandelde acromegaly, en in patiënten met een groeihormoon deficiëntie [GHD]), dit in vergelijking met controle personen die een gelijke BMI, leeftijd en geslacht hadden (**Hoofdstuk 1.2**).

Hoewel de plasma RLP-C waarden verhoogd zijn in het bloed van al deze groepen patiënten, ligt de oorsprong van de verhoging op verschillende niveaus van het vetmetabolisme. Deze verschillende niveaus zijn:

1. de activiteit van het enzym lipoproteine

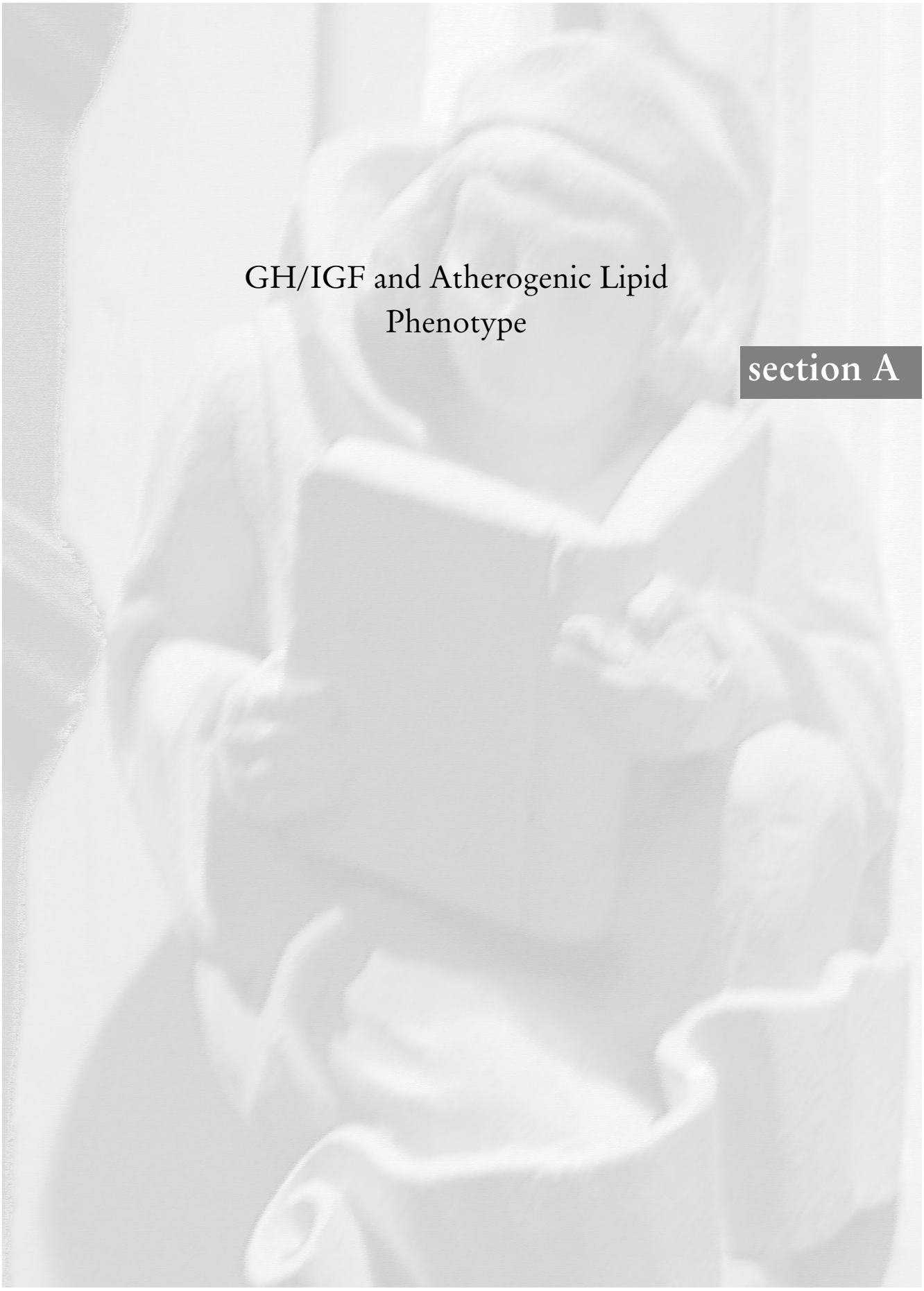
lipase (dat verlaagd is bij acromegalie; **Hoofdstuk 1.12**),
2. de secretie van het very low-density lipoproteins (dat verhoogd is bij GHD en FH; **Hoofdstukken 1.6, 1.7, en 1.9**),
3. de activiteit van het cholesteryl ester transfer protein (CETP; dat is verlaagd bij GHD), en
4. de expressie van de LDL receptoren op de lever (dat verlaagd is bij FH en bij GHD; **Hoofdstukken 1.7 en 1.9**).

Het proces dat leidt tot aderverkalking bestaat uit verscheidene stappen, waarvan disfunctie van de vaatwand er een is. Behandeling van GHD patiënten met recombinant groeihormoon (GH) verlaagt de plasma RLP-C waarden na een vet inname en verbetert de verminderde vaatwand functie in AGHD patiënten (**Hoofdstuk 1.6**). De verhoogde RLP-C waarden na een vet inname leidt tot een ontstekingsrespons in het bloed, een fenomeen dat ook gekoppeld is aan vroegtijdige aderverkalking (**Hoofdstuk 1.8**). De verhoogde nuchtere plasma RLP-C waarden zijn verbonden aan de dikte van de vaatwand van de halsslagader, hetgeen een veelgebruikte indicator is voor het vaststellen van de mate van aderverkalking (**Hoofdstuk 1.11**). In in-vitro onderzoek zijn de RLPs grotendeels samengesteld uit cholesteryl esters en triglyceriden. De RLPs hebben dan grotendeels de omvang van het VLDL-1 (namelijk 85%), ze kunnen makkelijk geoxideerd worden en geven aanleiding tot schuimcelvorming (**Hoofdstuk 1.3**).

Type 2 suikerziekte is in het algemeen te wijten aan een combinatie van insuline resistentie en een afname van de afgifte van insuline door bèta-cellen in de alvleesklier (het pancreas). De uiteindelijke expressie van een type II suikerziekte is gekoppeld aan het falen van de insuline afgevende cellen in hun poging de verhoogde plasma suiker waarden te compenseren. Een verstoring van het suiker evenwicht, die meestal beperkt en kort van aard is, wordt in het bloed gevonden na het begin van de GH behandeling bij patiënten met een AGHD (zoals te zien in **hoofdstukken 1.5, 1.6, 1.7, en 2.4**). De verstoring van het suiker evenwicht is nog duidelijker bij patiënten met een onbehandelde acromegalie (**hoofdstukken 1.12 en 3.2**). Naast een relatie tussen de GH as/ IGF systeem en de plasma RLP-C waarden, is er ook een relatie (maar zover enkel geobserveerd in dier experimenteel onderzoek) in het bloed te vinden tussen de GH as/ IGF systeem en het glucose evenwicht. Deze relatie bestaat ook tussen de secretie van het insuline door de alvleesklier en de GH as/ IGF systeem (**Hoofdstuk 2.1**). Een component van het IGF systeem, het IGF-2, is positief gekoppeld aan de insuline secretie bij gezonde volwassenen. Dit is onafhankelijk van het IGF-I, het IGF BP-3 en de BMI (**hoofdstukken 2.2 en 2.3**). Receptoren voor IGF-2 zijn aangetoond op insuline secernerende cellen in het pancreas. Het IGF-2 is een belangrijke factor in de prenatale ontwikkeling van het mesodermale weefsel, zoals het pancreas, de skeletspier, de lever en het hart. De variatie van de plasma IGF-2 waarden worden grotendeels genetisch bepaald en daardoor kan verondersteld worden dat de capaciteit van de insuline secretie reeds voor de geboorte wordt bepaald. Een afname van de insuline secretie is een belangrijk symptoom in type II suikerziekte, net als een toename van de suikervorming door de lever middels de zogenaamde gluconeogenese (GNG) route. Inderdaad vonden we een negatieve correlatie tussen de plasma IGF-2 waarden en de GNG in GHD patiënten (**Hoofdstuk 2.5**). De mate van afname van de insuline gevoeligheid (bepaald met de HOMA index) door de GH behandeling wordt bepaald door de waarden voorafgaand aan de behandeling van het plasma IGF-I (**Hoofdstuk 2.4**). De toename van de GNG gedurende de GH behandeling heeft zijn oorsprong in een toegenomen oxidatie van het hogere aantal circulerende vrije vetzuren dat de energie verschaft voor de GNG route (met het pyruvaat als dominant substraat) (**Hoofdstuk 2.5**). De verhoogde plasma suikerwaarden worden nagenoeg volledig gecompenseerd door de toegenomen hoeveelheid insuline.

Niet enkel de eerder besproken metabolismen (die van het suiker- en van het vetmetabolisme) zijn gekoppeld aan het IGF systeem. Ook functionele cellen van het hart (cardiomyocytes) staan onder invloed van het lokale en het systemische GH as/IGF systeem (**Hoofdstuk 3.1**). Hoge plasma waarden van het IGF-1, maar vooral van het GH, zijn verantwoordelijk voor hartfalen als gevolg van de cardiomyopathie bij patiënten die niet voor hun acromegalie worden behandeld. Na adequate verlaging van het GH is de afgenomen hartfunctie te herstellen in het merendeel van de patiënten met acromegalie (**Hoofdstuk 3.2**).

Kortom, de plasma waarden van de lipoprotein remnants die geïsoleerd worden middels de immuno-isolatie methode, zijn geschikt voor de analyse van de vatbaarheid van personen voor vroegtijdige ziekten en overlijden aan hart- en vaatziekten die worden veroorzaakt door aderverkalking. Afzonderlijke metabole verstoringen (zoals in het lipoprotein remnant en in het glucose metabolisme) en endotheel gerelateerde componenten die beschouwd worden als belangrijke factoren in het ontstaan van aderverkalking, zijn geassocieerd met verstoringen in de GH as en het IGF systeem.



GH/IGF and Atherogenic Lipid Phenotype

section A

1.1
Elevated Remnant-Like Particle cholesterol (RLP-C) concentration: a feature of the atherogenic lipoprotein phenotype
(review)

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Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the Western World. Although an increase in plasma low-density lipoprotein (LDL) concentration is an important factor in the etiology of coronary heart disease (CAD), oxidation of LDL is believed to play a key role in the development of atherosclerosis, based on *in vitro* studies demonstrating a role of ox-LDL in foam cell formation and endothelial dysfunction (1-4). Circulating levels of plasma ox-LDL are however low, and ox-LDL is primarily localized in the atheromatous plaque in autopsy studies (5-7). Therefore, additional circulating lipids and/or lipoproteins are likely to contribute to the onset and development of coronary atherosclerosis as well as other cardiovascular complications. Recently, in a large observational study, the calculated non-HDL plasma cholesterol concentration (LDL plus VLDL cholesterol) was a stronger predictor of cardiovascular events than plasma cholesterol alone (8-10). Improvement of CAD predictability upon inclusion of VLDL cholesterol emphasizes the proatherogenic role of triglyceride-rich lipoproteins (TRL). These lipoproteins may be directly atherogenic by affecting lipid-accumulation by cells in the artery wall. Alternatively, they may be indirectly atherothrombogenic by affecting the size and oxidizability of LDL, or by affecting levels of plasma HDL, fibrinogen or PAI-I. Smaller, partly lipolyzed TRL remnants are considered to be more atherogenic than larger newly secreted TRL since they can more readily penetrate the endothelial lining of the arterial wall. Disparity in the atherogenicity of large versus small TRL is best illustrated by the difference in prevalence of premature CAD in patients with different inherited forms of hypertriglyceridema. Type I or type V hyperlipoproteinemic patients have extremely high plasma triglyceride levels due to a deficiency in lipoprotein lipase or apo C-II. They characteristically have an accumulation in plasma of very large TRL, yet they are not at greatly increased risk of CAD (11; 12). In contrast, type III hyperlipoproteinemic patients have more moderate hypertriglyceridemia and due to defective remnant lipoprotein uptake by the liver have an accumulation in plasma of smaller TRL remnants (i.e. β-VLDL). These patients do have increased risk of CAD and also increased risk of peripheral vascular disease (13). TRL remnants are considered to be atherogenic. Accurate quantification of TRL remnants is, however, problematic (14), since: 1) remnants are difficult to differentiate from their larger and more triglyceride-rich precursors; 2) their plasma concentration is very low compared to other lipoproteins; and 3) they are biochemical difficult to isolate or detect because they represent a heterogeneous group of variable size and composition. An immunoaffinity method for isolation of TRL remnants has recently been used in a number of clinical and laboratory studies to study remnant lipoprotein metabolism, atherogenicity (15), and to asses its clinical

Triglyceride rich particles in the atherogenic phenotype

The importance of plasma TG as an independent risk factor for CAD was recognized after the publication of a meta analyses by Austin et al (16; 17) showing that plasma triglyceride levels predict relative risk for CVD mortality in relatives of familial combined hyperlipidemia (FCHL) patients. Epidemiological data from the Framingham study already showed that plasma TG is an important risk indicator of CAD in women (18). Additional evidence was obtained from by Yarnell et al in a ten-year follow-up study (19), and confirmed in several other studies (20; 21). The relationship between TG and prevalence of cardiovascular disease was dependent upon plasma HDL-cholesterol concentration in the Procam study (22) and was decreased when plasma TG levels were above 800mg/dL, whereas Criqui et al (23) could not demonstrate any relationship with plasma TG.

The analyses of plasma TG are complicated because of large inter- and intra-individual

variation, and heterogeneity of circulating triglyceride-rich lipoprotein subclasses such as intestinally derived apo B48-containing chylomicrons, and chylomicron remants and hepatic derived apo B100-containing very low density lipoproteins (VLDL) and their remnants. The remnant particles are the atherogenic fraction, as illustrated by the observation that fasting and postprandial triglyceride-rich remnants are associated with premature atherosclerosis (24-26). The concentration of these circulating remnant particles *in-vivo* have been determined with a number of different methods, based on their density, size charge, specific lipid components, apolipoprotein composition or apolipoprotein immunoaffinity (14). Isolation of apo B100 and apo B48 lipoprotein fractions was performed with the use of density-gradient ultracentrifugation based on the assumption that lipoprotein subfractions differ in density (27-29). Apolipoproteins are analysed by SDS-PAGE (28). Association between increased levels of apo B-48 and apo B-100 in Sf20-60 (small VLDL) and Sf 12-20 (IDL) and increased risk for cardiovascular disease were found in a number of studies (30-35). The fate of the fatty acid moiety of intestinal derived chylomicron particles can be analysed by in vivo labeling of the particles with retinyl esters (RE)(36) as a marker. Several studies have shown an association between abnormalities in the postprandial response of TG, RE, and premature atherosclerosis (31; 33; 37-41) The problem with this approach is that, at later time points in the postprandial test, exchange of RE between the different lipoprotein fractions occur (42-44). All these techniques are very laborious and are not useful in large clinical studies.

RLP-C analysis

Nakajima et al (45) introduced a new method for estimation of lipoprotein remnants (RLP) with specific monoclonal antibodies directed against epitopes of apo A-I and apo B-100, bound to Sepharose 4B gel. Plasma samples are incubated with the immunoaffinity gel. HDL, LDL and most of the VLDL particles bind to the gel, whereas in the unbound supernatant fraction, remnant-like particle cholesterol is measured (remnant-like particle cholesterol, RLP-C). The binding capacity of immunoaffnity sepharose gel is high with a binding of > 95% of both plasma HDL,LDL, and VLDL. The epitope of apo B-100 that is recognized by the monoclonal antibody is localized in the amphipathic helical region of the protein encompassing residues 291-2318, approximately apo B-51. The epitope for Mab JI-H (JIMRO-Havel) was mapped to 81 residues (2251 to 2331), so that all apo B-48 lipoprotein particles are recovered in the RLP fraction. In addition, a subfraction of TRL containing apo B-100 is also included (46) The K_a value for the antibody to apo A-I is $4.2*10^8$ mol/L. The K_a value of the antibody directed to apo B-100 of LDL is $5.5*10^8$ mol/L and that of VLDL $3.2*10^8$ mol/L (15). Saturation of the coated sepharose gel occurs at HDL-cholesterol levels above 100 mg/dl, resulting in a rapid loss of binding. For the monoclonal antibody directed against apo B-100 no saturation could be obtained so that 95% of LDL-cholesterol is bound to the immuno-affinitygel at plasma LDL-cholesterol levels up to 600 mg/dl (47).

RLP composition and Particle size

In fasting plasma of normolipidemic subjects, RLP consists of an equal number of TG and cholesterol molecules (3000 and 4000) for each apo B molecule (>95% Apo B-100), with an average of 33 molecules of apo C-III and 7 molecules of apo E per apo-B molecule (48;49). Separation of the RLP fraction by size exclusion chromatography showed that normolipidemic RLP was eluted at the size large LDL (average particle size of 27 nm) whereas 38% displayed a predominantly VLDL size (average particle size 38 nm)(15;50).In normolipidemics the concentration of plasma TG is determinant for its size and composition. In those individuals with low plasma TG the RLP is predominantly of LDL-size (46). With increasing levels of plasma TG, the peak of RLP size moves towards the larger VLDL fraction. In

normolipidemic individuals with low plasma TG levels, the contribution of IDL + LDL sized RLP is around 30% and depends upon the LDL pattern A or B (46). To compare, in the postprandial state, after a mixed meal, TRPs in the Sf 60-400 density fraction (larger TG-rich VLDL particles) that were subjected to immunoaffinity chromotography (with the use of specific monoclonal antibodies 4G3 and 5E11 against C terminal Apo B-100), contained 2.0 molecules apo E and 25 molecules apo C-III per particle, with 25000 mol TG and 3500 mol cholesterol per particle. In the Sf 20-60 fraction (small VLDL, IDL fraction) 2.0 mol apo E and 11 mol apo C-III per particle were found, with 10000 mol TG and 3000 mol cholesterol per particle (51). RLP from type III patients contained twofold higher triglycerides and cholesterol molecules for each apo B molecule (50% of apo B was apo B-48) than RLP in normolipidemic subjects ref. The content of apo CIII and apo E molecules was slightly increased, but not significantly different from normolipidemic subjects. In type IV patients an increased number of TG and apo CIII molecules per apo B in RLP were found, but the number of cholesterol and apo E molecules were unchanged (48), and the RLP particle size is on average larger than in normolipidemic subjects, and thus resembles that of triglyceride-rich lipoproteins in the d<1.006 g/ml density fraction. (48).

Dynamics of TRL

Triglyceride-rich lipoprotein particles originate either from the intestine (apo B48-contaning chylomicrons) or from the liver (apo B 100-containing VLDL). Upon secretion into the circulation TRL undergoes lipolysis by the enzyme lipoprotein lipase (LPL) that is anchored in the endothelial lining of the arterial wall (52). Most circulating TRL in plasma contain apo B-100, while in the postprandial period an increase of 20 % of apo B-48 containing TRL was observed. Catabolic pathways are mediated through receptors that are expressed at the hepatic surface. Apo B-48-containing TRL are taken up by LDL - and LRP receptors (53). Apo B-100 TRL are precursors for the denser LDL fraction. Isotope studies revealed that TG-rich VLDL-1, in contrast to cholesterol-rich VLDL-2, is a substrate for formation of intermediate density lipoproteins (IDL) and subsequently LDL by further modulation through LPL and hepatic lipase (HL) and by exchange of TG for cholesterylesters from HDL by the transfer protein: cholesterol ester transfer protein (CETP). This small dense LDL fraction possesses atherogenic properties (54). It is of interest that the coronary system, the aortic arch and the carotid arteries are the initial arterial beds that are in contact with TRL and TRL remnants, including lung and its alveolar macrophages. Early atherosclerotic disease is frequently found in these arterial regions.

Fasting plasma RLP-C levels in normolipidemic subjects

Normal levels of RLP-C are obtained from a number of different studies and summarized in Table 1.The mean level of RLP-C in a healthy Caucasian population is less than 0.20 mmol/l. Despite the fact that anthropometric and lipid parameters of normolipidemic Caucasian and Japanese control subjects are similar, plasma RLP-C levels in Caucasian populations are higher. A real explanation for the differences can not be deducted from the literature since no data on life style were reported. Differences in plasma RLP-C concentration between different ethnic groups may account for the increased prevalence of cardiovascular disease that is

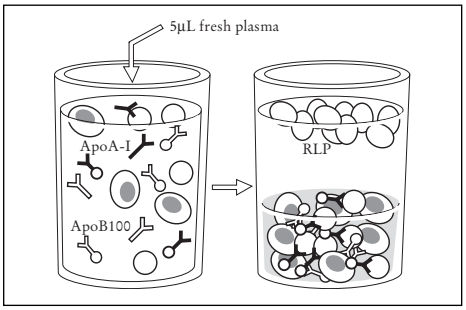

Figure 1. RLP isolation method. Free, unbound fraction contains RLP. the bound fraction consists of ApoB-100 and Apo A-I particles.

clustered in distinct ethnic populations, such as in African Americans (55). A number of studies have demonstrated significant differences between RLP-C levels in both men and women, with higher levels in men (56;57). In the Framingham study, fasting plasma RLP-C levels in men were higher than in women (0.21±0.10 mmol/L versus 0.18±0.06 mmol/L) (56;58). Plasma RLP-C concentrations significantly increase at older age as was shown by Leary et al (57). Additional analysis of RLP-C levels in postmenopausal women in the Framingham study demonstrated plasma RLP-C levels of 0.19 mmol/l and plasma RLP-TG of 0.25 mmol/l (59). Plasma RLP-C levels increase with age in both males and females (for example: in men with age < 30 years, mean levels are 0.18±0.09 mmol/l; in men aged between 50-59 years, 0.22±0.10 mmol/l and in men aged between 70-79 years, 0.22±0.12 mmol/l) (56).

In a large Framingham cohort of women without CVD, of whom 57% were in a postmenopausal state, plasma RLP-C and RLP-TG levels were 16% and 27% lower than in those women diagnosed with active CVD. In the total cohort, the prevalence rate for CVD in the upper quartile of plasma RLP levels with an age adjusted estimate is 85 per 1000 women. The lower three quartiles result in an age adjusted estimate of 32-50 per 1000 women. Moreover, plasma RLP-C levels, and not the total plasma TG levels, were an independent risk factor for cardiovascular disease (59).

Postprandial plasma RLP-C levels

Plasma triglyceride-rich lipoproteins (TRL) and RLP accumulate in the postprandial period, with an abnormal lipid profile in dyslipidemic patients. Indeed, an increased postprandial accumulation of TRP is a feature of the atherogenic lipid phenotype. With the use of an oral fat load test with cream (50 g fat/m^2) or a mixed meal, the postprandial lipid profile is estimated. Maximal postprandial RLP-C levels in mildly obese normolipidemic subjects, aged 50 years, were reached between 2 and 4 hours postprandial (0.48 mmol/l) (60;61). In healthy young male volunteers plasma RLP-C levels increase from 0.15 mmol/l to 0.23 mmol/l 4 h postpandially

Table 1: Fasting plasma RLP-C levels in normolipidemic subjects.

	N	Age	BMI	C	LDL-C	TG	RLP-C
Oda, H. etal (120)	106	30.2±5.6	np	3.9-6.0	1.9-4.2	0.4-1.8	0.07±0.03
Okazaki, M. et al (50)	49	np	np	4.8±0.9	3.0±0.6	1.0±0.3	0.09±0.04
Marcoux, C. et al (49)	10	42.7±5.1	24.5±0.8	4.7±0.3	3.0±0.2	1.2±0.2	0.22±0.01
Marcoux, C. et al (48)	8	41.5±5	24±1	4.57±0.26	2.7±0.26	1.21±0.1	0.21±0.02
Devaraj, S. et al 1998 (95)	23	49.3±3.3	27.7±5.8	4.91±0.78	3.25±0.9	1.20±0.6	0.15±0.05
Ohnishi, H. et al (83)	352	64.4±10.0	22.6±2.7	5.21±0.80	3.14±0.9	1.21±0.6	0.09±0.06
Saigo, M. et al (121)	128	53.7±12.7	np	5.37±0.78	np	1.33±0.64	0.12±0.06
Koba, S. et al (122)	142	60±11	23.2±3.0	5.2±0.8	3.2±0.7	1.2±0.6	0.12±0.07
Schaefer, E.J. et al (58)	88	62±9	26±4	5.6±1.2	3.5±1.2	1.5±0.9	0.21±0.1
Ooi, T.C. et al (123)	10	55±4	24±1	4.55±0.15	2.75±0.10	1.34±0.13	0.17±0.01
Sakata, K. et al (98)	66	60±9	22.9±3.4	5.2±1.3	3.2±1.0	1.5±0.7	0.06±0.06
Ai, M. et al (63)	10	33±1	22.9±0.5	4.8±0.21	2.8±0.2	0.9±0.1	0.09±0.01
Twickler, Th.B. et al (61)	7	47.7±6.9	25.2±1.6	5.11±0.46	3.18±0.64	0.91±0.3	0.20±0.08

Abbreviations: np=not presented, TC: total cholesterol, LDL-C: LDL-cholesterol, TG: triglycerides. Plasma levels are expressed as mmol/l and as mean ± S.D.

(62), whereas atorvastatin intervention attenuates the postprandial RLP-C increase in contrast to placebo and gemfibrozil treatment. Ai et al (63) found an postprandial RLP-C increase from 0.08 mmol/l to 0.13 mmol/l in normolipidemic Japanese subjects after an oral fat load with only 17 g fat/m^2. It has been shown that the amount of fat given orally will influence the height of the postprandial response. Chylomicron fatty acid moiety (reprented as retinyl palmitate (RE) in the <1.006 g/L density fraction) in normolipidemic French, non-obese (BMI: 20-24 kg/m^2) subjects did not increase 3 hours after ingestion of either a non-fat or a 15 g/m^2 low fat meal, whereas significant increases in chylomicron RE were induced 3 hours after ingestion of meals with a fat content of 30, 40, and 50 g/m^2 (64).

RLP-C levels in patients with dyslipidemia

Familial hypercholesterolemia (FH) is characterized by mutations in the LDL-receptor gene with a lipoprotein phenoype that is characterized by elevated plasma cholesterol and LDL-cholesterol levels. In a subset of heterozygous FH patients elevated plasma TG levels were found (65), and plasma RLP-C levels were increased independent of plasma LDL-cholesterol (FHExPRESS study, (66)) with a median of 0.47 mmol/l. Moreover, a post-hoc analysis of the ExPRESS study revealed a significant association between baseline plasma RLP-C levels and carotid artery intima media thickness, a surrogate marker for atherosclerosis (Twickler et al, unpublished data). Both plasma apo B-48 and RLP-C levels were increased in heterozygous FH population in Australia (67). Additional kinetic analyses using a stable isotope breath test revealed no difference in chylomicron catabolism in patients with FH as compared to controls (68). Earlier studies showed a delayed chylomicron remnant clearance in the postprandial state (39; 69) using ultracentrifugal analyses and RE as a marker for chylomicron clearance. These observations support the growing interest for RLP as an atherogenic lipid component, even in lipid disorders such as FH.

Patients with Type III dyslipidemia are characterized by elevated plasma levels of remnant particles. These patients have a genetically abnormal apo E genotype (apo E2/apo E2) resulting in abnormal apoE mediated TG-rich lipoprotein uptake via hepatic lipoprotein receptors resulting in accumulation of remnant particles in the circulation (13). Only a small proportion of individual

Table 2: Postprandial plasma RLP-C in normolipidemic healthy subjects

authors	intervention	age	BMI	Baseline TG	Postprandial TG	Baseline RLP-C	Postprandial RLP-C
Twickler, Th.B. et al (61)	Cream 40% fat (50 g/m^2 BSA)	48±7	25±2	0.91±0.3	1.66±0.53	0,20±0,08	0,34±0,05
Schaefer, E.J. et al (124)	Mixed meal, 57% fat	62±9	26±4	1.5±0.9	2.36±1.4	0.20±0.01	0.33±0.2
Wilmink, J. et al (62)	Cream 40% fat (50 g/m^2 BSA)	25±4	23±3	1.1±0.5	2.0±1.0	0.15±0.06	0.23±0.07
Schaefer, E.J. et al (58)	Mixed meal, 57% fat	62±9	26±4	1.5±0.9	2.36±1.4	0.21±0.1	0.34±0.16
Schreuder, P.C.N.J. et al (60)	Cream 40% fat (50 g/m^2 BSA)	49±9	25±3	0.9±0.4	1.8±1.2	0.18±0.06	0.48±0.31
Ai, M. et al (63)	17 g fat/m^2 BSA	33±1	23±1	0.86±0.6	1.17±0.1	0.08±0.01	0.11±0.01

Results are expressed as mean ± SD (mmol/l)

with apo E2/E2 genotype will develop type III hyperlipidemia. It is therefore necessary to have an additional clinical marker. Measurement of RLP-C and the RLP-C/TG ratio, an estimation of the enrichment of TRL in cholesterol (a characteristic of beta-VLDL), provides an alternative for diagnosis of lipid abnormalities in these patients (70;71). The sensitivity 0.95 (0.85-1.00), specificity 0.94 (0.85-1.00) and accuracy 0.94 (0.88-1.00) (with a cut off point of >0.23) was much better in comparison to agarose gel electrophoresis of the VLDL fraction obtained after ultracentrifugation. Plasma RLP-C was increased five-fold in type III subjects (1,39 mmol/L). RLP in patients with type III hyperlipidemia had a slow beta VLDL mobility on agarose gels, but their size was smaller (32 nm). In an Asian population, plasma RLP-C levels in type III hyperlipidemia were increased compared to control subjects, but were lower than in Caucasian subjects (72).

In patients with type IIb hyperlipidemia plasma RLP-C levels were two-fold increased (0,46 mmol/L) and in type IV subjects almost threefold (0,53 mmol/L), as compared to control subjects (14; 48; 70). Plasma RLP particles were larger with a particle size of 41 nm in patients with severe hypertriglyceridemia (TG>4,5 mmol/L) (73)

Patients with familial combined hyperlipidemia (FCHL) are characterized by elevated levels of plasma TG, plasma apo B and/or plasma cholesterol. Lipoprotein metabolism in FCHL is disturbed, leading to remnant accumulation in the circulation (74-76). The level of plasma RLP-C may be an additional marker for the characterization of patients with FCHL. Male FCHL patients had a six-fold increased plasma RLP-C concentration than male normolipidemic controls (1.5 mmol/l vs 0.2 mmol/l), whereas the plasma RLP-C concentration was only two-fold higher in FCHL female patients as compared to controls (0.4 mmol/l vs 0.2 mmol/l) (77). No significant differences in plasma apo B, HDL-C and total cholesterol levels were found, but plasma TG was strongly correlated with plasma RLP-C in both the FCHL men and women. Moreover, the waist hip ratio was higher in the FCH men than in FCH women and control subjects. No data are available yet from a large study in FCHL.

Postprandial RLP-C abnormalities in patients with primary hyperlipidemia

In patients with elevated plasma TG levels, postprandial plasma TG increase after an oral fat load was in the range of 30-50% in type IIb, type III, and type IV dyslipidemic patients, with no significant differences between the maximal postprandial TG in all the dyslipidemic groups. Although postprandial RLP-C elevations at 4, 6 and 8 hours from baseline in type III patients tended to be smaller and in type IV patients tended to have a greater postprandial RLP-C increase, no significant differences between the groups were found. The ratio RLP-C/ RLP-TG during the postprandial period decreased due to a relative increase in RLP-TG in patient with

Table 3: Plasma RLP-C levels in primary dyslipidemie

Authors	Type IIa	Type IIb	Type III	Type IV
Okazaki, M. et al (50)	0,11±0.04	0,21±0.11	0,78±0.13	0,33±0.23
Wang, T. et al (70)		0,46 [0.37, 0.63]	1,39 [1.04, 2.75]	0,53 [0.39,0.76]
Marcoux, C. et al (48)	0.21±0.01	0.50±0.07	0.58±0.11	
Marcoux, C. et al (125)		0.51±0.03	1.31±0.14	0.56±0.07
Ooi, T. et al (123)		0.29±0.02	0.67±0.1	

Plasma levels are expressed as mmol/l and as mean ± S.D. or as median [95th percentile, 5th percentile].

type IIb and IV, and an concomitant enrichment of cholesterol only in type III patients at all postprandial time points (78). In patients with FCHL, abnormalities in postprandial lipoprotein metabolism have been extensively described (79; 80), indicating the presence of elevated plasma levels of apoB-48-containing and apo B-100 containing TG-rich lipoprotein fractions. No data on plasma RLP-C levels are yet published, but we may speculate that RLP-C will be an additional marker for the postprandial lipid response.

RLP in secondary dyslipidemia

Insulin Resistance and type II diabetes mellitus

The insulin resistance state is associated with abnormalities in lipid metabolism, such as elevated plasma TG and decreased plasma HDL-C levels, and in glucose homeostasis. Metabolic defects includes an impaired Free Fatty acid (FFA) metabolism, saturation of TRL remnant removal and disruption of the regulation of hepatic secretion of VLDL particles into the circulation. Kinetic studies have shown that acute hyperinsulinemia in healthy men suppresses hepatic VLDL-1 production. This phenomenon does not occur in patients with type 2 diabetes mellitus (81; 82). Elevated plasma RLP-C (RLP-C>7.5 mg/dl) concentrations were found more frequently in individuals with insulin-resistance (IR) than in healthy individuals. Moreover, in a multiple regression analysis, the HOMA-ratio (an index for insulin resistance) was closely related to plasma RLP-C (83). In insulin-resistant, postmenopausal women, the postprandial elevations of plasma RLP-C were lower than in the insulin-sensitive women. ref

Type II diabetes mellitus is associated with a marked increase in cardiovascular disease. A characteristic clinical feature of type 2 Diabetes Mellitus is dyslipidemia with elevated plasma levels of TG, presence of small dense LDL, and decreased levels of HDL-C (84). Patients with the metabolic syndrome (i.e. patients with visceral obesity, hypertension and insulin resistance) have a similar abnormal lipoprotein profile. It is therefore not surprising that a number of studies have been published to analyse the contribution of RLP-C in the atherogenic lipoprotein profile in DM. In patients with type II diabetes mellitus, postprandial RLP-C levels were elevated (58; 63; 85-90). Interestingly, the impact of type 2 DM on the lipoprotein phenotype and the risk on CAD is relatively worse in women as compared to men. Women with type II DM have a higher proportion of small dense LDL (91), relatively higher plasma RLP-C levels (58). Both parameters significantly contribute to the athreogenic lipoprotein phenotype as seen in patients with type II DM.

In patients with renal disorder the expression of an atherogenic lipid phenotype is associated with increased plasma levels of TRL, and consequently increased plasma RLP-C concentrations Indeed, glomerular disease and the presence of albuminuria (defined as >2 grams / 24 hour collected urine), lead to an increase in plasma RLP-C levels (18,9 mg/dl vs 7,7 mg/dl in matched control subjects). Plasma RLP-C concentration was not related to renal function (assessed as calculated creatinine clearance), but associated closely with plasma TG levels (92). Patients with a fatty liver or steatosis had significantly higher plasma RLP-C levels as compared to control subjects (16 mg/dl vs 5 mg/dl, respectively) (93).

RLP and atherosclerosis

It has been shown in a number of studies that the presence of elevated plasma RLP-C levels and endothelial dysfunction, as a marker for atherosclerotic disease, are significantly associated (94). Patients with established coronary heart disease have elevated plasma levels of RLP-C (95; 96). The intima media thickness of the carotid artery was positively related to baseline plasma RLP-C concentrations in a secondary intervention study with patients, aged 50 years and older, after their first cardiovascular event (94). This association was independent of plasma TG and LDL-C levels. In the LOCAT study (97) the mean plasma on-treatment RLP-C concentration was significantly associated with the

Table 4: Baseline fasting plasma RLP levels in insulin resistance, and type 2 DM patients.

authors	Design study	TG	LDL-C	RLP-C
Watanabe, N. et al (126)	Observational transversal (75-g OGTT)	NGT (n=392): 1.2 ±0.7 IFG (n=16): 1.3±0.5 IGT(n=93): 1.7±1.3 DM+ (n=40): 1.7±0.9	NGT:3.3 ± 0.8 IFG:3.4 ± 0.7 IGT: 3.2 ± 0.8 DM+: 3.2 ± 0.8	NGT :0.11±0.09 IFG: 0.12±0.05 IGT:0.16±0.19 DM+: 0.16±0.11
Koba, S. et al (122)	Case control	CAD- DM-: 1.2±0.6 CAD- DM+: 1.3±0.5 CAD+ DM-: 1.5±0.7 CAD+ DM+: 1.7±1.2	CAD- DM-: 3.2±1 CAD- DM+: 3.1±0.9 CAD+ DM-: 3.0±0.8 CAD+ DM+: 2.8±0.7	CAD- DM-: 0.12±0.07 CAD- DM+: 0.16±0.1 CAD+ DM-: 0.15±0.0.12 CAD+ DM+ 0.16±0.13
Ai, M. et al (63)	Case control	C:0.9 ± 0.1 N-DM: 1.3 ± 0.1 H-DM:1.7 ± 0.3	C:2.8 ± 0.3 N-DM :3.0±0.2 H-DM: 3.4 ± 0.3	C:0.09±0.01 N-DM:0.091±0.05 H-DM:0.13±0.01
Sone, H. et al (127)	Intervention study (pitavastatin 2mg od)	2.3 ± 0.5	4.4 ± 0.8	0.17±0.07
Hirano, T. et al (86)	Case control intervention study (Doxazosin 2mg od)	HT DM +: 1.9 ± 0.9 HT DM -: 1.7 ± 0.7	HT DM +: 3.1 ± 0.8 HT DM -: 3.1 ± 0.7	HT DM +: 0.22±0.1 (minus 18%) HT DM -: 0.18±0.08 (minus 28%)

N-DM: normoinsulinemic patients with type 2 DM, H-DM: hyperinsulinemic patients with type II-DM, C: control subjects, P: patients, DM+: presenting type II DM; NGT: normal glucose tolerance IFG: impaired fasting glucose; IGT: impaired OGTT, HT: hypertension, CAD: coronary artery disease. Plasma levels are expressed as mmol/l and as a mean ± S.D. or as median [95th percentile, 5th percentile].

progression of minimum luminal diameter (P<0.004). However, this association was not independent upon plasma TG levels. In addition, a significant association was found between the plasma RLP-C concentration and occurrence of new lesions in vein grafts. In patients with vasosoatic angina with or withour myocardial infarction, plasma RLP-C was a major risk factor for predictio of myocardial infarction (98). Elevated levels of plasma RLP-C were predictive for future coronary events in Japanes patients with CAD independent of other risk factors (99;100). In the postprandial period, arterial relaxation (as assessed by flow mediated dilation) in healthy young normolipidemic subjects decreased in parallel with the plasma peak concentration of TG and RLP-C (101). Additional evidence for a relationship between postprandial increase in plasma RLP-C and endothelial function was observed in a small study by Wilmink et al (62), wherein the increase in plasma RLP-C and decrease in endothelial function was attenuated by short term statin treatment, independent upon the differences in plasma TG and cholesterol. Human studies therefore suggest a direct effect of RLP on atherogenesis.

In-vitro studies have further elucidated the role of RLP the atherosclerotic process. Incubation of triglyceride rich particles with isolated rabbit aorta showed that RLP inhibited arterial relaxation by acetylcholine in a dose-dependant manner (102). Pretreatment of rat aortas with L-NAME completely downregulated vasorelaxation of thoracic rat aortas, indicating the involvement of a nitric oxide (NO) mediated pathway. Moreover, inhibition of cell surface glycoproteins with heparin and lactoferrin did not affect RLP-induced impairment of vasorelaxation. These results suggest that RLP exert a direct effect on endothelial function without binding to glycoproteins that are located at the apical site of the endothelium. Incubation with RLP (0,1 mg cholesterol/ml), but not VLDL TG-rich lipoproteins (d<1,006) or LDL (1,019<d<1,063) that are separated by ultracentrifugation, induced an increased expression of ICAM-I, VCAM-I and Tissue Factor (TF) in a HUVEC cell model partly through a redox-sensitive mechanism (103). Incubation with alpha-tocopherol suppressed the RLP-induced mRNA and protein expression of these molecules in a dose dependent manner. In addition, treatment of subjects with higher plasma RLP-C levels (RLP-C > 5,1 mg cholesterol/dL) for 4 weeks with alpha-tocopherol (300 mg/day) prevent the rise in plasma levels of adhesion molecules, such as sICAM-I and sVCAM-I. (103). Incubation of RLPs with HUVEC and monocytes in a flow conditioned model results in enhanced expression of CD11a, CD18 and CD49d and IL1β (104), indicating a role of remanant lipoproteins in vascular inflammation. Pro-inflammation is a key step in progressive atherosclerosis and a pro-inflammatory phenotype is closely associated with a high cardiovascular mortality. Macrophages and subsequently foam cells are frequently found in both human and animal atherosclerotic plaques. The interaction of RLP with macrophages and the consequent atherogenic response is still under investigation, but RLP may induce foam cell formation according to previous studies in animal models ref.

Effect of treatment on plasma RLP-C

Only limited intervention studies are available to evaluate potential therapeutic reductions of increased plasma levels of RLP-C.

Effect of statin treatment

Although evidence exist that statin treatment result in a reduced cardiovascular mortality that may be explained by plasma LDL-cholesterol lowering, the effect of statin treatment on elevated plasma RLP-C levels is not consistently observed. Pravastatin, atorvastatin or simvastin exert its own effect on plasma RLP-C lowering in a randomized cross-over study in patients with combined hyperlipidemia (105). Plasma RLP-C reduction did not occur after pravastatin (40 mg/d), whereas Simvastatin (20 mg/d; median reduction of 6%) and Atorvastatin (10

Table 5: Plasma RLP-C in diseased state

authors	Design study	Disease	TG	LDL-C	RLP-C
Saigo, 2000	Case control	CHD	C:1.3±0.6 P:1.0±0.6	np	C:0.12 ± 0.06, P:0.11±0.7; Positive correlation with factor VIIa,(r=0.32) and VII a/FVIIag ratio (r=0.24)
Oda, 1997	Case control	Chronic renal insufficiency; 10 year period of hemodialysis	np	np	C: 0.07 ± 0.03 M: 0.07 ± 0.03, F:0.07 ±0.04 P: 0.21 ±0.17 M: 0.18 ± 0.15,F:0.25 ±0.19
Schaefer, 2001	Case control post-hoc analysis of placebo controlled randomized trial	CHD	C (n=88): 1.5± 0.0 P (n=88): 2.2 ± 1.0	C: 3.5±1.1 P: 3.8±1.6	C: 0.20±0.09 P: 0.27±0.29
Takeichi, 1999	Postmortem pathological study	Sudden cardiac death; with (A+) vs without atherosclerosis (A-)	A-: 4.7 (3.4-5.8) A+: 4.1 (3.2-5.6)	A-: 3.0 (2.1-3.9) A+: 3.6 (2.4-4.4)	A-: 0.22 (0.13-0.48) A+: 0.35 (0.21-0.65)
Schaefer, 2002	placebo controlled randomized intervention trial	CHD, LDL-C >3.4 mmol/l	P: 2.2 ± 1.3	P: 4.7 ± 1.6	P: 0.29 ± 0.29
Deighan, 2001	Randomized cross over study; fenofibrate(feno) vs cerivastatin (ceri)	Renal disease with proteinuria	P: 3.6 ± 2.6, Feno: - 41%. Ceriva: - 14 %	P: 5.2 ± 1.2, Feno: - 8%, Ceriva: - 23%	P: 1.17 (0.83-1.74), Feno: -21%, Ceriva –2%
Masuoka, 1998	Observational ; angiographic stenosis (S)	CHD	np	np	S-: 0.18±0.15 S+: 0.10±0.07
Masuoka, 2000	Observational ; angiographic stenosis (S)	CHD (n=208; NC=151,HC=57)	HC S+: 1.7 ± 0.6 HC S-: 2.9 ± 0.4 NC S+: 1.6 ± 0.8 NC S-: 1.3 ± 0.5	HC S+: 4.5± 0.6 HC S-: 4.0± 1.0 NC S+: 3.0 ± 0.6 NC S-: 2.8 ± 0.7	HC S+: 0.18±0.13 HC S-: 0.16±0.9 NC S+: 0.18±0.15 NC S-: 0.09±0.06
Fukushima, 2001	Case control angiography study	CHD	PM CHD- 1.4± 0.2 PM CHD+ 1.5 ± 0.9 CHD+ M: 1.4 ± 0.6	PM CHD -: 3.1 ± 0.9 PM CHD +: 3.3 ± 1.0 CHD+ M: 3.2 ± 0.7	PM CHD-: 0.07(0.05-0.1) PM CHD+: 0.09 (0.07-0.14) CHD+ M 0.08 (0.42-1.27)
Karpe, 2001	Posthoc analysis of Randomized, placebo controlled fibrate (F) intervention study	CHD	np	Baseline: 3.5 (3.0-3.8) F+ (-6%), F- (+3%)	Baseline: 0.24 (0.17-0.39) F+(-33%), F- (-7%)

A: Atherosclerosis; Coronary heart disease: CHD, C: control subjects, p: patients, F: female, M: male, HC: hypercholesterolemic, NC: normocholesterolemic NP: Not presented PM: postmenopausal, M: male, F: female. minus (-): not present plus(+): present. Plasma levels are expressed as mmol/l and as a mean ± S.D. or as median [95th percentile, 5th percentile].

mg/d; median reduction of 26%) lowered plasma RLP-C levels significantly. Although high dose Simvastatin therapy in heterozygous FH patients also reduces plasma RLP-C concentrations significantly, only 25 % of FH patients reached plasma RLP-C levels comparable with aged and sex matched normolipidemic subjects (66). In a small FH population, a significant reduction in baseline and postprandial RLP-C levels were found by statin treatment (106; 107). No dose-dependent decrease in plasma RLP-C (-33%, -34%, -32%) from atorvastatin (20 mg/day, 40 mg/day and 80 mg/day) in patients with CHD was found, in contrast to plasma levels of LDL-cholesterol (-38%, -46%, and -52%) and TG (-22%, -26%, and -30%) (108). This is not surprising since the primary target for lipid lowering with a statin will be plasma LDL-cholesterol. The decrease in plasma TG after statin therapy is much less pronounced and is mostly not dose-dependent (109; 110). Additional intervention studies are needed to compare different statins with respect to their potency to reduce plasma RLP-C levels in distinct patient groups with disturbances of lipid metabolism.

Fibrates

The principal effect of fibrates is lowering of plasma TG and increase of plasma HDL-C. The working hypothesis is that fibrates alter the expression of PPAR-α. Several genes involved in triglyceride metabolism, such as apo C-III and LPL have a PPAR regulating element in the promoter region. Apo C-III expression is downregulated by fibrates, whereas LPL gene expression is upregulated (111). As stated before, plasma RLP-C concentrations are closely associated with plasma TG, VLDL-cholesterol and VLDL-TG levels, but not with LDL-cholesterol. It is therefore likely to expect a significant lowering effect of fibrate therapy on plasma RLP-C levels. In patients after coronary artery bypass grafting, a two year treatment with Gemfibrozil (LOCAT study; 1200 mg/day) reduced median plasma RLP-C levels with 34 % (from baseline 0.24 [0.17-0.42] to 0.15 [0.13-0.20] mmol/l) (97). In patients with proteinuric renal disease, no effect of cerivastatin (100 or 200 μg once a day for 2 months) on plasma RLP-C was found, in contrast to fenofibrate (200 mg once a day for 2 months) that results in a 35 % decrease in plasma RLP-C levels (112). In line with the reduction of plasma TG during fenofibrate treatment, the TG reduction correlated with a decrease in VLDL1 and and a shift towards less dense, less atherogenic LDL subfraction. In addition, the reduction in VLDL-1 was associated with the decrease in plasma RLP-C. Therefore, treatment with fibrate is favored above treatment with statins with respect to plasma RLP-C reduction in patients with a nephritic-range proteinuria. In patients with combined hyperlipidemia the threat of rhabdomyolysis occurs when both a statin and a fibrate are subscribed. Therefore a direct comparison was made between the effect of statin versus gemfibrozil on plasma lipid phenotype. However there was a significant decrease in plasma TG on gemfibrozil treatment and no effect with the statin. Statin treatment results in a significant decrease in plasma LDL-C, whereas no change was seen in gemfibrozil treated group. Plasma RLP-C decrease was similar in both treatment groups (113).

Diet intervention

The effect of diet intervention on plasma RLP-C levels are still not studied extensively. In contrast to long-term intervention studies, short-term dietary intervention studies with either a high fat or a high carbohydrate intake in healthy adults did not result in changes in plasma TG levels (114). Moreover, in type IV hyperlipidemic patients who consume a low fat diet for three months, no changes in fasting plasma TG levels were found (115). In a short-term diet intervention study, in which carbohydrate was added to mixed meals, even in large amounts, no increase in de-novo lipogenesis was found (116;117). A diet of 20 grams of soy protein isolate during 3 weeks reduced baseline plasma RLP-C levels by 9,8% (118). A replace-

ment of diacylglycerol by triacylglycerol in the oral fat load, increased postprandial plasma RLP-C levels (119).

Conclusion

The importance of atherogenic lipoprotein remnants in the development of premature and progressive atherosclerosis, beyond LDL-cholesterol have now been extensively documented. It remains however to be studied in large epidemiological studies to what extend the measurement of plasma RLP-C provides additional knowledge for the treatment goals of the individual patients. In comparison to a plasma TG measurement, plasma RLP-C analyses is expensive and time-consuming. The samples should be stored at -80°C and not for a period longer than one or two years. Furthermore, the standardization of the assay is very important and critical for the outcome of the plasma RLP-C levels. It is still striking that plasma RLP-C levels in Japan are so much lower than in Caucasian countries. In conclusion, plasma RLP-C analyses further completes the atherogenic profile of the patient at risk and can be used as a specific target for treatment. Assessment of RLP-C, in addition to LDL-cholesterol and the calculated non-HDL cholesterol, may therefore complete the atherogenic lipid profile with a view to estimation of the risk of coronary events.

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1.2
Isolation of remnant particles by immunoseparation: a new approach for investigation of postprandial lipoprotein metabolism in normolipidemic subjects

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Abnormal postprandial lipoproteins are associated with an increased risk for cardiovascular disease. Postprandial remnant lipoproteins were usually analyzed indirectly using retinyl esters (RE) as a chylomicron core label during an oral fat loading test. Apo B-100 containing VLDL remnants in addition to apo B48 containing chylomicron remnants can also be directly quantified using the RLP-Cholesterol Immunoseparation Assay. This recently available method uses monoclonal antibodies to apo A-I and apo B-100 to remove non-remnant lipoproteins and quantifies cholesterol in the remaining apo E-rich remnant fraction. In the present study we compared the analysis of retinyl ester with the immuno-based RLP-Cholesterol (RLP-C) analysis in measuring postprandial remnant lipoproteins in healthy normolipidemic subjects.

Sixteen healthy normolipidemic subjects were selected for this study. Postprandial plasma retinyl esters peaked at 5.0 ± 1.2 hr, whereas plasma RLP-C showed a peak significantly earlier ($P<0.001$) at 3.5 ± 0.6 hr. In comparison, postprandial plasma TG and FFA peaked at 3.3 ± 1.1 hr ($P<0.005$ compared to retinyl esters).

In conclusion, levels of RLP-C changed, during the postprandial phase, in parallel with plasma TG and FFA concentrations and peaked significantly earlier than retinyl esters. Postprandial measurements of RLP-C can be considered as a fast alternative method for the more laborious retinyl ester analysis in clinical studies.

Abnormal postprandial lipoproteins are associated with an increased risk for cardiovascular disease (1; 2). Dietary fat enters the small intestine and is packed in apoB48 containing chylomicrons in the enterocyte (3;4). These particles are heterogeneous of composition and size. Upon secretion into the circulation chylomicron triglycerides are lipolyzed by the direct action of lipoprotein lipase (LPL) in peripheral tissues, leading to the formation of free fatty acids and remnant particles (1). Remnants have a reduced triglyceride content and are enriched in cholesterol and apolipoproteins B and E (5). Increased levels of these remnant particles have already been associated with the presence and progression of cardiovascular disease.

Most studies on the relationship between cardiovascular disease and lipoproteins metabolism have been based on fasting lipoprotein levels. Given the fact that a person is in the postprandial state a great period of the day, it appeared to be more appropriate to analyze lipoprotein metabolism postprandially using a standardized fat intake or a standardized meal. Postprandial remnant lipoprotein metabolism was typically measured in humans by incorporation of retinyl esters into chylomicron particles as a core label during an oral fat loading test (6; 7). As chylomicrons undergo lipolysis and become a smaller remnant particle the palmitate ester label remains in the core and is cleared from the plasma. Therefore, monitoring the retinyl ester label during an oral fat loading test provides data about chylomicron appearance and clearance. The evaluation of data using retinyl esters as marker have been discussed extensively, because of the possible exchange of the label with other non-intestinally derived lipoprotein fractions (8; 9). It is thus important to search for a more reliable method to assess remnant lipoprotein concentrations. Recently, the development of a novel immunoseparation method by Nakajima et al (10) enables a new approach for remnant analysis both in fasting and postprandial plasma samples. This assay is based on the specificity of two antibodies used to isolate non-remnant lipoproteins. The apo B100 monoclonal antibody (mab JI-H) recognizes

an epitope near apo B51 enabling the isolation of all LDL and most VLDL particles. A monoclonal antibody against apo AI removes all HDL particles. In the remaining unbound fraction apoB 48 remnant particles and a small portion of apo E containing VLDL remnant particles are present. This fraction is called 'remnant-like particles' (RLP). Cholesterol RLP-C) and triglycerides (RLP-T) are measured using an enzymatically assay. Increased levels of RLP-C has been found in a number of disorders related to increased risk for atherosclerosis like diabetes, type III dyslipoproteinemia, chronic kidney insufficiency, and hypertriglyceridemia.
In the present study we evaluate the classical approach of postprandial lipid kinetics by retinyl palmitate incorporation into intestinally derived lipoprotein particles with remnant analysis using the new immunoseparation technique in measuring postprandial remnant lipoproteins in normolipidemic individuals.

Methods and Materials

Subjects
Normolipidemic healthy control subjects, matched for age, gender, BMI and apo-E genotype, were selected for this study by advertisement in the local newspaper. Exclusion criteria included the presence of diabetes, hepatic -, renal -, thyroid dysfunction and a positive family history for cardiovascular diseases. The human investigation review committee of the University Medical Center Utrecht approved the study protocol and written informed consent was obtained from all participants.

Oral fat loading test
After an overnight fast of 12 hrs, participants were admitted to the metabolic ward at 7.30 h am. Cream (consisting of 40% fat (w/v) with a P/S ratio of 0.06, 0.001% cholesterol (w/v) and 2.8% carbohydrates (w/v)), supplemented with 120.000 IU Vitamin A, was given as a single fat load in a dose of 50 g fat per m^2 body surface area. Prior to the test meal blood samples were obtained for baseline values. After ingestion of the cream venous blood samples were taken hourly from an indwelling catheter in the ante cubital vene during 8 hours. During the test only water or tea without sugar were allowed to drink. Blood was collected in EDTA (2mM) containing tubes. All blood samples were immediately put on ice and immediately centrifuged at 4°C, 3000 rpm for 15 min.

Analytical Methods
Plasma triglyceride and total cholesterol were measured by enzymatic assays (Monotest cholesterol kit no. 237574 and GPO-PAP no. 701912, Boehringer Mannheim, Germany). HDL cholesterol was determined using heparin-manganese precipitation of non-HDL lipoproteins (11). Plasma free fatty acids (FFA) were analyzed by an enzymatic assay (WAKO chemicals, Neuss, Germany). Plasma apo E and plasma apo CIII concentrations were analyzed with a commercial available immunoelectrophoretic assay (Hydragel LP E and LP CIII, Sebia, Issy-les-Moulineaux, France). Apo E genotype was determined as described before (12). ApoB was measured using an enzymatic assay (UNIMATE 3 APOB kit, Roche Diagnostics Systems, France) using a Cobas Mira S auto-analyzer (ABX Diagnostics, Montpellier, France). Retinyl esters were analyzed on the HPLC (LC 10A, Shimadzu, Japan) (13) using retinyl palmitate as a standard.

Preparation of chylomicron and non-chylomicron fractions
Lipoproteins were separated in a single ultracentrifugation step by flotation in a Sf>1000 fraction which contains chylomicrons, large chylomicron remnants and large hepatic triglyceride rich lipoproteins, and a remaining infranatant fraction (Sf<1000) containing small chylomicron remnants and all the other lipoproteins (14). Plasma samples were adjusted to d=1.006 g/L using and overlayered with d=1.006 g/L NaCl solution. Ultracentrifugation was performed in a SW 50.1Ti

swinging bucket rotor at 40.000 rpm for 4h at 15°C in a Beckmann Optima Ultracentrifugation.

Analysis of postprandial RLP-C.
The RLP fraction was prepared using an immune separation technique described by Nakajima et al (15). Briefly, 5 μl of serum was added to 300μl of mixed immunoaffinity gel suspension containing monoclonal anti-human apo A-I (H-12) and anti-human Apo-B-100 (JI-H) antibodies (Japan Immunoresearch Laboratories, Takasaki, Japan). The reaction mixture was gently shaken for 120 minutes at room temperature on a special mixer (RLP-mixer J100-A, Photal, Otsuka Electronics, Japan). After 15 minutes, 200μl of the supernatant was used for the measurements of cholesterol (RLP-C) by an enzymatic assay included in the assay kit using a Cobas Mira S auto-analyzer (ABX Diagnostics, Montpellier, France). The interassay variability was 1.3 %.

Statistical Analysis
Data are presented as means ± SD, unless shown otherwise. Paired student's t-test was used to test the significance of the difference between the peak times of the various postprandial analyses. Pearson correlation test was used for correlation analysis. $P<0.05$ was considered statistically significant. An Area under the integrated Curve (AUC) was calculated using GraphPad Prism software (version 3.1, San Diego, California, USA).

Results

Population characteristics
All subjects were normolipidemic according to the latest European recommendations. The baseline characteristics are presented in (**Table 1**). The subjects were middle-aged men (8) and women (8) on a free-living diet. No difference in age, body mass index, plasma lipid and apolipoprotein levels was observed between male and female subjects. As a consequence we analyzed the data for males and females together.

Postprandial profiles in normolipidemic subjects
Postprandial plasma, chylomicron (Sf>1000) and non-chylomicron (Sf<1000) levels of TG, FFA, retinyl esters, apo B and RLP-C were analyzed. Plasma TG and FFA levels increased after ingestion of the cream (**Figure 1**, panel A and B). The maximal postprandial plasma TG concentration (peak: 1.8 ± 1.2 mmol/l, baseline: 0.9 mmol/L; $p<0.001$) and maximal postprandial plasma FFA concentration (peak: 0.62 ± 0.23 mmol/L; baseline: 0.36 ± 0.23 mmol/L $p<0.05$) occurred between 3 and 4h, respectively. After 8h the plasma triglyceride curve returned to its baseline levels, whereas the plasma FFA curve remained elevated throughout the whole test period. Plasma apo B levels did not change during the postprandial test (**Figure 1C**). Plasma retinyl ester concentration (baseline: 0.62 ± 0.73 mg/L) increased, with a delay over the first two hours, (**Figure 2**, panel A) to the maximal postprandial plasma retinyl ester response of 7.65 ± 5.15 mg/L ($p<0.0001$) at around 5h. Plasma retinyl ester concentrations remained elevated until 7h after the start of the oral fat load test, and did not return to its baseline levels after 8h. The retinyl ester concentrations in the Sf<1000 fraction (baseline: 0.33 ± 0.23 mg/L) and the Sf>1000 fraction (baseline: not detectable) showed curves comparable to the plasma retinyl ester curve (**Figure 2**, panel A). The maximal postprandial retinyl ester concentra-

tion in the Sf<1000 (2.59 ± 2.00 mg/L; p<0.001 in comparison with baseline concentrations) and in the Sf>1000 (5.08 ± 2.43 mg/L) was reached around 5h.

Plasma RLP-C (baseline: 0.18 ± 0.06 mmol/L) and RLP-T concentration (baseline: 0.25 ± 0.14 mmol/L) gave an immediate response upon ingestion of the dietary fat load (**Figure 2**, panel B). The maximal postprandial plasma RLP-C concentration (0.48 ± 0.31 mmol/l; p<0.05 versus baseline) was reached at 3.6 h, comparable to the plasma triglyceride peak. The RLP-T and the RLP-C profile, are similar, although we were unable to measure the 3-hour RLP-T point. The peak of Sf<1000 retinyl ester was significantly later than the plasma RLP-C peak (**Figure 3 and Table 2**, p<0.0001), and plasma TG peak (p<0.0001) (**Table 2**). The baseline values, integrated area under the curve (AUC) and incremental AUC of plasma RLP-C, plasma TG, Sf<1000 retinyl ester are presented in **Table 3**.

To determine which parameters were best predictive for plasma RLP-C levels a correlation analysis was performed (**Table 4**). Baseline RLP-C was positively correlated with BMI (0.54; p<0.05), plasma TG (0.79; p<0.001), plasma cholesterol (0.52; p<0.05), apo B (0.73; p<0.001) and apo E (0.61; p<0.05). The integrated AUC of RLP-C showed similar correlations but an additional correlation with apo CIII (0.59; p<0.05) was found. The incremental AUC of RLP-C was positively correlated with apo CIII (0.53; p<0.05) and with baseline plasma cholesterol (0.49; p=0.05). The integrated AUC of RE (Sf<1000) was positively correlated with plasma TG (0.55; p<0.05), plasma cholesterol (0.60; p<0.05) and apo B (0.64; p<0.05). The incremental AUC of RE (Sf<1000) was positively correlated with plasma TG (0.53; p<0.05) and apo-B (0.61; p<0.05).

Table 1: Baseline characteristics of the subjects

Parameter	
N	16
Age (y)	49 ± 9
BMI (kg/m^2)	25.30 ± 3.22
WHR	0.83 ± 0.09
Cholesterol (mmol/L)	5.16 ± 0.70
HDL-chol (mmol/L)	1.53 ± 0.37
Triglycerides (mmol/L)	0.90 ± 0.38
ApoB (g/L)	0.95 ± 0.25
ApoAI (g/L)	1.20 ± 0.22
ApoE (mg/dL)	4.76 ± 1.1
Apo CIII (mg/dL)	3.0 ± 0.7
NEFA (mmol/L)	0.36 ± 0.16
ApoE genotype	
E3E3	10
E3E4	2
E4E4	3
E2E3	1

Values are expressed as mean ± SD.[1]

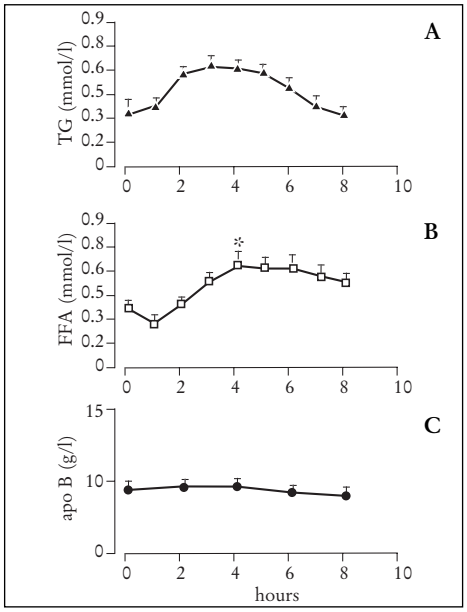

*Figure 1. Postprandial plasma TG (panel A), plasma FFA (panel B) and plasma apo B (panel C) curves. All data are expressed as mean ± SEM. Peak versus baseline: * p<0.05 and ** p<0.001 as tested with a student's T-test.*

Table 2: Differences in peak time of plasma TG, Sf<1000 RE and plasma RLP-C during the oral fat load.

	Time (hr)	p-value
RLP-C vs. TG	3.56 ± 0.63 vs. 3.25 ± 1.13	ns
Sf<1000 RE vs. TG	5.14 ± 1.10 vs. 3.25 ± 1.13	0.0001
RLP-C vs. Sf<1000 RE	3.56 ± 0.63 vs. 5.14 ± 1.10	<0.0001

Values are expressed as mean ± SD. P-values were determined using the Student's t-test.

Table 3: Baseline values and postprandial integrated areas under the curve (AUC) of plasma RLP-C, plasma TG, Sf<1000 RE and plasma TG/Apo-B ratio.

	RLP-cholesterol	TG	Sf<1000 RE
Baseline	0.18 (0.06)	0.89 (0.37)	0.33 (0.23)
AUC 0-8 h	2.59 (0.89)	11.41 (4.81)	13.36 (9.50)
ΔAUC 0-8 h *	1.14 (0.61)	4.49 (2.41)	10.70 (8.68)

Values are expressed as mean ± SD. P-values were determined using the Student's t-test. * ΔAUC means incremental area under the curve. RLP-C and TG are expressed as mmol/L, retinyl esters as mg/L.

Table 4: Correlation coefficients of variance of baseline lipid parameters and BMI with plasma RLP-C (Baseline, AUC and incremental AUC) Sf<1000 RE (AUC and incremental AUC).

		RLP-C		Sf<1000 RE	
	Baseline	AUC 0-8 h	ΔAUC 0-8 h †	AUC 0-8 h	ΔAUC 0-8 h †
BMI	0.54 *	-	-	-	-
TG	0.79 **	0.53 *	-	0.54 *	0.54 *
Cholesterol	0.52 *	0.61 *	0.49 *	0.60 *	-
Apo-B	0.73 **	0.56 *	-	0.64 *	0.61 *
Apo-CIII	-	0.59 *	0.53 *	-	-
Apo-E	0.61 *	0.58 *	-	-	-

† Δ AUC 0-8h indicates area under the curve incremental curve after substraction of baseline concentrations. Correlations were determined using Pearson's correlation coefficient test. *P<0.05, ** P<0.001.

Discussion

In the present postprandial study, the characterization of chylomicron/VLDL-remnants by immunoseparation or by retinyl ester analysis in the Sf<1000 fraction after an one-step ultracentrifugal separation revealed different postprandial chylomicron remnant profiles in normolipidemic subjects. Fasting plasma RLP-C concentrations in our normolipidemic subjects (mean RLP-C 7.0 mg/dl, 0.18 mmol/L) were in the higher range of the 75th percentile RLP-C concentrations when compared to a general North American population (6.6 mg/dL, 0.17 mmol/L) (16) and were in the lower range when compared to the Framingham study population (7.2 mg/dL, 0.19 mmol/L) (17). Post-hoc analysis in the Framingham study population resulted in gender differences for fasting RLP-C concentrations (males vs. females: 8.02 mg/dL (0.21 mmol/L) vs. 6.80 mg/dL (0.18 mmol/L) respectively). In our study, fasting RLP-C tended to be higher in males than in females, but due to the small number of subjects it did not reach significance. Fasting RLP-C was positively correlated with BMI, fasting plasma TG, and fasting plasma cholesterol (17). In the present study BMI, plasma TG and cholesterol were lower compared to the Framingham study population and could explain the relatively lower RLP-C levels.

Enhanced postprandial chylomicron-remnant levels are a feature of premature atherosclerosis (18). Measurement of retinyl esters in the triglyceride-rich lipoprotein fraction has been proposed as a marker for chylomicron remnants (6; 19-22). However, retinyl ester label does not specifically resembles chylomicron clearance due to the exchange of the label between lipoprotein species in plasma (8; 9; 23). Up to 25% of the retinyl ester in the postprandial state was due to retinyl ester in the apo B100 TRL fraction. The apo B100 peak occurred at a later time point than the apo B48 peak that is in favor of a precursor - product relationship. Brunzell et al (24) already proposed that VLDL and chylomicrons are catabolized by a common pathway by competition for the enzyme lipoprotein lipase at the capillary endothelium surface. Increased presence of apo B48 containing remnants could explain the increased levels of triglycerides in apo B100 particles. On the other hand evidence is available that in the fed state an increase in the production rate of triglyceride-rich apo B100 containing lipoprotein particles occurred (25).

In the enterocyte, retinyl esters are incorpo-

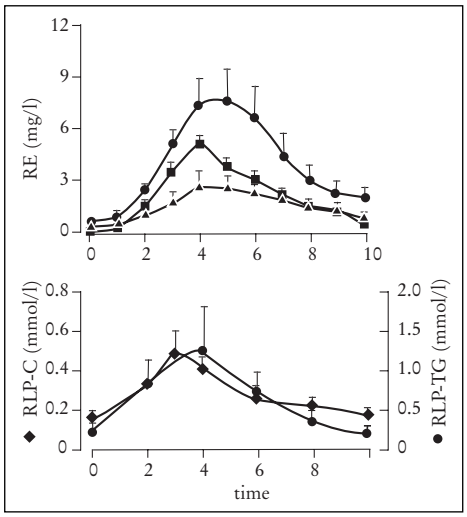

*Figure 2. Plasma retinyl esters (▼), Sf>1000 RE(▫) and Sf<1000 RE (▫)responses(panel A). Postprandial RLP-C (▫; left y-axis) and RLP-T (▼ right y-axis) (panel B). All data are expressed as mean ± SEM. Peak versus baseline: * p<0.05*

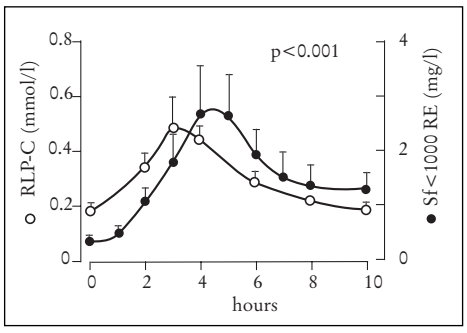

*Figure 3. Postprandial RLP-C (left y-axis) curve and the postprandial Sf<1000 RE (right y-axis) are shown. All data are expressed as mean (SEM). ** P<0.001 compared to baseline value. The peaks of the RLP-C and RE curve are significantly different (p<0.0001).*

rated in chylomicrons proportionally to the size of chylomicrons (26). Postprandially, the numbers of chylomicron particles, measured as apo B48 concentration, do not increase. However, during the postprandial phase the size of chylomicrons is distributed in a bimodal way (27). In the early postprandial phase smaller primordial lipoproteins (apo-B48) are assembled independently from the formation of triglyceride-rich lipid droplets, whereas at later time points (6-8 hours) larger chylomicrons, formed by fusion of TG-rich lipid droplets and primordial lipoproteins, are secreted by the enterocyte. This means that the postprandial retinyl ester peak in most studies, with an average at 5 hours after ingestion of the test meal, reflected the capacity of large chylomicron particles to incorporate the retinyl esters. The postprandial retinyl ester response, with a delay in the first hour in oral fat-loading test, in our normolipidemic subjects confirmed this hypothesis. An additional support was derived from the fact that plasma retinyl esters and retinyl esters in the remnant fraction (Sf<1000) peaked significantly later than plasma triglycerides and the RLP-C peak. Moreover, the postprandial RLP-C curve changed in parallel with plasma TG and FFA concentrations.

In summary, we have presented a novel application for the elegant and fast immunoseparation technique to quantify remnant particles in the postprandial phase. Although both RE and RLP-C can be used to study postprandial lipoprotein metabolism, they measure different remnant lipoprotein fractions.

Acknowledgement

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1.3
Physicochemical Properties of the Remnant-Like Particle Fraction and its Susceptibility to Oxidative Stress

Preliminary report

Remnants of triglyceride-rich particles, including chylomicrons and very low-density lipoprotein (VLDL), (TRP) are associated with an increased risk for cardiovascular disease. A recent method to analyse these lipoprotein remnants (RLP) in a suitable way was introduced by Nakajima et al (1). Elevated plasma levels of RLP-C are found in patients with coronary artery disease. In patients with an adult-onset growth hormone deficiency (AGHD), an atherogenic lipoprotein phenotype was reported, together with elevated plasma RLP-C levels that have similarities with a mild form of type II-B dyslipidaemia (2). This atherogenic lipid phenotype is characterized by elevated plasma levels of total cholesterol and triglyceride (>250 and > 200 mg/dL, respectively) (3), reflecting elevated plasma VLDL and IDL concentrations, and in addition, subnormal plasma HDL-cholesterol levels. An increased mortality due to cardiovascular disease is observed in these patients. Although direct negative effects of the RLP fraction on endothelial function was found, the mechanism underlying this effect remains to be established Moreover, the physical-chemical properties of the RLP fraction and its relation to the atherogenic properties of this specific TRP fraction are not totally elucidated.

In this preliminary report, our objective was to analyse the physicochemical composition of the RLP fraction and to evaluate its susceptibility to oxidative stress in Type IIB patients.

Patients/Methods

Patients

Patients (n= 50) were selected who had a combination of hypercholesterolemia and hypertriglyceridemia, a phenotype termed combined or "mixed" hyperlipidemia (type II-B). Plasma samples were obtained from the patients after an overnight fast and pooled. Mean plasma cholesterol and triglyceride (TG) levels were 296 ± 28 mg/dl and 253 ± 32 mg/dl, respectively. Plasma samples were stored at minus 80°C for subsequent analyses.

Lipoproteins

The lipid content of plasma and isolated lipoprotein fractions was quantified enzymatically by using kits from Roche Molecular Biochemicals (Meylan, France) for total cholesterol (TC) and free cholesterol. CE mass was calculated as (TC-FC) x 1.67 and, thus, represents the sum of esterified cholesterol and fatty acid moieties (4). Kits from Bio-Mérieux (Marcy-l'Etoile, France) were used for determination of TGs and phospholipids. Bicinchoninic acid assay reagent (Pierce Chemical Co., Rockford, IL) was used for protein quantification. Lipoprotein mass was calculated as the sum of the mass of the individual lipid and protein components for each lipoprotein fraction.

TRP fraction analysis

Subfractions of triglyceride-rich lipoproteins, i.e. VLDL-1 (Sf 60-400), VLDL-2 (Sf 20-60) and IDL (Sf 12-20) were isolated from plasma (2 ml) by cumulative flotation following non-equilibrium density gradient ultra centrifugation using a Beckman SW41 Ti rotor (5). All lipoprotein fractions were analysed for their protein and lipid content.

RLP isolation

Isolation of the RLP fraction was performed as described before, using an immunoseparation technique described by Nakajima et al (1) with some modifications (1; 6). From each pool of plasma, 10 ml plasma was added

to 20 ml mixed immunoaffinity gel suspension containing monoclonal anti-human apo A-I (H-12) and anti-human Apo-B-100 (JI-H) antibodies (Japan Immunoresearch laboratories, Takasaki, Japan). The reaction mixture was gently shaken during the night at 4°C. The gel mixture was applied in a column, and washed with PBS EDTA, pH 7.4. The unbound fraction was collected, and further isolated by ultracentrifugation (40.000 rpm, 24h) in a SW 60 rotor in a Beckman ultracentrifuge at a density of 1.063 g/ml to reduce contamination with plasma proteins, such as albumin. The top fraction (5 ml) was collected and concentrated five fold by using Millipore centrifugal filter device (Mw cutt off 100.000) and dialysed extensively against PBS pH 7.4 (24 h, 4°C). The RLP fraction was analysed for protein and lipid content.

Oxidation studies

The same procedure was performed, as described earlier (7). Lipoproteins were extensively dialysed against PBS pH 7.4 (three changes over 24 h, 4°C) before the oxidation studies. In short, lipoproteins were incubated at a final concentration of 50 μg protein/ml; with 5.0 μM $CuSO_4$ at a final volume of 500 ml. Conjugated diene absorption (234 nm, each 10 min, at 37°C) was measured by spectrophotometry over a period of 20 h. As a positive control, a LDL-3 fraction from normolipidemic subjects was oxidised in parallel. The oxidative curve typically reveals three phases; specifically the lag, propagation and decomposition phase (8). Propagation rate is the average oxidation rate in the propagation phase.

Chromatographic size exclusion

To estimate the size range of RLP, in comparison with VLDL-1, VLDL-2 and IDL, we used the Duo Flow system (Biorad), that consists of two Superose 6HR 10/30 columns in series with a flow rate of 0.4 ml/min. Both the RLP fraction and the distinctive TRP fractions (each 200 μL) were injected separately in the Duo Flow system. Fractions of 2 ml were collected and used for the determination of TG and cholesterol content. From the RLP profile, the total area under the curve (AUC) and partial AUCs, corresponding to the TRP subfraction, was calculated with GraphpadPrism (version 3.1, 2002).

Statistical Analysis

All values are presented as means ± SD. Two sided paired student t-tests were used with a p value of <0.05, as significant. The GraphpadPrism (version 3.1, 2002) was used as statistic software.

Results

Plasma lipoprotein profile and chemical composition

Table 1 summarizes the percent weight chemical composition of the different TRL fractions (VLDL-1, VLDL-2 and IDL) and RLP, isolated from four different plasma pools obtained from type II-B patients. The RLP fraction is distinguished by its high cholesteryl ester content, compared to the other TRP fractions.

Physical characteristics

Figure 1A and 1B show distinctive chromatographic (size exclusion) profiles from the RLP fraction and each fraction of TRPs (VLDL-1, VLDL-2 and IDL). The first peak that could be detected was RLP, followed by VLDL-1, VLDL-2 and IDL. Although the elution position of the peak of RLP-TG was similar to that of VLDL-1, the RLP cholesterol peak was broader and overlapped partly the VLDL-2 and IDL fractions. The fractions were well separated by the column in the different runs. The RLP-C peak was subsequently detected in the same chromatographic (size exclusion) profiles (fraction 77 ± 2). The part of the RLP curve that is in the size range of VLDL-1 (fraction 76-89) is 56% (RLP-C), and 82% (RLP-TG), 25% (RLP-C) and 23% (RLP-TG) is in the size range of VLDL-2 (fraction 86-118); and 12 % (RLP-C) and 14% (RLP-TG) in the size range of IDL (fraction 99-126). These data indicate that the particle size range of RLP closely resembles that of the VLDL-1 subfraction.

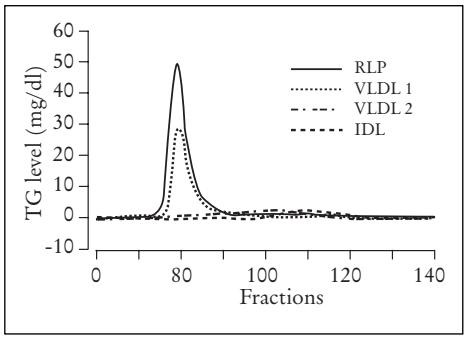

Figure 1A. The RLP-TG profile of RLP and VLDL-1, VLDL-2 and IDL in type II-B dyslipidemic subjects after size exclusion chromatography.

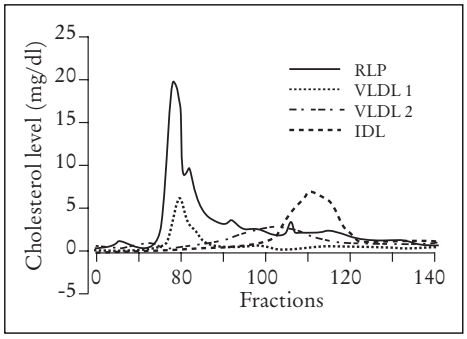

Figure 1B. The RLP-C profile of RLP and the content of cholesterol in the VLDL-1, VLDL-2 and IDL in type II-B dyslipidemic subjects after size exclusion chromatography.

Oxidizability

Both the RLP fraction and the TRP fractions, like LDL-3, were susceptible to oxidative modification after exposure to $CuSO_4$ as assessed by the formation of conjugated dienes (figure 2). The conjugated diene curves were analysed for lag time, T1/2 and maximal conjugated diene concentration. The RLP fraction (compared to LDL-3 from normolipidemic subjects) was found to have a six fold longer lag-phase (Table 2), with no

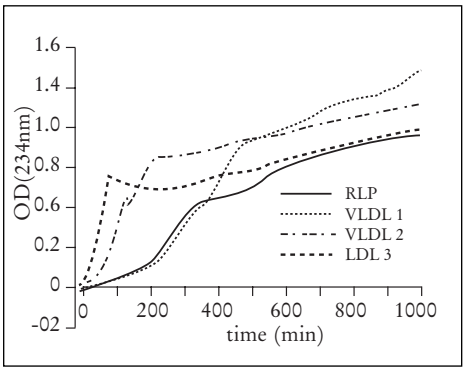

Figure 2. Conjugated diene curves generated from the oxidation of RLP, VLDL-1, VLDL-2 and LDL-3. The conjugated diene curves were produced by incubating (room temperature) the lipoprotein preparations (50 mg protein/ml) with CuSO4 (5mM) in EDTA-free PBS and continuously monitoring the changes in absorbance at 234 nm for 20h.

difference in maximal diene production. Oxidation of RLP produced a conjugate diene curve that had a similar lag time , and took the same time to reach maximal diene production, as that of VLDL-1 and VLDL-2. The overall pattern of oxidative susceptibility increased in the order of LDL-3>VLDL-2>RLP(VLDL-1

Discussion

This preliminary report shows that RLP , among the other TRP fractions including VLDL-1, VLDL-2 and IDL, is characterized by increased content of cholesteryl esters (CE) and a size range that overlapped mostly with the VLDL-1 fraction. Moreover, this RLP fraction is susceptible to oxidative stress, but to a lesser degree as the LDL-3 fraction from normolipidemic subjects.

Remnant lipoproteins, such as RLP, originate from chylomicrons and VLDL in the circulation where they are processed intravascularly by lipoprotein lipase, hepatic lipase and cholesteryl ester transfer protein (CETP).

Table 1: Chemical composition of plasma RLP , and plasma RLP of normolipidemic healthy subjects (RLP N), and VLDL-1, VLDL-2 and IDL fractions from subjects with Type II-B dyslipidemia.

	% Weight					
	Free cholesterol	Cholesteryl ester	tri-glycerides	phospho-lipids	protein	Total mass (mg/dl)
RLP (N)	4.2±0.9	8.9±1.2	72.2±5.9	7.8±2.9	6.7±1.3	93.2±1.7
RLP (II-B)	3.6±0.7	18.9±5.4	52.5±9.5	16.8±3.3	9.4±1.7	150.0±5.3
VLDL-1	1.8±0.2	2.5±0.5*	70.4±0.8	20.5±0.9	4.8±1.2	194.2±27.7
VLDL-2	3.4±0	4.5±0.4*	60.6±0.5	20.6±1.2	13.5±0.5	127.7±32.8
IDL	12.7±1.8*	15.7±1.3	18.1±2.4*	24.8±6.5	28.8±2.0*	68.8±14.6*

Values are expressed as means ± SD of four different fasting plasma pools in type IIB and 2 pools in the normolipidemic healthy subjects. * $p<0.05$ vs. RLP (IIB)

Table 2: Comparison of oxidative susceptibility of triglyceride-rich lipoprotein particle subfractions in type II-B subjects, and LDL-3 from normolipidemic healthy subjects.

	Lag phase (min)	Propagation rate	Propagation time (min)	Max diene production
RLP	251.6 ± 83.7*	1.1± 0.75	154.1± 84.8	181.1±111.7
VLDL-1	302.6±52.2**	1.48±0.81	153.1±42.2	232.2 ± 83.6
VLDL-2	121.5±41.4*	3.7±1.8	59.6±14.1	231.6 ± 76.4
LDL-3	39.6±0.35	3.2±0.3	51.0±1.0	162.8 ± 15.9

Values are expressed as means ± SD of four different plasma pools. * $p<0.05$ and ** $p<0.01$ vs. LDL-3,

Indeed, the TG content in RLP is lower (although not significantly) and the CE content is higher than that of VLDL-1 and VLDL-2 in type II B patients. Indeed, the effect of CETP and LCAT on an incubation with mildly hypertriglyceridemic plasma showed a marked increase in CE content and a decrease in TG content in the TRP fraction (9).

The size of RLP from type IIB patients is close to the VLDL-1 and chylomicron fraction. Also in type III, and type IV dyslipidemia, the size of the RLP was within the range of TRP (6; 10). In case of lipoprotein disturbances (such as in insulin resistance), the regulation of the amount of VLDL-1 (that is a precursor for dense LDL) towards VLDL-2 is often disturbed, with consequently a higher plasma pool of VLDL-1 (11;12). The increased plasma levels of RLP-C that are found in these populations with an increased cardiovascular risk may therefore reflect an increased plasma VLDL-1 pool. Moreover, the elevated content of CE in RLP make the RLP more atherogenic than VLDL-1, as CE is the lipid which accumulate in macrophage foam cells. Recently, native VLDL-1 was shown to induce foam cell formation without first being oxidized, in contrast to LDL-cholesterol that results in less intracellular cholesterol accumulation in its native form (13-15). So far, no reports exist about the effect of RLP on foam cell formation. We found that RLP (compared to VLDL-1) leads to a similar accumulation of TG and cholesterol in a human monocyte macrophage model. However, incubation of human monocytes macrophages with RLP tends to increase cell cytotoxicity (compared to VLDL-I), with no increase in apoptosis. (preliminary results, ThB Twickler). These results support a relation between the lipoprotein remnants and atherogenesis.

Oxidative stress is an important event in atherogenesis. In contrast to chylomicrons, lipoprotein remnants can accumulate preferentially in the subendothelial space due to their smaller size. After accumulation in the extracellular matrix, their susceptibility to oxidative stress may contribute to the development of atherosclerotic plaques. The RLP fraction from type IIB patients was less susceptible to oxidation, than LDL-3 from normolipidemic subjects. Nonetheless, it is still easily oxidized *in vitro*. Our observation is in line with Huff et al who showed that artificially produced remnants in vitro could be oxidized, but that oxidation of TRP remnants was not a key factor for the induction of foam cells (16). The explanation of the mechanism behind the oxidative profiles that we found is not simple. Additional analyses that will focus more in extents on the separate levels of fatty acids and antioxidants which are major factors determining lipoprotein oxidizability, will give more insight; this work is under investigation now. A first step to explain may be in the elevated surface free cholesterol/protein ratio in both VLDL-1 and RLP, compared to VLDL-2. Increased free cholesterol content is known to decrease the susceptibility of lipoproteins for in-vitro oxidation (17; 18). However, the CE/protein ratio, that is closely associated to the susceptibility for oxidative stress is higher in RLP (19). Therefore, although a definite answer on the mechanism is not there, this observation indicated that RLP are readily oxidized in-vitro and this support their effect in the process of atherosclerosis.

In conclusion, our results show that the RLP fraction obtained from type II-B patients, have atherogenic properties and may be an important lipid in addition to the atherogeniclipid phenotype.

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1.4
Adult-onset Growth Hormone Deficiency: Relation of Postprandial Dyslipidemia to Premature Atherosclerosis
(review)

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A complex relationship exists between disease of the cardiovascular system and a spectrum of neural and humoral factors. Recently, the modulating role of hormones, such as thyroid hormone (1-4), in the atherosclerotic process has been emphasized. However, several other hormones, in addition to thyroid hormone, may contribute to atherogenesis, thereby constituting a key element in the concept of cardiovascular endocrinology (5). Recently, evidence has been provided to suggest that disturbances of the pituitary growth hormone (GH) axis and its mitogenic partners, including insulin-like growth factor-1 (IGF-1) and IGF-binding proteins (IGFBP), are critical actors in the initiation of the atherosclerotic process (6-8). Indeed, disturbances in the GH axis/ IGF system appear to be intimately related to the perturbed postprandial lipoprotein metabolism which is typical of subjects presenting premature atherosclerosis (9;10). Furthermore, a high baseline plasma GH concentration is associated with increased cardiovascular mortality (11;12). These observations suggest a U-shaped relationship between disturbances in the GH axis/IGF system and increased cardiovascular morbidity and mortality.

In the present review, we explore the key pathways of lipid metabolism in adult-onset GH deficiency (AGHD) that may be involved in the initiation of atherosclerosis, and which result in elevated cardiovascular risk.

AGHD and the GH axis/IGF system

In the last 15 years, GH deficiency in adulthood has been focused on (pan-) hypopituitarism (depletion of (all) hormones that originate in the pituitary as a consequence of pituitary damage) or on transition from childhood-onset GH deficiency. In such cases of GH deficiency, one deals with an absolute deficiency state. Nowadays, a new group of patients with disturbances in the GH/IGF system have been defined that are characterized by a relative deficiency in optimal GH secretion, and which includes subjects displaying obesity and NIDDM (13). Moreover, ageing is associated with reduction in plasma GH and IGF-1 levels; indeed, subjects with a low serum IGF-1 level display an increased risk of ischemic heart disease (IHD) (14). In addition, patients with heart failure in a catabolic condition, or with progression of cancer, or with end-stage renal failure (15;16) were characterized with GH resistance.

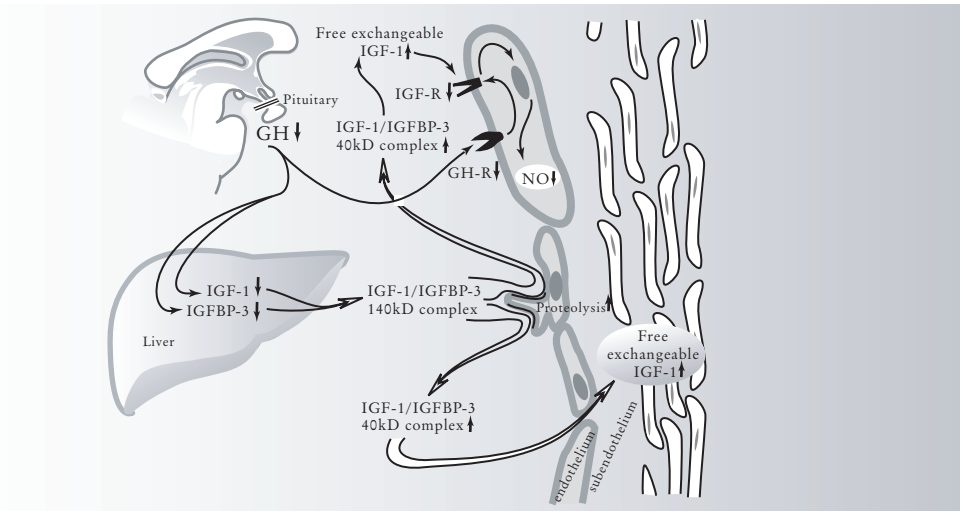

Figure 1. The GH axis IGF system in GH deficiency. The GH axis in AGHD is disrupted and the inducible effect on IGF-1 secretion is defective with consequences for the proportion of exchangeable IGF-1 that acts as a local paracrine/autocrine growth factor. IGF, insulin-like growth factor; GH, growth hormone; IGFBP, IGF-binding-protein; NO, nitric oxide.

With the growing clinical importance of GH deficiency, knowledge of the physiological function of the GH/IGF system has progressed. The GH axis originates in the cerebrum with the hypothalamus and the pituitary as regulation centers (figure 1). GH, whose release is induced by growth hormone-releasing-hormones (GHRH) from the hypothalamus, is secreted by the pituitary in a diurnal pattern with the highest serum GH levels occurring early during the night. After secretion into the circulation, GH is primarily bound to GH binding protein (GHBP). The circulating bound fraction of GH is thought to be biologically inactive, and for expression of biological activity, GH must be present in an unbound configuration. GH stimulates a spectrum of metabolic processes and is a major factor in the generation of IGF-I and its binding proteins. The liver secretes IGF-I into the circulation; by contrast GH stimulates IGF-I gene expression in a wide range of tissues, leading to local autocrine and paracrine actions of the biological active IGF-1 in tissues such as the endothelium, and is active in cellular processes such as cell proliferation and protection from apoptosis. Recent data from hepatospecific IGF-I knock-out mice indicate that circulating IGF-I should be considered more as a 'marker' of GH action in the liver.

The IGF system consists of two major growth factors, IGF-I and IGF-II, and six IGF binding proteins (IGFBP1-6) (figure 1). IGF-1 (17) and IGF-II are primarily synthesized in the liver. IGF-I and IGF-II are biological active in the unbound form. When IGF-I or IGF-II are bound to IGFBPs, then loss of biological activity occurs. Recently, it has been shown in a twin study that serum levels of IGFBPs are primarily genetically determined (18), but fine-tuning control of the GH axis is needed in order to respond to local tissue requirements of IGF-I and IGF-II (19;20). The majority of circulating IGF-1 in healthy subjects is bound to IGFBP-3 together with the acid labile subunit (ALS) in a 140 kDa complex with a relatively long residence time. In GH deficiency, IGF-1 shifts toward a IGFBP-IGF complex with a molecular weight of 40kDa, which does not contain the acid labile subunit, and which is capable of crossing the capillary endothelium (21). In addition to elevated circulating levels of IGFBP-3-bound IGF1, local tissue levels of biologically active IGF-1 are increased by proteolysis of the IGF/IGFBP3 complex; this process is mediated by proteases on the apical side of the capillary endothelium and in plasma. Plasma levels of biologically active IGF-I are inversely related to age. In AGHD, this relationship is not present. Despite the fact that total IGF-1 levels are lower in AGHD than in healthy subjects, the IGF-1/intact IGFBP-3 ratio is similar (22).

Accelerated atherosclerotic disease in AGHD

In several retrospective studies, cardiovascular mortality in AGHD is increased in comparison with a matched healthy population. The first report by Rosèn and Bengston revealed increased cardiovascular mortality in subjects with panhypopituitarism substituted with adrenal, gonadal and thyroid hormones, as compared to an age- and gender-matched control population (standard mortality rate, 1.8) (23). Subsequent reports have confirmed this observation (24-26). In another Scandinavian population displaying panhypopituitarism supplemented with adrenal, gonadal and thyroid hormones, the standard mortality rate was increased (2.2), but female GH-deficient subjects displayed a higher mortality rate than male subjects, while atherosclerosis-related events originated more in cerebrovascular than in coronary arteries (27). These results were confirmed in a Swedish population with pituitary adenoma in which the increased cardiovascular mortality was higher among women than men, and in which atherosclerosis was preferentially located in the cerebrovascular than in the coronary vascular region (24).On the other hand, no increase in cardiovascular mortality was observed in an AGHD population in the United Kingdom (28). In conclusion, most retrospective observational reports noted an increased cardiovascular mortality in subjects

with panhypopituitarism substituted with adrenal, gonadal and thyroid hormones, but with GH deficiency. One prospective observational study found a limited effect of GH on the increased mortality in hypopituitarism. However, no extended data on GH status were presented (29). These limitations may have underestimated the effect of GH. Long term randomized follow-up GH intervention trials will definitively answer the question as to whether GH treatment will result in reduced cardiovascular mortality in AGHD.

Mechanisms

Direct proatherogenic effects of components of the GH/IGF system

Atherosclerotic disease is a complex disorder involving several co-existing features, such as dyslipidemia, inflammation and a pro-thrombotic state (30). Disturbances in the GH/IGF system may be directly related to the progressive development of atherosclerosis. In GH deficiency, increased intima-media thickness in both the femoral and carotid artery were found (30) and endothelial function is impaired (31; 32). Endothelial dysfunction is considered as an early feature of atherogenesis (33-35) and involves reduced availability of endothelial NO, a vasodilatatory compound (36; 37). It has been shown that IGF-I has a direct NO-releasing effect on NO in cultured human endothelial cells (38), and that low basal IGF-I levels in serum are associated with low basal urinary nitrate and cyclic camp excretion (17). Decreased biological activity of NO in the endothelium may account for the increased occurrence of hypertension in AGHD, another risk factor for development of premature atherosclerotic disease (39).

The composition of the extracellular matrix is relevant to the development of atherosclerosis (40; 41). Proteoglycans, key components of the extracellular matrix, accumulate in the intimal layer of the artery. Based on earlier studies, it has been proposed that the small IGF-I/IGFBP-3 complex in GH deficiency is capable of crossing the capillary endothelium, leading to enhanced concentrations of IGF-I in the subendothelial space (22). However, *in-vitro* studies with cultured human smooth muscle cells have revealed that GH increased the accumulation of both hyaluronan and chondroitin sulfate proteoglycans (42), whereas administration of IGF-I in cell cultures *in-vitro* is without effect (43). Thus, IGF-1 does not affect the composition of the extracellular matrix. On the other hand, paracrine and autocrine IGF-1 and insulin (44) play a role in smooth muscle cell hypertrophy and the local secretion of angiotensinogen. This observation is supported by studies in a diabetic rat model, with the restriction that only high systemic levels of IGF-1 promote growth of SMC. In addition, no change in elastin and collagen content of the thoracic aorta media layer was found (45). As stated above, plasma IGF levels in AGHD are decreased, but the biologically active free IGF-1 available in AGHD is not reduced, thereby suggesting that local IGF-I concentrations in tissues are distinct from plasma levels. The induction of smooth muscle cell growth may therefore account for the increased intima-media thickness of the carotid artery in GH deficiency.

Impact of GH treatment on direct proatherogenic effects of the GH/IGF system

The rapid regression of the thickened intima media layer measured with carotid IMT at three and six months post GH treatment is impressive (7 46; 47). In comparison with previous results from dyslipidemic populations, such a reduction can only be obtained after extensive lipid lowering involving a decrease of 30-40% in LDL-cholesterol (48-50). In general, generation of a similar reduction in endothelial dysfunction by LDL-cholesterol reduction requires three to four years of therapy (48; 50).

After 6 to 12 months of GH treatment in AGHD, brachial artery function (measured by Flow Mediated Dilation) is improved compared to pre-treatment FMD values

(51). In patients with long term GH substitution, improvement in arterial performance is maintained (46).
It is established that GH directly affects the expression of the hepatic LDL-receptor and of key enzymes implicated in bile acid metabolism with effects on intracellular cholesterol homeostasis in the liver. This may be linked to metabolism of triglyceride-rich particles (VLDL and remnants particles) (52). The indirect, metabolic effects of the GH deficient state correspond more closely to factors that continuously interact with local arterial structures and may have pro-atherogenic effects, such as the induction of elevation in plasma VLDL- and chylomicron remnant levels during the postprandial period.

The GH/IGF system, atherogenic lipoprotein phenotype and the arterial wall (fig. 2).
The dyslipidemia of adult-onset GH deficiency is characterized by lipid disturbances in LDL-cholesterol and triglyceride-rich lipoprotein particles as illustrated in figure 2 (53).

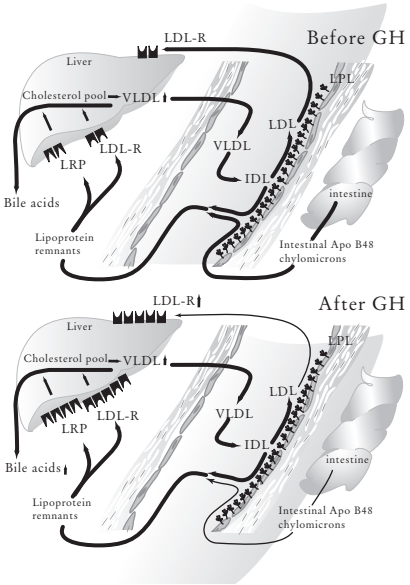

Figure 2. The effect of GH therapy on lipid metabolism in AGHD before and during GH therapy . Abbreviations: VLDL: very low density lipoprotein, LPL: lipoprotein lipase, TRP:triglyceride-rich proteins, LDLr, LDL receptor, LRP, LDL-receptor like protein, 7-OH –ase: 7 alpha hydroxylase, LDL, low density lipoprotein.

LDL-cholesterol
In most published studies, plasma levels of LDL-cholesterol are only moderately increased (3.5-4.5 mmol/l) in adult-onset GH deficiency, as compared to healthy age-, and gender-matched control subjects (54-59). In GH-deficiency, hepatic LDL-receptor activity is decreased, a process which can be reversed by GH substitution. A small (10%), but significant decrease in plasma LDL-cholesterol levels occurs during GH therapy (60-64). Several studies detected no effect of short term GH therapy on total and LDL cholesterol levels (64-68), whereas one report even noted no difference in plasma lipid levels in AGHD after 7 years of GH therapy (69). The LDL-cholesterol profile shifted from atherogenic dense particles towards larger, less-dense particles after GH therapy (70). All these beneficial changes contribute to attenuation of the atherogenic phenotype (71), which is characterized by elevated levels of LDL cholesterol, small dense LDL, triglycerides, and decreased levels of HDL cholesterol.

Triglyceride rich particles
In AGHD, elevated levels of baseline plasma TG were found, which are considered to constitute an independent risk factor for cardiovascular disease (72). Elevated plasma levels of TG and TG-rich lipoprotein particles (TRP), consisting of VLDL containing apo B100 of hepatic origin and of chylomicrons containing apo B48 of intestinal origin, are associated with increased carotid intima-media thickness and cardiovascular mortality (73-77). It has been shown in AGHD that VLDL apo B100 secretion is increased and that VLDL particles are enriched in TG (78). Enrichment of VLDL with TG in AGHD gives rise to a dyslipidemic lipoprotein pattern including the presence of more small dense LDL (79). Zilversmit (80) postulated some decades ago that the increased susceptibility to premature atherosclerosis may be related to atherogenic factors occurring as a result of a continuous postprandial state that is a consequence of the consumption of a

minimum of three meals a day in Western Societies (81). Indeed, elevated postprandial levels of TRP and especially of TRP remnants have been observed in subjects with cardiovascular disease, as compared to age-, gender- and BMI-matched control subjects (76; 82-85) and their postprandial lipemia is positively associated with atherosclerotic disease (86). The analyses of determinants of postprandial lipemia requires distinctive and laborious techniques, such as the determination of apoB48 or apoB100 in VLDL density fractions isolated by ultracentrifugation. The availability of an immuno-isolation method based on a Sepharose gel coated with apoAI and apoB100 monoclonal antibodies greatly improved the analysis of TRP remnants (remnant-like particle cholesterol; RLP-C) (87). In a Japanese population with coronary artery disease, fasting plasma RLP-C levels above the 90th percentile predicted the occurrence of cardiovascular events more strongly than LDL-cholesterol. In an *in-vitro* study, RLP induced a dose-dependant impairment of vasorelaxation in rat thoracic aorta (88). RLP may interact with the endothelial nitric oxide system, thereby accounting for the observed endothelial dysfunction (89). The postprandial rise in RLP-cholesterol levels in healthy normolipidemic subjects is associated with impairment of endothelial function (90). In patients with AGHD, postprandial levels of RLP-cholesterol are higher than in matched healthy control subjects. Postprandial plasma RLP-C levels in GH deficiency rose from 0.41 mmol/L to 0.70 mmol/L (from baseline to 4 h) after ingestion of an oral fat load. Such levels induced endothelial dysfunction in isolated rat aortas (91) and in healthy human subjects (90). Such elevated levels of postprandial RLPs are in accordance with the observations of Al-Shoumer et al (92) , who noted an increase in postprandial plasma TG levels in AGHD after three consecutive meals during the day. A significant reduction in postprandial plasma RLP-C (towards 0.42 mmol/L) was observed during GH substitution (93).

To account for the marked postprandial increase in RLP-C levels in adult-onset GH deficiency (AGHD), several steps in postprandial TRP metabolism require evaluation. Although hepatic apoB100 secretion is increased (70) in AGHD, no decrease in postheparin LPL activity in AGHD humans has been found (94). The accumulation of postprandial RLP-C in AGHD may be explained by a decrease in their removal from the circulation via hepatic lipoprotein receptors (95; 96). Indeed, the expression of several hepatic surface receptors, such as LDL- and LRP receptors, is lower in GH deficient states than in healthy subjects (97). The substitution of GH results in increased expression of these surface receptors. For example, the GH deficient LDL-receptor knock-out mouse benefits from GH substitution despite lack of the LDL-receptor. Thus treatment with GH resulted in increased expression of key enzymes involved in cholesterol synthesis (HMG co-A reductase) and bile acid metabolism (7 α-hydroxylase), resulting in an increased transport of intracellular cholesterol towards the bile acid pool with an enrichment in the faeces with bile acids (98). The intracellular pathway of cholesterol excretion is positively linked to the synthesis and secretion of VLDL in hepatocytes. In man, reduction of intracellular hepatic cholesterol content may reduce secretion of the cholesterol-rich VLDL-2 subfraction but not the larger TG-enriched VLDL-1 (99).

HDL

Decreased levels of HDL-cholesterol are associated with an increased risk of CAD (100). Plasma levels of HDL-cholesterol in AGHD are decreased as compared to healthy controls (101) or remained unchanged (93). It has also been shown by Beentjes et al (102) that mainly HDL cholesteryl ester concentration is significantly decreased in AGHD, whereas free cholesterol remained unchanged. HDL plays a major role in the process of reverse cholesterol transport, thereby transporting cholesterol from the peripheral tissues towards the liver for excretion into the bile. CETP and PLTP are key

lipid transfer proteins which play an important role in intravascular HDL remodelling. CETP transfers TG from TG-rich lipoprotein particles to HDL in exchange for cholesteryl esters from HDL. Plasma CETP activity is lower in AGHD than in healthy subjects, whereas no difference was found for LCAT and PLTP activity(102). We confirmed the presence of lower circulating CETP mass in AGHD and found a significant increase during GH therapy (figure 3). This strongly suggest that the decrease in CETP activity resulted in impaired transfer despite higher concentration of TG-rich lipoprotein particles, resulting in impaired reverse cholesterol transport from peripheral cells back to the liver (103). Upon treatment with rhGH, plasma HDL cholesterol levels increase with accompanying increase in HDL cholesteryl ester concentration and increase in plasma CETP mass (figure 3). This is in contrast to data from Beentje et al (104) who showed a decrease in cholesteryl ester transfer activity upon long term GH replacement therapy. It is evident that more studies have to be performed to establish the role of GH treatment in AGHD upon improvement of the atherogenic lipoprotein profile.

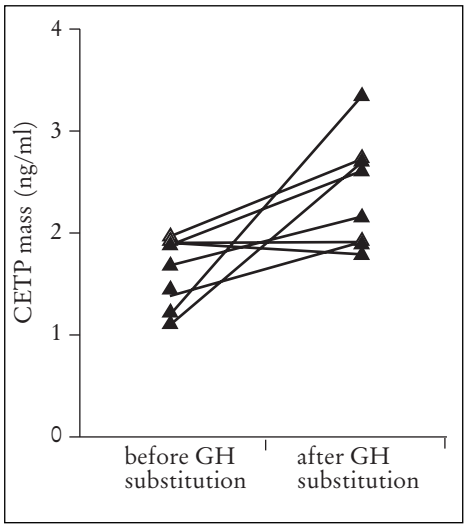

Figure 3. Plasma CETP mass was increased significantly after rh-GH substitution (from 1.63 ± 0.34 to 2.38 ± 0.54 mg/mL, P<0.05)

Arterial wall and lipoprotein remnants

Interaction of postprandial atherogenic lipoprotein particles with the endothelium may result in an inflammatory response. Indeed, addition of antioxidants in the form of α-tocopherol leads to a decrease in the inflammatory response after incubation of endothelial cells with RLP with subsequent attenuation of endothelial dysfunction (105; 106). RLP particles are able to penetrate the endothelium with retention in the subendothelial or extracellular matrix (107). Consequently, elevated amounts of lipoproteins retained in the subendothelium space can no longer be efficiently removed, resulting in enhanced formation of macrophages (foam cells), and induction of local vascular inflammation (108; 109). Indeed, when elevated concentrations of RLP are present as in the postprandial state, plasma Il-6 and TNF-α levels in GHD are increased (figure 4.) (110). RLP may bind to human monocyte-macrophages through surface receptors (for example LRP), but uptake of RLP via a receptor-independent mechanism may also occur (111).

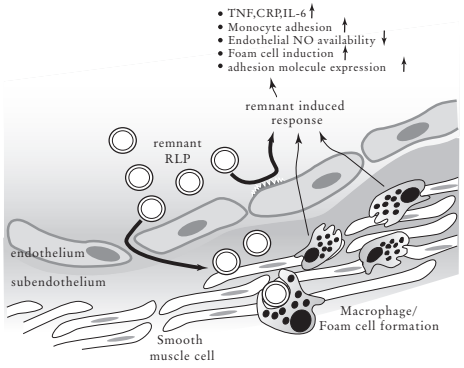

Figure 4. The hypothetical atherogenic pathway of RLP that gives rise to (a) retention in subendothelial space of RLP, and (b) a direct interaction with the endothelium resulting in induction of vascular inflammatory response. After retention in the subendothelial space, macrophages engulf the RLP with subsequent foam cell formation that gives rise to proinflammatory and prothrombogenic responses interacting with surrounding local vascular structures.

GH/IGF Axis in association with other hormonal balances

To understand the role of the GH/IGF axis in the pathogenesis of accelerated atherosclerosis, knowledge of the interplay of this axis with other hormonal systems is essential. We limit the discussion of this topic to the interaction with the glucocorticoid system.

Glucocorticoid system (25).

Patients with AGHD who present a defect in the GH-IGF-1 axis additionally display a deficit in circulating corticosteroid levels. Daily cortisol production, measured in stable isotope studies, is overestimated by a factor of 2 to 3 (112; 113). Based on recent research, physiological cortisol levels are in the range of 6-7 mg/m^2/day. As a consequence, AGHD patients are often treated with doses of corticosteroids far above daily requirements. In addition, glucocorticoid metabolism is altered in AGHD. The enzyme 11-β-hydroxysteroid dehydrogenase 1 (11β-HSD 1) activates cortisol via its action on inactive cortison and enforces the action of cortisol in the liver and adipose tissue. GH inhibits 11β-HSD activity. Therefore, the increased activity of 11β-HSD 1 in GH deficient patients (not substituted with GH) results in an increase in central omental fat subsequent to increased conversion of cortison to cortisol (114). The increase in abdominal fat creates additional active metabolic compartments that are discussed below.

Postprandial action of adipose tissue

A decade ago, adipose tissue was considered as an inactive tissue. Recent research however has shown that adipose tissue, especially in the intra-abdominal cavity, possesses elevated metabolic activity and may secrete factors (such as adiponectin and TNF-α) with hormonal action. Moreover, in terms of their differentiation and maturation, adipocytes are under the control of many growth factors, such as IGF-I (115). These cellular processes in adipocytes may therefore be directly influenced when disturbances in the GH/IGF system occur. In the postprandial period, there is an influx into adipocytes from free fatty acids and catecholamines, in addition to efflux from adipocytes of other biologically active factors, including adiponectins, TNF-α and IL-6 (116; 117). The postprandial balance of adipocyte influx/efflux is mainly regulated by the adrenergic system and insulin. Increased amounts of visceral fat in AGHD patients have been reported, but little is known about the degree of differentiation and maturation of adipocytes in these patients, and consequently the properties of adipocytes in this GH deficient condition. Due to the lipolytic effects of GH therapy on the accumulated visceral fat, intra-abdominal fat decreases significantly during GH therapy (118). Despite quantitative decrease in intra-abdominal fat in AGHD as a result of GH treatment, the influence of GH substitution on adipocyte capacity is not totally clear and therapy with GH may therefore exert additional effects to that of lipolysis. Studies in obese subjects have shown that increased amounts of intra-abdominal fat are associated with higher plasma TNF-α levels, and that plasma TNF-α levels are higher in obese than in lean subjects (119). TNF-α secretion by adipocytes is a function of differentiation and maturation, with optimal secretion capacities limited to highly differentiated adipocytes. Although the amounts of intra-abdominal fat in AGHD patients are equal to those of obese patients, baseline plasma TNF-α levels in AGHD are lower than in obese patients. However, after GH therapy, baseline plasma TNF-α levels reach similar levels in both AGHD and obese patients. The induction of increased expression of TNF-α suggests additional effects of GH substitution on adipocytes, such as an increase in adipocyte maturation. In addition, the metabolic activity of the intra-abdominal fat may be influenced by circulating GH levels. Increased intra-abdominal fat may therefore exert an additional aggravating effect on accelerated atherosclerosis.

Atherosclerosis is considered to be a part of a pro-inflammatory condition (120-122) and the increased postprandial release of these

cytokines by the adipose tissue in GH deficiency could influence glucose homeostasis via an increase in insulin resistance, a decrease in macrophage response and an altered differentiation of fibroblasts into smooth muscle cells (123; 124). Moreover, secretion of *de novo* remnant particles from visceral adipose tissue may contribute to the catabolism of RLP (125). Therefore, part of the beneficial effect of GH on the atherogenic lipoprotein phenotype may be due to altered metabolic properties of intra-abdominal adipocytes. Moreover, the visceral adipocyte compartment in AGHD is in continuous interplay with the metabolic profile that is attained in the postprandial period. Both aspects may therefore be considered as a therapeutic target in order to attenuate an atherogenic lipid phenotype in GH deficiency.

In conclusion, epidemiological observational studies in AGHD patients have revealed increased cardiovascular morbidity and mortality, due to accelerated atherosclerotic disease. The precise atherogenic mechanisms in GH deficiency are not, however, fully elucidated, but a dyslipidemic phenotype, which is exacerbated during the postprandial period together with a lack of endothelial NO are considered to be key factors. The role of hormonal and growth factor disturbances in the development and progress of atherosclerotic disease is part of a new and rapidly growing field in medical practice, that of cardiovascular endocrinology.

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1.5
Effect of growth hormone therapy in adult-onset growth hormone deficiency on home measured capillary triglyceride status.

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Adult-onset growth-hormone deficiency (AGHD) is characterised by presence of an atherogenic lipoprotein profile and associated with an increased cardiovascular mortality. Recently, an individual self-monitoring method for triglycerides became available. We evaluated this method in a case controlled intervention study in an out-of-hospital setting (AGHD patients: n=10 versus controls: n=10), in relation to dietary intake and composition in patients with adult growth hormone deficiency (AGHD), and the effect of 6 months of rhGH therapy.

Baseline plasma cholesterol and HDL-cholesterol levels were similar, but baseline plasma triglycerides were significantly elevated in AGHD patients versus controls (2.30 ± 0.9 mmol/l vs 0.92 ± 0.3 mmol/l, P<0.05). Fasting capillary TG (TGc), measured with the Accutrend®, was similar in controls and patients at baseline (1.60 ± 0.59 mmol/l vs 1.49 ± 0.46 mmol/l). Area under day Curve of the TGc (AUC TG-c) was significantly elevated in AGHD patients (14.6 ± 7.7 mmol*h/L) compared to controls (7.14 ± 3.8 mmol/L*h; p=0.01). After rhGH therapy, the incremental AUC TG-c decreased towards control levels (11.88 ± 7.5 mmol*h/L). At baseline, AGHD patients consumed less total calories (1703 Kcal vs 2210 Kcal), mainly as carbohydrates (208±86 g/d vs 309 ± 127 g/d; P<0.05) and fat (56.3 ± 22.2 g/d vs 86 ± 32g/d; P<0.05) than the control subjects. After rhGH therapy the dietary intake of carbohydrates, and fat was comparable to controls.

In conclusion, the increased capillary TG profile during the day in AGHD, in spite of lower calorie and fat intake, improved upon rhGH treatment.

Adult-onset growth hormone deficiency (AGHD) is associated with premature atherosclerosis and an increased cardiovascular mortality (1-4). Although LDL-cholesterol has been considered as the most atherogenic factor, the importance of triglyceride-rich lipoprotein particles is recently emphasized (5). Premature atherosclerosis is associated with elevated fasting plasma TG levels (6; 7) and postprandial TG levels (8; 9). As a result of our daily food intake, plasma levels of circulating TG-rich lipoprotein particles have the property to accumulate (10). Therefore, an assessment of a daytime TG profile provides us with data on the actual lipid load during the day.

Postprandial levels of plasma TG are elevated in GH deficiency after one single oral fat load, and remain elevated during GH substitution, although GH treatment improves the atherogenic phenotype (11). These observations are in line with Al-Shoumer et al (12) who found elevated postprandial TG levels in AGHD, that were even more prominent in female AGHD. In both studies, disturbances in TG profiles in GH deficiency were found. These studies have both been performed in a metabolic ward in a strictly controlled in-hospital setting under fasting conditions. Under these circumstances, it is not possible to study variation in TG throughout the day in ordinary life. Recently a new method to assess the capillary TG (TGc) levels during the day by self-measurements with the Accutrend apparatus became available (13). In the present study we evaluated the day time capillary TG levels before and during GH treatment in GH deficiency (in comparison to matched control subjects).

Materials and Methods

Subjects

Ten adult-onset GHD patients from the outpatient clinic of the Utrecht University Hospital were included after giving written informed consent. The protocol had been approved by the local ethical committee. The results were calculated on the basis of 9 patients. One patient dropped out of the follow up due to recurrence of the pituitary. All GHD patients suffered from hypopituitarism after surgical removal of a pituitary adenoma. Deficient pituitary hormones, except GH, were adequately supplemented for a minimum of three months. GH deficiency was diagnosed with a growth hormone releasing hormone-arginin test and an insulin tolerance test.

After baseline measurements, all subjects participated in the TG self measurement protocol, followed by a period of 6 month rhGH treatment (0.8-1.1 IU/day for GH deficient patients with a repeat of the TG self measurement protocol. The definite daily dosage of rhGH was aimed at reaching the recommended plasma IGF-1 levels, adjusted for age and sex. All patients were stable with regard to GH status during the last three month. Exclusion criteria for patients were: smoking, alcohol intake > 2 U/day, renal and liver disease, apo E2/E2 genotype, use of lipid lowering drugs and a family history for premature cardiovascular disease. Normolipidemic matched (for body mass index (BMI), age, sex and apo E genotype) controls without a family history of cardiovascular and metabolic diseases, were included. None of the participating subjects was unable to perform in the TG protocol due to cognitive, visual or motility disabilities.

Study design

Anthropometric values, lipid, glucose and hormone levels were determined in the control subjects at the start of the study and in the AGHD patients before and after six months of rhGH treatment. Venous blood samples were collected into EDTA-containing tubes, were put on ice immediately, centrifuged and stored at - 80°C for further analysis.

TG protocol

At the first visit, all subjects were thoroughly trained to use the Accutrend GCT (Roche, Basel, Switzerland) according to the manufacturer's instructions, and to administer the dietary records. The Accutrend GCT (Roche) is a home glucose meter that has been modified to measure adequately triglycerides (TGc) (14). TGc's are measured in fresh capillary blood using a reflection-photometric method. A sample volume of 12-45 μl is required and the operating time is 45-174s, depending on the TG concentration. A range of measurement between 0.8 mmol/l (70 mg/dl) and 6.86 mmol/l (600 mg/dl) is achieved. At six fixed time points,1) at wake-up, 2) at lunch, 3) three hours after lunch, 4) at dinner, 5) three hours after dinner, 6) before sleep, the capillary triglyceride levels (TGc) were measured for two separate days (except week-end days and Monday).

Fasting blood samples at start of the study

Plasma was obtained by centrifugation at 3000 rpm for 15 min at 4°C. TG and cholesterol were analysed in duplicate in total plasma with a colorimetric assay (Monotest cholesterol kit no. 237574 and GPO-PAP no. 701912, Boehringer Mannheim, Germany). HDL-Cholesterol was determined after precipitation of apoB-containing lipoproteins with the heparin-$MnCl_2$ dextrane-sulphate precipitation method (15). Low-density lipoprotein (LDL) cholesterol was calculated with the Friedewald Formula (16). Apolipoprotein (apo) B concentrations were analysed automatically on a Cobas Mira autoanalyzer (Unimate 3 Apo B, Roche Diagnostics, Basel, Switzerland). Plasma Insulin and IGF-1 concentrations were determined with a radio-immuno assay test (17). Insulin resistance was estimated by calculating HOMA-index (fasting insulin * fasting plasma glucose/22.5) (18). Apo E genotype was determined as described before (19).

Body composition measurements
Body fat was assessed by bioelectric impedance analysis (BIA) (tetrapolar BIA-101 analyzer: RJL-Systems, Detroit), on the basis of resistance and reactance measurements. Resistance and reactance were measured (in Ω) after application of an alternating current of 800 μA at 50 kHz with the electrodes placed (20). Body fat was calculated with the use of the manufacturer-supplied equation.

Analysis of dietary intake
During the days of TG measurements, the intake of food and beverages (including alcohol) were carefully recorded in a diary. The food record was reviewed by a physician in a personal consultation within one week of return. Energy and nutrient intakes from the food items were calculated using Becel nutrition program, version NL03a, 1987 (Unilever, Vlaardingen) based on the Dutch Nutrient Data Base. The evaluation of the results was performed in collaboration with the department of Clinical Nutrition UMC Utrecht. Estimated nutrients (total fat, unsaturated fat, saturated fat, carbohydrates, protein and alcohol) were grouped for the different time points at the two consecutive days of TGc measurements and averages were calculated.

Statistical analyses
All values are presented as means ± SD. The capillary TG concentrations of the two consecutive days were grouped for each time point and averaged. The area under the daytime TGc curve (AUC TGc) was calculated as a total (AUC TGc) and as an incremental (or delta) AUC (dAUC TGc) with the use of GraphPad Prism Software (Inc, San Diego,1994). Testing for differences between the patients and controls were performed with an unpaired Student t-test. Paired student t-tests were used to test if differences before and after treatment exist. Parameters were log transformed before analyses in case of non-normality, if appropriate. A $p<0.05$ was considered as significant. The SPPS PC program (version 8.0, SPPS, Inc, Chicago, USA) was used as statistic software.

Results

Population characteristics
No differences were observed in the anthropometric characteristics (BMI, WHR, fat mass (FM) and FM as a percentage of total body weight (FM%) between the AGHD patients, before and after rhGH treatment, and control subjects (table 1). Two AGHD patients had an apo E3/4 genotype and eight subjects had an apo E3/3 genotype. The control subjects were matched for apo E genotype. Average duration of hypopituitarism was 3 years. All patients were substituted with thyroxin, 8 patients with hydrocortison, 2 patients with cortison acetate, 8 patients with sex hormones and 8 patients with desmopressin. All patients were treated with rhGH during minimally 3 months with an average of 7 ± 2 months.
Fasting plasma TG concentrations were significantly higher in the AGHD patients compared to controls (2.30 ± 0.92 mmol/l vs 0.9 ± 0.3 mmol/l, $p<0.05$), without any difference in fasting plasma total cholesterol, HDL-cholesterol and LDL-cholesterol levels. Upon treatment with rhGH, both plasma TG and cholesterol concentrations decreased. Fasting plasma glucose levels were similar in the AGHD patients and controls. The HOMA index increased significantly ($P<0.05$) after rhGH treatment. In AGHD patients. Plasma IGF-1 levels were significantly lower than in the control subjects. After treatment with rhGH, plasma IGF-1 levels increased towards normal levels.

Daytime capillary triglyceride concentrations
The mean daily capillary TG concentrations (TGc) are presented in figure 1. The TGc levels at 8.00 am were similar in AGHD patients and controls. This is in contrast to the plasma TG measurements. The daily TGc profiles were different in AGHD patients than in controls. In AGHD patients, the TGc increased after the breakfast meal, and remained elevated during the day. At 10 pm the maximum TGc level (3.22 ± 0.49 mmol/l) was reached. In controls no increase

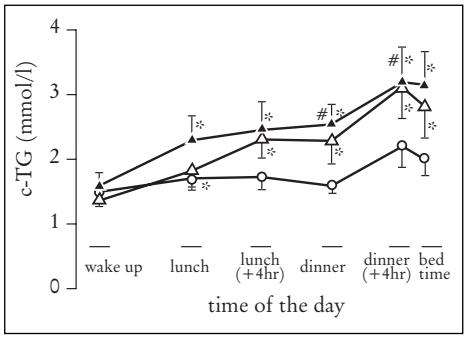

Figure 1. Capillary triglyceride concentrations (TGc) during the day in controls (open circles) and adult-onset GHD patients before (closed triangles) and after GH (open triangles) (P< 0.05, TGc in the day vs baseline, # P < 0.05, TGc in the day in GHD patients vs TGc in the day in control subjects). TGc concentrations are expressed as a mean of a two-day measurement (±sem).*

in TGc was observed until 10.00 pm (maximum: 2.22 ± 0,23 mmol/l). Treatment with rhGH treatment only resulted in a small improvement in daily TGc profile. The curves were still higher than in the control subjects.

The integrated areas under the TG curve (AUC TGc) were significantly elevated in the AGHD patients (+ 47%; P<0.05) compared to controls (table 2). After correction for fasting TGc concentrations, the dAUC TGc in AGHD was still significantly elevated (P<0.05). After rhGH therapy, a 19% reduction in dAUC TGc was found (p<0.05).

Daily dietary intake and composition

In AGHD patients, total daily energy (E) intake was lower than in controls (1720 ± 603 vs. 2209 ± 650 kcal; p=0.09) (table 3). The absolute intake of total dietary fat, monounsaturated fat and polyunsaturated fat were significantly lower in AGHD patients than in the control subjects (P<0.05), but as a percentage of total daily energy intake, no differences were found. Upon rhGH therapy,

Table 1: Baseline characteristics of AGHD patients before (GH-) and after GH treatment (GH+), and control subjects

	controls	AGHD GH (-)	AGHD GH (+)
N (M/F)	10 (6/4)	9 (5/4)	9 (5/4)
Age (y)	48 (7.2)	48 (7.8)	48 (7.8)
BMI (kg/m^2)	27.7(2.3)	27.6(3.9)	26.5(4.1)
WHR (cm/cm)	0.89	0.92	0.93
Cholesterol (mmol/l)	5.09(0.93)	5.48(1.1)	5.19(0.72)
Triglycerides (mmol/l)	0.92(0.3)	2.30(0.9)*	1.62(0.44)*
HDL-cholesterol (mmol/l)	1.17(0.29)	1.15(0.48)	1.17(0.51)
LDL-cholesterol (mmol/l)	3.51 (0.32)	3.21 (0.63)	3.35 (0.44)
Total IGF-1 (nmol/l)	173(56)	105(52) *	204 (57)#
FT4 (nmol/l)	nd	16.1(3.1)	17.5(4.6)
Glucose (mmol/l)	5.80(0.59)	4.70(0.34)	5.02(0.11)
HOMA-index	2.85(2.27)	1.55(0.94)	3.14(0.82)#
FM (kg)	22 (5.6)	19.7(5.2)	18.45(6.7)
FM %	25.8 (6.7)	24.3 (6)	22.96(6.7)

Values are presented as mean (SD). * p< 0.05 vs. control subjects, # p<0.05 vs. before GH treatment.

Table 2: Fasting capillary TG (TGc) and daytime capillary TG profiles calculated as integrated area under the triglyceride curve (dAUC TGc; mmol/l*h) in AGHD before (GH -) and after GH (GH+) treatment, and in control subjects.

	controls	AGHD GH (-)	AGHD GH (+)
TGc	1.49 (0.46)	1.60 (0.59)	1.41 (0.68)
AUC TGc	23.55(4.67)	34.71(13.05)†	28.77(12.97)
dAUC TGc	7.14 (3.78)	14.61(7.67)†	11.88 (7.46)

Values are presented as mean (SD). †$p < 0.05$ vs. control subjects.

Table 3: Daily nutrients intake in AGHD before (GH -) and after GH (GH+) treatment, and in control subjects.

	controls	AGHD GH (-)	AGHD GH (+)
Energy (Kcal)	2209 ± 649	1720 ± 535	1929 ± 787
Fat			
(g)	86 ± 32	56 ± 24#	76±45*
(% of energy)	*34*	*29*	*34*
Saturated			
(g)	31 ± 12	22 ± 11	32 ± 20*
(% of energy)	*12*	*11*	*14*
Polyunsaturated			
(g)	15 ± 6	9 ± 4#	9 ± 5*
(% of energy)	*6*	*5#*	*4*
Monounsaturated			
(g)	33 ±13	21 ± 8#	31 ± 18*
(% of energy)	*13*	*11*	*15*
Carbohydrates			
(g)	264 ± 73	213 ± 89	217 ± 95
(% of energy)	*48*	*49*	*47*
Protein			
(g)	96 ± 32	85 ± 18	88 ± 33
(% of energy)	*18*	*22*	*19*
Cholesterol (mg)	216±67	150±76	197±120

Values are based on the daily food records. Values are presented as mean (SD) # $p<0.05$ vs. control subjects, * $p< 0.05$ vs. before GH treatment.

the intake of total calories increased although the difference was not statistically significant. The absolute intake of total dietary fat, saturated fat and monounsaturated fat were significantly higher (56 g, 22 g and 21 g vs. 76 g, 32 g and 31 g, respectively; $p<0.05$). On a percentage basis, only the intake of PUFA remained significantly decreased. No association was found between the difference in day time TGc profiles and the daily food intake in AGHD patients after rhGH treatment.

Discussion

In the present study, the day-time TGc profile in AGHD patients, measured with the Accutrend in a natural home situation, was impaired as compared to healthy matched control subjects with a slight, but significant, improvement during rhGH therapy. This improvement in TGc profile was independent of the dietary intake.

Dyslipidemia, characterized by elevated plasma TG and low plasma HDL levels, is thought to be an important risk factor for initiation of premature atherosclerosis in GH deficiency (21). Indeed, fasting plasma TG levels in AGHD are most frequently slightly elevated (1.5-3.0 mmol/l). In the present study different levels of baseline TGc and plasma TG were found. A similar variability in capillary and plasma TG levels has previously been noted (22). The explanation for this observation is not yet known, but may be due to the use of different analytical methods. The analytical method for TG analyses is standardised, whereas the dry chemistry technology used in the Accutrend methodology is new. The observed difference in plasma and capillary TG is too large to be explained only by differences in fatty acid trapping in the capillary beds (23). Critical evaluation of the method of self-measurement of TGc levels in AGHD patients reveals a day to day TGc variation of 25% (range 1.44-72.71) (24), that may limit a general use of this method. In general, the use of out-of-hospital TGc method looks very promising despite the methodological caveats that have to be improved.

TG-rich lipoproteins, reflected by total plasma TG levels, accumulate during the day. An exaggerated profile is found when disturbances in lipoprotein metabolism occurs (25). Because the close association between an atherogenic lipoprotein profile and increased cardiovascular mortality, a day-time TG profile may provide additional information for the characterization of the atherogenic lipoprotein phenotype (26). Indeed, several studies found elevated levels of postprandial plasma TG in patients with coronary artery disease (27; 28). Moreover, postprandial hypertriglyceridemia is related with enhanced platelet aggregation (29; 30), endothelial dysfunction (31) and with intima media thickness (IMT) of the carotid artery (32).

Beneficial effects of rhGH treatment in AGHD on the proatherogenic phenotype have already been reported (33; 34). Despite an increase in insulin resistance (determined by HOMA-index), we observed a decrease in day-time TGc profile during rhGH treatment. A transient decrease in insulin sensitivity with improvement of the initial dyslipidemia has also been reported in AGHD during rhGH treatment (35-37), however the exact mechanisms remained unclear.

The amount of TG accumulation during the day may be dependent upon the daily food intake. Increased fasting plasma TG levels were found when diets were restricted in fat content (38), because a shift towards increased carbohydrate intake occurred to meet the adequate daily energy intake (39). However, in the present study, the GH deficient patients consume less total calories per day with a normal proportion of fat and carbohydrates. Snel et al (40) reported a lower daily energy intake and a lower basal metabolic rates (BMR) in AGHD. In contrast to the long-term intervention studies, the short-term dietary intervention studies in healthy adults, show no change in plasma TG levels with either a high fat or a high carbohydrate intake (41). In type IV hyperlipi-

demic patients who consume a low fat diet for three months, no changes in fasting plasma TG levels were found (42). The lack of association between day-time TGc profile and diet composition is therefore in accordance with previous reports (43).

In conclusion, despite a lower total daily food intake, an increased day-time TGc profile was observed in AGHD patients that contributes to a proatherogenic phenotype. During rhGH treatment, the day-time TGc profile improved. Although promising, the feasibility of TGc measurement in a natural home environment needs further investigation before it is implemented in daily clinical practice as part of the assessment of cardiovascular risk.

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1.6
Growth hormone treatment decreases postprandial remnant-like particle cholesterol concentration and improves endothelial function in adult-onset growth hormone deficiency

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Premature atherosclerosis is a clinical feature in adult-onset growth hormone deficiency (AGHD). Evidence is accumulating that disturbances in triglyceride metabolism, reflected by abnormalities in circulating remnant lipoproteins, are associated with increased atherogenic potential. In a case-controlled intervention study, we investigated postprandial lipoprotein metabolism using a new remnant lipoprotein method based on immunoseparation principle (RLP-Cholesterol). In addition we analyzed retinyl ester (RE) analysis in plasma and in Sf<1000 fraction. Endothelial function was assessed as flow mediated dilatation (FMD). Eight patients diagnosed with acquired adult-onset growth hormone deficiency and eight controls matched for gender, age, BMI and apo E genotype were enrolled in the study. Oral vitamin A fat loading tests were performed at baseline in both groups and after six month of treatment with rh-Growth Hormone (rh-GH) in the AGHD patients. AGHD patients had significantly higher fasting RLP-C, postprandial RLP-C concentrations (plasma RLP-C: 0.29 ± 0.14 mmol/L and incremental AUC-RLP-C: 2.13 ± 1.60 mmol*h/L, respectively) than controls (0.19 ± 0.06 mmol/L and 1.05 ± 0.72 mmol*h/L (P<0.05), respectively). They also had significantly higher postprandial RE in plasma and Sf<1000 fraction. Treatment with rh-GH significantly reduced postprandial RLP-C concentrations (incremental AUC-RPL-C 0.73 ± 0.34 mmol*h/L; p<0.05) but had no effects on the fasting RLP-C concentrations (0.317 ± 0.09 mmol/L, P<0.05), nor on the postprandial RE in plasma and in Sf<1000 fraction. Endothelial function measured as FMD was improved from 5.9 ± 3.3% to 10.2 ± 4.0% (p<0.05) in patients treated with rh-growth hormone.

It is concluded that patients with AGHD have increased levels of fasting and postprandial RLP-Cholesterol and an impaired endothelial function as measured as FMD. Treatment with rh-GH resulted in a decrease of postprandial RLP-C concentration thereby improving the postprandial atherogenic lipoprotein profile and improvement of endothelial function, however the clearance of large chylomicron particles as reflected by RE remained disturbed.

Adult-onset growth hormone deficient patients (AGHD) suffer from premature atherosclerosis in the coronary and in the peripheral arteries (femoral and cerebral arteries) (1; 2) and increased cardiovascular mortality (3). After six month of substitution with synthetic growth hormone (rh-GH) regression of lesions have been found (4). Abnormalities in plasma cholesterol and LDL cholesterol levels have been observed in AGHD patients which could be improved by treatment with Growth hormone for at least three months (5-9). However, improvement of endothelial dysfunction and decrease in intima-media thickness in AGHD patients after growth hormone substitution could not fully be explained by decreased concentrations of plasma LDL cholesterol (4). Triglyceride-rich lipoproteins, particularly lipoprotein remnants, have been shown to be involved in atherogenesis (10; 11). Disturbances in postprandial lipoprotein remnant levels were found in patients with premature coronary atherosclerosis like Familial Combined Hyperlipidemia, Familial Hypercholesterolemia and type 2 diabetes (12-14). Al-Shoumer et al (15) observed an increase in plasma triglyceride (TG) levels in AGHD patients during daily regular meals, suggesting abnormalities in postprandial clearance of lipoprotein particles. No data are yet available on detailed analyses of postprandial remnant lipoproteins in AGHD patients.

In the present study, we used both the classical RE analysis and a new remnant lipoprotein method based on the immunoseparation principle to study remnant metabolism developed by Nakajima et al (16). Apo AI-containing and apo B100-containing particles were bound to a sepharose gel coupled with specific monoclonal antibodies against Apo B100 and Apo AI. In the remaining supernatant fraction, remnant particles were found with only apo B48 or with apoB100/apoE. Lipoprotein remnants isolated with this method maintained their pathological properties in *in-vitro* studies (foam cell formation, decreased endothelial dilatation) (17). Remnant lipoprotein particles, isolated in this way, were associated with endothelial-dependent vasomotor function (17) and restenosis of coronary arteries after PTCA (18). The clearance of remnant particles was also assessed by incorporation and analysis of exogenous vitamin A (retinyl esters, RE) as core label for lipoprotein particles of intestinal origin. The suitability of vitamin A as a marker for chylomicrons and its remnants has been criticized (19; 20). Incorporation of RE occurs mostly in the late postprandial period as reflected by the delayed postprandial RE response compared with apoB48 analysis in the VLDL/chylomicron fraction (21). Furthermore RE label has been shown to exchange to other lipoprotein particles at later postprandial time points.

In this case-controlled intervention study, we investigated whether disturbances in postprandial lipoprotein metabolism were associated with the atherogenic lipoprotein profile observed in AGHD patients, and whether rh-GH treatment was capable of improving the atherogenic profile thereby decreasing the risk for coronary artery disease in AGHD patients.

Methods

Subjects

Patients with adult onset growth hormone deficient (AGHD) were recruited from the out patient clinic of the Department of Endocrinology from the University Hospital Utrecht. All patients had acquired growth hormone deficiency in adult life due to recent (within one year) treatment of a pituitary adenoma with surgery and/or radiotherapy. Other deficient pituitary hormones were supplemented for at least six months and were at a stable level at the start of the study. GH deficiency was defined as a peak plasma growth hormone concentration < 5 μg/L after the arginine infusion test. Recombinant human growth hormone (rh-GH was) substituted to plasma IGF-1 levels within the age-related normal range (22; 23). Exclusion criteria were presence of lipoprotein disorders such as familial hypercholesterolemia and familial combined hyperlipidemia, Body Mass Index (BMI) > 30, renal and/or liver disease, diabetes mellitus, Apo E2/E2 genotype and a positive family history of premature atherosclerosis. Eight healthy control subjects, matched for age, gender, BMI and ApoE genotype, were selected for this study by advertisement. They had no diabetes, no hepatic, renal, thyroid or cardiac dysfunction and a negative family history for cardiovascular disease. Post-heparin lipoprotein lipase (LPL) and hepatic lipase (HL) activities were measured at baseline in controls and before and after 6-month rh-GH treatment in AGHD patients. Oral fat load tests with RE were performed on a separate day.

The human investigation review committee of the University Hospital Utrecht approved this protocol and written informed consent was obtained from all participants.

Oral fat loading vitamin A test

After an overnight fast (12 hrs), participants were admitted to the metabolic ward at 7.30 am. Cream (consisting of 40% fat (w/v) with a P/S ratio of 0.06, 0.001% cholesterol (w/v) and 2.8% carbohydrates (w/v)) was given as

a single fat load in a dose of 50 g fat per m^2 body surface area, After ingestion of the cream supplemented with 60.000 IU aqueous vitamin A per 125 ml cream, 10 hourly venous blood samples were collected from an indwelling catheter in the ante cubital vene into EDTA containing tubes. All blood samples, protected from light, were immediately put on ice, centrifuged and analyzed. During the postprandial period, the subjects were allowed to drink only water or tea without sugar. None of the subjects experienced gastrointestinal complaints after drinking the cream.

Laboratory measurements

Plasma was obtained by centrifugation at 3000 rpm for 15 min at 4°C. TG and cholesterol were measured with a colorimetric assay (Monotest cholesterol kit no. 237574 and GPO-PAP no. 701912, Boehringer Mannheim, Germany). Cholesterol was analyzed in the HDL fraction isolated by the heparin-$MnCl_2$ dextran-sulphate precipitation method (24). LDL cholesterol was calculated with the Friedewald Formula (25). Plasma apo B concentrations were analyzed automatically on a Cobas Mira autoanalyzer (Unimate 3 Apo B, Roche Diagnostics). Plasma Apo E and Apo CIII concentrations were determined with a commercial test kit using the immunoelectrophoresis technique (Sebia Inc. USA). The coefficient of variance for plasma Apo E and Apo C III analysis was < 7.5%. The plasma Insulin and IGF-1concentrations were determined with a radioimmuno assay (26). Apo E genotype was determined as described by Dallinga-Thie et al (27). Plasma for LPL and HL was obtained 20 minutes after an intravenous injection of 50 IU/kg of heparin. Postheparin Lipoprotein Lipase and Hepatic Lipase activity were assayed as described previously (28; 29). Non esterified fatty acids (expressed as nmol free fatty acids (FFA) min^{-1} (mU)/mL) were measured with an enzymatic assay (WAKO chemicals, Neuss, Germany). HOMA-index (fasting glucose*fasting insulin/22.5) was calculated to estimate the insulin sensitivity. Body composition was assessed with bioimpedance analysis.

Assessment of lipoprotein remnants

Lipoproteins were separated by flotation using a single ultracentrifugation step in a Sf>1000 fraction which contains chylomicrons, large chylomicron remnants and large hepatic triglyceride-rich lipoproteins, and a remaining infranatant fraction (Sf<1000) containing small chylomicron remnants and all the other lipoproteins (30; 31). Retinyl ester concentrations in plasma and in the Sf > 1000 and Sf<1000 fraction were measured with high-performance liquid chromatography (HPLC) as described by Ruotolo et al (32). Recoveries of retinyl esters in the Sf>1000 and Sf<1000 were between 80 and 105%.

The RLP fraction was prepared using an immunoseparation technique described by Nakajima et al (16; 33). Briefly, 5 μl of serum was added to 300 μl of mixed immunoaffinity gel suspension containing monoclonal anti-human apo A-I (H-12) and anti-human apo B-100 (JI-H) antibodies (Japan Immunoresearch Laboratories, Takasaki, Japan). The reaction mixture was gently shaken for 120 minutes at room temperature followed by standing for 15 minutes. Then 200 μl of the supernatant was withdrawn for the assay of RLP-C and RLP-TG. Cholesterol (CV <3%) and triglycerides (CV<3%) in the RLP fraction were measured by an enzymatic assay using an automatic chemistry analyzer (ABX Diagnostics, Montpellier, France).

Forearm vasomotion study

Prior to the test meal blood samples were obtained for baseline values and a forearm vasomotion test was performed. The ultrasound measurements were performed in a supine position at the elbow of the right arm using a vessel wall-movement system (Wall Track System, Pie Medical, Maastricht, The Netherlands) consisting of an ultrasound imager with a 7.5 MHz linear array transducer connected to a data acquisition system and a personal computer. In short, an optimal

two dimensional B-mode image of the brachial artery was obtained. An M-line perpendicular to the vessel was selected. Next, the ultrasound system was switched to M-mode, after which storage of data started. The vessel movement detector system repeatedly registered end-diastolic vessel diameter during a period of 5 to 6 cardiac cycles. This procedure was performed three times. The measurements were averaged to provide for a baseline diameter measurement. By inflation of a blood pressure cuff for 4 minutes at a pressure of 100 mmHg above the systolic blood pressure, ischemia was applied to the forearm distal to the location of the transducer. Ultrasonography continued for 3 minutes after cuff release with measurements at 30 seconds intervals. The widest lumen diameter was taken as a measure for maximal diameter. Measurements were obtained for another 5 minutes, at 1-minute intervals. Flow Mediated Dilatation (FMD) was expressed as a percentage change relative to baseline diameter. The intersession variability was 3.4%.

Statistical Analysis

Data are presented as means ± SD, unless shown otherwise. Area under the integrated curve was calculated using data from the first 8 hours after the start of the oral fat loading test for postprandial TG, retinyl esters and RLP-C using GraphPad Prism software (version 3.1, San Diego, California, USA). Effects of rh-GH substitution in AGHD patients and differences between untreated AGHD patients and controls were analyzed by two-tailed unpaired Student's t-test. Pearson's correlation or Spearman's rank correlations were applied to evaluate relationships between parameters. A two-sided p-value of 0.05 was considered to be significant. Statistical analysis was performed with Graphpad InStat version 3.00 for Windows 95, Graphpad Software, San Diego, California, USA.

Results

Characteristics of the subjects

Table 1 shows characteristics of the subjects. Average duration of hypopituitarism was 40 ± 8 month. All 8 patients were substituted with thyroxine, 6 patients with hydrocortisone, 2 patients with cortisone acetate, 7 patients with sex hormones (males: testosterone esters; females: cyclic estrogen and progesterone) and 7 patients with desmopressin. Seven patients had an apo E3/E3 genotype and 1 patient had an apo E3/E4 genotype. All patients were treated with rh-GH during minimally 6 months with an average of 7 ± 1 month.

Fasting plasma TG and LDL cholesterol levels were significantly elevated in AGHD patients as compared to control subjects. After treatment LDL cholesterol levels decreased, whereas plasma TG levels remained significantly elevated. Plasma apo B, apo E and apo CIII levels were similar in all groups. The fasting plasma insulin levels, plasma IGF-1 levels and the HOMA-index were significantly lower in the AGHD patients than in controls ($P<0.05$). After rh-GH treatment plasma insulin levels and the HOMA-index increased and were not distinguishable from control levels. In contrast plasma IGF-1 levels increased and were significantly higher than in controls ($P<0.05$). In AGHD patients, fasting plasma IGF-1 concentrations were positively correlated with the HOMA-index ($r=0.67$; $p<0.01$), fasting plasma insulin concentration ($r=0.63$; $p=0.01$) and fasting plasma glucose concentration ($r=0.52$; $p=0.046$). Plasma LPL activity was similar in AGHD patients and controls, but treatment with rh-GH resulted in a significant decrease in LPL activity. No differences were observed for plasma HL activities.

Postprandial TG responses

After the oral fat load, maximum postprandial plasma TG levels were reached at 3 hours in control subjects and between 4 and 5 hours in AGHD patients (Figure 1).

They were significantly higher in AGHD patients than in matched control subjects, 3.17 ± 1.51 mmol/L vs. 1.93 ± 0.69 mmol/L ($P<0.05$). Area under the TG curve (AUC-TG), analyzed over an 8-hour postprandial interval, was significantly higher in AGHD patients (21.29 ± 8.91 mmol*h/L; $p<0.05$) than in controls (11.65 ± 3.66 mmol*h/L, $P<0.05$, Table 2). After correction for baseline TG levels (incremental AUC-TG) this difference disappeared (8.62 ± 5.92 mmol*h/L versus 4.71 ± 2.42 mmol*h/L). Treatment with rh-GH did not result in improvement fasting plasma TG levels.

Postprandial RLP-Cholesterol response

Fasting RLP-Cholesterol concentrations were significantly elevated in AGHD patients (0.29 ± 0.14 mmol/L) as compared to in control subjects (0.19 ± 0.06 mmol/L; $p<0.05$, Figure 2). The maximum postprandial RLP-C concentration was reached between 2 and 4 hr in control subjects and between 4 and 6 hr in AGHD patients and was significantly higher in AGHD patients. The AUC-RLPC (table 2) and the incremental AUC-RLPC were significantly elevated in AGHD patients (4.46 ± 2.0 mmol*h/L respectively 2.13 ± 1.60 mmol*h/L) than in control subjects (2.59 ± 1.08 mmol*h/L respectively 1.05 ± 0.72 mmol*h/L; $p<0.05$). Growth hormone treatment resulted in a significant decrease of AUC-RLPC and incremental AUC-RLPC (Table 2), suggesting an improvement of postprandial clearance of remnant-like particles after normalization of the growth hormone axis, although the peak time for RLP-C was unchanged (Figure 2). It is important to note that despite the fact that a strong correlation existed between baseline TG and RLP-C, they seemed to have different metabolic properties in AGHD patients. In AGHD patients, positive correlations were observed for incremental AUC-RLPC and baseline plasma cholesterol concentrations ($r = 0.63$; $p<0.05$), plasma TG concentrations ($r = 0.62$; $p<0.05$), LDL cholesterol ($r = 0.62$; $p<0.05$), and Apo B concentrations ($r = 0.63$; $p<0.05$), whereas IGF-1 concentrations showed a negative correlation ($r = -0.54$, $p<0.03$).

Postprandial retinyl ester response

Maximum postprandial plasma RE concentrations were reached at 4 hours in control subjects and between 5 and 8 hours in AGHD patients and were higher, albeit not statistically significant, in AGHD patients (Figure 3A). Similarly the area under the retinyl ester curve (AUC-RE) was higher in AGHD patients (Table 2) but the difference did not reached the level of statistical significance. Growth hormone treatment resulted in even higher maximal postprandial RE levels and the difference with the control subjects now reached the level of significance

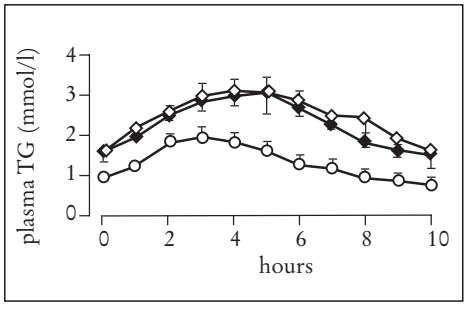

Figure 1. Postprandial response for plasma TG in untreated AGHD patients (ŧ), AGHD patients with rh-GH treatment(u) and control subjects (m). Data are presented as mean ± SEM.

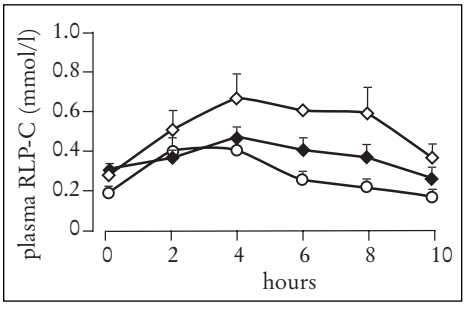

Figure 2. Postprandial remnant-like particle cholesterol (RLP-C) response in untreated AGHD patients (ŧ), AGHD patients with rh-GH treatment (u) and control subjects (m).

Table 1: Characteristics of AGHD patients before and after treatment with rh-GH and matched control subjects

	AGHD rhGH (-)	AGHD rhGH (+)	Controls
N	8	8	8
Male/Female	5/3	5/3	5/3
Age (yrs)	49 (8.0)	49 (8.0)	47 (7.7)
BMI (kg/m^2)	27.5 (2.3)	27.5 (3.7)	25.7 (1.6)
FM (kg)	20.58 (4.2)	22.64 (6.67)	19.7 (4.6)
FM%	25.4 (5.6)	27.1 (6.7)	24.9 (6.4)
Waist to hip ratio	0.92 (0.04)	0.93 (0.03)	0.88 (0.06)
Cholesterol (mmo/L)	5.51 (0.96)	5.04 (0.66)	4.98 (0.72)
TG (mmol/l)	1.55 (0.68)$^{\#}$	1.75 (0.37)$^{\#\#}$	0.91 (0.27)
HDL-cholesterol (mmol/L)	1.31 (0.43)	1.24 (0.52)	1.42 (0.32)
LDL-cholesterol (mmol/L)	3.63 (0.92)$^{\$}$	3.07 (1.12)	3.07 (0.70)
Apo-B (mg/L)	1.01 (0.28)	0.97 (0.09)	0.90 (0.21)
Apo-CIII (mg/L)	27.63 (9.76)	26.17 (5.8)	27.80 (8.67)
Apo-E (mg/L)	43.9 (8.17)	43.67 (7.32)	53.6 (10.7)
Insulin (mU/L)	5.57 (0.79)$^{\#}$	9.43(4.65)	9.25 (3.4)
HOMA-index	1.17 (0.29)$^{\# \$}$	2.09 (1.01)	2.14 (0.89)
IGF-1 (nmol/L)	111 (49)$^{\# \$}$	222 (43)$^{\#}$	169 (28)
fT3 (nmol/L)	1.35 (0.44)	1.34 (0.35)	1.52 (0.27)
LPL activity (mU/mL)	149 (40)$	112 (27)$^{\#}$	149 (26)
HL activity (mU/mL)	472 (268)	499 (316)	357 (131)

All values are expressed as mean ± SD. rhGH (-) indicates without rh-GH treatment and rhGH(+) indicates with rh-GH treatment. AGHD vs controls: $^{\#}$ P<0.05 and $^{\#\#}$ P<0.01; AGHD patients treated vs. not treated: $^{\$}$ P<0.05.<0.01

Table 2: Postprandial data for triglycerides, RLP-C, and retinyl esters in the Sf<1000 fraction.

	AGHD rhGH (-)	AGHD rhGH (+)	Controls
Fasting TG	1.55 (0.70)$^{\#}$	1.75 (0.37)	0.91 (0.27)
AUC-TG	21.29 (8.91)$^{\#}$	19.99 (6.72)	11.65 (3.66)
ΔAUC-TG*	8.62 (5.92)	7.51 (2.88)	4.71 (2.42)
AUC-RE	56.26 (35.41)	66.11 (14.47)$^{\#}$	29.91 (21.88)
Sf<1000 AUC-RE	18.36 (12.93)	25.71 (6.04)$^{\#}$	12.69 (9.66)
Fasting RLP-C	0.29 (0.14)	0.32 (0.09)$^{\#}$	0.19 (0.06)
AUC-RLPC	4.46 (2.0) $^{\# \$}$	3.21 (1.07)	2.59 (1.08)
ΔAUC –RLPC*	2.13 (1.60) $^{\# \$}$	0.73 (0.34)	1.05 (0.72)

All values are expressed as mean ± SD. rhGH (-) indicates without rh-GH treatment and rhGH(+) indicates with rh-GH treatment. AGHD vs controls: $^{\#}$ P<0.05; AGHD patients treated vs. untreated: $ P<0.05. AUC and incremental (Δ) AUC were calculated over a period from 0 – 8 hours. *ΔAUC indicates area under the incremental curve after correction for baseline concentrations.

(P<0.05). The AUC-RE in the Sf<1000 fraction increased after rh-GH treatment in AGHD patients and was significantly elevated as compared to control subjects (P<0.05, Table 2).

Endothelial function in AGHD patients

To assess the atherosclerotic burden of the AGHD patients we measured the flow-mediated diameter of the brachial artery. No measurements were performed in control subjects. Rh-GH treatment in AGHD patients resulted in an increase in the flow mediated diameter in the brachial artery as shown in Figure 4 (from 5.9 ± 3.3% to 10.2 ± 4.0%, P<0.05). The basal diameter of the artery was similar in AGHD patients before treatment: 4.4 ± 0.8 mm and after rh-GH treatment: 4.3 ± 0.7 mm.

Discussion

In the present study postprandial lipoprotein remnant concentrations, measured as remnant-like particle cholesterol was significantly increased in AGHD patients. Rh-GH treatment resulted in a significant decrease in postprandial remnant-like particle cholesterol, and improvement of endothelial function, although due to the small samples size the correlation did not reach the level of significance. Other pituitary hormone deficiencies were sufficiently treated throughout the study and no shift in plasma free T3 concentrations were found, stressing the effect of the rh-GH intervention.

Postprandial remnant lipoprotein particles have to be considered important mediators into the atherogenic process (34; 35). We used two different approaches to assess lipoprotein remnant metabolism. A new isolation method based on immunoseparation of remnant lipoproteins with sepharose coated with specific antibodies against apo B100 and Apo AI was applied, resulting in the scavenging of all HDL and most of the LDL and VLDL particles from the plasma. In the supernatant fraction the remnant-like particles (apo B48-containing and apo B100/Apo

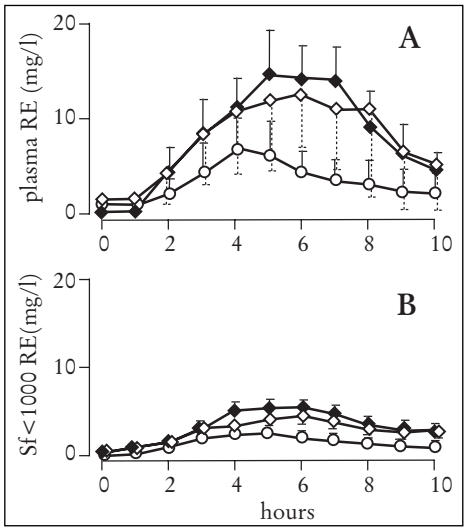

Figure 3. Postprandial RE responses for untreated AGHD patients (▾), AGHD patients with rh-GH treatment(▵) and control subjects (▪). Presented as mean ± SEM.The incremental plasma RE (panel A) and the RE level in the Sf<1000 fraction (panel B)

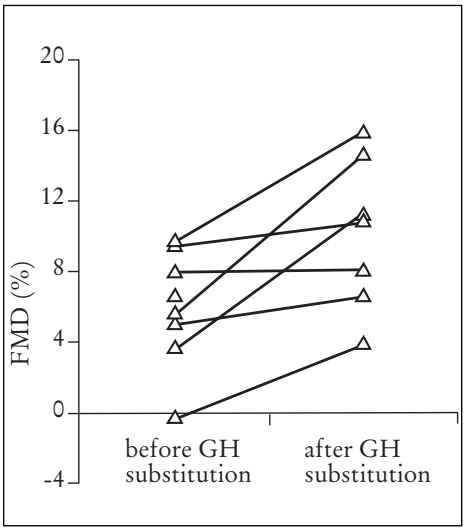

Figure 4. Flow mediated dilatation (%) before and after rh-GH substitution in 8 AGHD patients.

E enriched particles) were recovered. Analysis of postprandial remnant-like particles consisting mostly of Apo B48 particles is in agreement with assessment of postprandial apo B48 in Very-Low Density lipoprotein/ intermediate density fractions (Dallinga-Thie, unpublished data). Furthermore, the classical analysis of vitamin A (retinyl esters), which is incorporated into the core of the newly synthesized chylomicron particle, was used. Assessment of retinyl ester concentrations in the plasma and in the Sf<1000 and Sf>1000 fractions over a period of 8 hours will provide evidence for *in-vivo* chylomicron remnant clearance.

Our results show for the first time that in AGHD patients abnormalities in postprandial response were characterized by decreased clearance of RLP-C and retinyl esters in the Sf<1000 fraction, whereas fasting levels of RLP-C were also significantly increased. Treatment with rh-GH resulted in significant improvement of RLP-C clearance but no effects were observed in retinyl ester metabolic behavior and in fasting plasma RLP-C. Incorporation of vitamin A occurred mostly at later time points during the postprandial phase into larger chylomicron particles (36). After ingestion of dietary fatty acids the volume of the newly formed chylomicron particles increased thereby improving the capacity to carry vitamin A. Larger chylomicrons and its remnants were considered to be less atherogenic particles than smaller, early postprandial remnants (35). It has been shown that in rat enterocytes the secretion of chylomicrons occurred in a bimodal way. De *novo* secreted chylomicrons in the late postprandial period and the continuous secretion of smaller chylomicrons predominantly in the fasting and early postprandial period, albeit some degree of secretion of smaller particles remained throughout the later period. We hypothesize that apo B 48 and RLP-C reflects particles with identical behavior, whereas RE marks the properties of intestinal postprandial lipoprotein particles with a different metabolic behavior. Our results suggest that the secretion of large chylomicron particles remained abnormal after growth hormone substitution. Growth hormone therapy resulted in an upregulation of the hepatic expression of the LDL-receptor (37), resulting in improved clearance of apo B containing lipoprotein particles. In fact we observed a significant decrease in plasma LDL-cholesterol level and concomitant decrease in VLDL-cholesterol. Both apo B100 and apo B48 containing particles share the same degradation pathways (38), involving either the LDL-receptor pathway or alternative pathways involving the LDL receptor related protein (LRP) and proteoglycans. The preferential improvement of only the RLP-C fraction is in support of a role of the LDL-receptor pathway in apo B48 remnant particle clearance. No correlations were found between changes in LPL activity and postprandial parameters. It has been reported that growth hormone supplementation results in a specific decrease of LPL activity in adipose tissue but not in a change in LPL activity in skeletal muscle tissue (39; 40), that has been shown to be correlated with a beneficial lipoprotein profile.

We hypothesize that fasting plasma RLP-C levels resembles the presence of circulating apoB100 remnant particles enriched with apoE. Due to conformational changes in the particles they do not bind to the monoclonal antibody and remained in the supernatant fraction (16). The fact that its concentration remained elevated after growth hormone treatment is in favor for a different metabolic behavior of these remnant particles as compared to apo B48 containing remnants. Further studies are required to dissect the metabolic behavior of these different remnant fractions. Increased cardiovascular mortality due to accelerated atherosclerosis is a clinical feature in AGHD (1-3). Impaired endothelial dependent vasodilatation in response to flow is associated with early atherogenesis (41.42) Different interventions in the atherosclerotic process, including growth hormone substitution in AGHD, have shown to directly modulate endothelial functions (43-45). In the present study a significant

increase in flow mediated dilatation after treatment with rh-GH was observed, which is in agreement with an improvement of the atherogenic profile in response to growth hormone treatment in AGHD patients (4). It has been recognized that RLP-C offered independent assessment for CHD risk in addition to TG. Incubation with an isolated remnant-like particle fraction reduced endothelial-dependent vasorelaxation *in vitro*, which could be reversed by intervention with α-tocopherol (46; 47). In humans, plasma RLP-C concentrations were negatively associated with plaque regression of the coronary artery (17).

In conclusion, we showed a significant decrease in postprandial RLP-C concentration and improvement of the endothelial function in AGHD patients after growth hormone replacement therapy. We were not able to show a direct relationship between the improved endothelial function and improvement of the plasma lipoprotein profile, probably due to the small sample size. Improved postprandial lipoprotein remnant levels and improved endothelial function reflect a less atherogenic state and support the beneficial effect of early growth hormone replacement therapy in AGHD patients.

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1.7
GH treatment in adult-onset Growth Hormone deficiency leads to a decrease in plasma LDL-cholesterol despite an increased cholesterol synthesis

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Adult-onset Growth Hormone deficiency (AGHD) is associated with an atherogenic lipid profile that ameliorates during growth hormone therapy. We aim to study the effect of adequate GH substitution in AGHD patients on lipids and total cholesterol synthesis (by lathosterol/cholesterol ratio; LC ratio).

Nine AGHD patients (5 males, 4 females, age: 49.3 ± 7.8 y, BMI: 26.9 ± 2.7 kg/m2), and 13 matched (for age, sex, BMI and apolipoprotein E genotype) control subjects were included. Plasma levels of TG, RLP-C and of the L/C ratio were significantly higher in GH deficient subjects compared to control subjects. During GH therapy, plasma TG (1.62 ± 0.41 mmol/L), L/C ratio (1.31 ± 0.40), VLDL-cholesterol (0.83 ± 0.38 mmol/L) and RLP-C (11.8 ± 3.5 mg/dL) levels remained elevated, while plasma LDL-cholesterol decreased significantly from 3.63 ± 0.93 to 3.05 ± 1.12 mmol/L.

In conclusion, GH treatment in AGHD resulted in a 30 % decrease in plasma LDL-cholesterol levels, but TG levels, total cholesterol synthesis and plasma RLP-C levels did not change and persisted during GH therapy. Beneficial effects of GH therapy in AGHD is mostly due to increased expression of LDL receptors, although with minimal effect on plasma levels of atherogenic lipoprotein remnants.

Patients with an adult-onset GH deficiency (AGHD) have a higher risk on cardiovascular and cerebrovascular death due to progressive atherosclerotic disease. This increased susceptibility for atherosclerosis may be explained by the presence of a dyslipidemia (1). Although dyslipidemia in GH deficiency is characterized by a moderate elevated plasma LDL-cholesterol, also plasma levels of triglyceride-rich particles (TRPs) are increased (2). Recent developments show that elevated plasma levels of TG and apolipoprotein B levels complete the atherogenic lipid phenotype and are therefore an independent risk factor for atherosclerotic disease (3;4). Among the triglyceride-rich particles, lipoprotein remnants or Remnant-Like Particle-Cholesterol (RLP-C) are increasingly considered as high atherogenic particles (5-7)and their plasma levels are found increased in populations with a high risk for cardiovascular disease, such as familial hypercholesterolemia (8). In a previous report, we showed that GH therapy had no effect on fasting plasma RLP-C levels, while postprandially the plasma RLP-C accumulation decreased (9). In the background of an increasing interest of the TRPs in the atherogenic lipid phenotype, the effect of GH therapy in AGHD on both plasma levels of LDL-cholesterol and TRP need to be elucidated.

In order to focus more on the mechanism behind the plasma levels of LDL-cholesterol and RLP-C in AGHD and the effects of GH therapy on it, the relation between cholesterol synthesis and VLDL-cholesterol, as a common precursor of LDL-cholesterol and RLP-C, needs to be analyzed. Hepatic secretion of VLDL apo B100 is modulated by cholesterol synthesis (10). In GH deficient subjects, synthesis of VLDL apo B100 is elevated (11; 12)which may give rise to elevated plasma levels of both LDL-cholesterol and TRPs. However, in hepatocytes, expression of HMG co A reductase is determined by several growth factors, such as IGF-1, which is decreased in AGHD (13). Whole body cholesterol synthesis is in general reflected by plasma levels of the several cholesterol precursors, such as lanosterol, mevalonic acid (MVA), squalene, and/or lathosterol. Previous reports noted that plasma levels of squalene reflect plasma VLDL-cholesterol concentrations, whereas plasma levels of lathosterol (or lathosterol expressed per cholesterol) is a most reliable marker for whole body cholesterol synthesis (14; 15).

Moreover, plasma lathosterol levels are independent of cholesterol synthesis induced by dietary cholesterol (16).

We performed a case control intervention study, with the aim to determine the effects of GH therapy on the atherogenic lipid phenotype in adult-onset GH deficient adults and its relation with whole body cholesterol synthesis.

Subjects and Methods

Subjects

Patients and normolipidemic controls

Adult-onset growth hormone deficient (AGHD) patients were recruited from the out patient clinic of the department of Internal Medicine and Endocrinology from the University Medical Center Utrecht. The GH deficient patients and control subjects participated in a larger project that concerns the atherogenic metablic phenotype in GH deficiency and were also part of a previous published study (9). All patients had an acquired AGHD due to recent (within one year) neurosurgery (pituitary adenoma) and/or irradiation. Other deficient pituitary hormones than GH were supplemented for at least six months and were at a stable level at the start of the study. Three months stable GH therapy was reached before patients were tested again for the lipid profile. GH deficiency was defined as a peak plasma GH concentration $< 5\ \mu g/L$ after the combined administration of Arginin plus GH-Releasing Hormone (GHRH) intravenously. GH was substituted to plasma IGF-1 levels within two standard deviations of age- and gender related normal range (17;18). Exclusion criteria were lipoprotein disorders (as familial hypercholesterolemia and familial combined hyperlipidemia), Body Mass Index (BMI) > 30 kg/m^2, renal and/or liver disease, diabetes mellitus (DM), apolipoprotein E genotype (apoE): E2/E2 and family history of premature atherosclerosis.

Healthy control subjects, matched for age, gender, BMI and apoE genotype, were selected for this study by advertisement. They had no diabetes, no hepatic-, renal-, thyroid- or cardiac dysfunction and had a negative family history for cardiovascular disease. The protocol had been approved by the human investigation review committee of the University Medical Center Utrecht and written informed consent was obtained from all participants.

Methods

Fasting blood samples were obtained for baseline values. Plasma was obtained by centrifugation at 3000 rpm for 15 min at 4°C. TG and Cholesterol were measured with a colorimetric assay (Monotest cholesterol kit no. 237574 and GPO-PAP no. 701912, Boehringer Mannheim, Germany). Coefficient of variance for TG and Cholesterol was <5%. Cholesterol was determined in the HDL fraction isolated by the heparin-$MnCl_2$ dextran-sulphate precipitation method. Low-density lipoprotein (LDL) -cholesterol was calculated with the Friedewald Formula. Apolipoprotein (apo-) B concentrations were analysed automatically on a Cobas Mira autoanalyzer (Unimate 3 Apo B, Roche Diagnostics). VLDL-cholesterol was calculated with the equation: (Total cholesterol-(HDL-cholesterol + LDL-cholesterol)). The plasma Insulin and IGF-1 concentrations were determined with a radio-immuno assay (19). HOMA-index (fasting glucose x fasting insulin/22.5) was calculated to estimate the insulin sensitivity. Apo E genotype was measured as described (20). The amount of fat mass (FM) was assessed with bio-impedance analysis. The RLP fraction was prepared using an immunoseparation technique described by Nakajima et al (21; 22). Briefly, 5 μl of serum was added to 300 μl of mixed immunoaffinity gel suspension containing monoclonal anti-human apo A-I (H-12) and anti-human apo-B-100 (JI-H) antibodies (Japan Immunoresearch Laboratories, Takasaki, Japan). The reaction mixture was gently shaken for 120 minutes at room temperature followed by standing for 15 min-

utes. Then 200 µl of the supernatant was withdrawn for the assay of RLP-C. Cholesterol (CV < 6%) in the RLP fraction were measured by an enzymatic assay using a Cobas Mira S auto-analyzer (ABX Diagnostis, Montpellier, France).

Cholesterol Precursors

Plasma samples were collected and stored frozen at -20°C in the dark until use. For the extraction of the sterols, 500 µl of plasma was mixed with 100 µl 0.01 mg/ml stigmasterol and saponified for 60 min. at 60°C in 1 ml of 4 % (w/v) KOH in 90 % ethanol. After saponification, the samples were mixed with 1 ml of water and extracted two times with 2 ml of hexane. The pooled hexane extracts were dried under nitrogen and derivatized with 50 µl BSTFA/pyridine (v/v 5:1) at 60°C for 60 min.

For SIM-GC-MS, 2 µl of the derivative mixture were delivered by automatic injection to an HP-5890 gas chromatograph split injection port (1:20) leading to a 0.2 mm x 25 m Chrompack CP-sil 19 CB (WCOT Fused Silica) capillary column. The injection port contained a glass wool liner. The carrier gas was helium at a linear rate of 1 ml/min. The oventemperature starts at 120°C and was raised to 260°C at 20°C/min, then to 280°C at 2°C/min and finally to 300°C at 40°C/min and held for 5 min. An HP-5989B mass spectrometer was used as detector. Measurements were done in the electron impact mode at 70 eV with an ion source temperature of 250°C. The quadropole temperature was 150°C. Mass spectrometric data were collected in the selected ion mode at m/z = 136 and 341 for squalene, m/z = 325 and 351 for 7-dehydrocholesterol (7-DHC), m/z = 255 and 213 for lathosterol, m/z = 306 and 355 for dihydrocholesterol, m/z = 241 and 393 for lanosterol, and m/z = 255 and 394 for stigmasterol. Calibration curves were constructed by mixing 100 µl of 0.01 mg/ml stigmasterol with a series of 0 to 500 µl samples of a standard solution containing 30 µmol lathosterol, 10 µmol desmosterol, 2 µmol squalene, 2 µmol lanosterol, 15 µmol dihydrocholesterol and 1.5 µmol 7-DHC.

High purity solvents were purchased from Merck, Germany. Bis-trimethylsilyltrifluoroacetamide (BSTFA) was obtained from Sigma-Aldrich (the Netherlands) and pyridine from Pierce (USA). Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaeen), dihydrocholesterol (5α-cholestan-3β-ol), lathosterol (5β-cholest-7en-3β-ol) and lanosterol (3β-hydroxy-8,24-lanostadieen) were purchased from Sigma, cholesterol (5δ-3β-cholestenol) from ICN Biomedicals Inc. (the Netherlands), 7-dehydrocholesterol (5,7-cholestadien-3β-ol) from Sigma-Aldrich and stigmasterol from Lacoclau AB Sweden.

Statistical Analysis

Data are presented as mean ± SD, unless shown otherwise. Comparison of effects after GH substitution in AGHD patients was assessed by a paired Student's t-test. Comparisons between AGHD and control subjects were performed by two-tailed unpaired Student's t-test. Spearman's rank correlation were applied to assess the relationship between different variables and cholesterol precursors. $P<0.05$ (two-tailed) was considered to be significant. Statistical analysis was performed with Graphpad InStat version 3.00 for Windows 95, Graphpad Software, San Diego, California, USA.

Results

Characteristics of the subjects

Table 1 shows characteristics of the subjects. The AGHD patients were strictly matched with the control subjects for age, gender, BMI and apo-E genotype (table 2). Average duration of hypopituitarism was 3.4 years ± 9 months. All 9 patients were substituted with L-thyroxine. Additionally, 7 patients were substituted with hydrocortisone, 2 patients with cortisone-acetate; 7 patients were substituted with sex hormones and 5 with desmopressin. All patients were treated with GH for 6 months. The fasting plasma insulin levels, plasma IGF-1 levels and the HOMA-index were lower in AGHD patients before GH substitution compared with control subjects and significantly lower compared to the value obtained after GH treatment.. After GH treatment, insulin and HOMA-index increased to the control levels, while IGF-1 concentration increased into the age-and gender related normal range.

Plasma lipids

Table 2 shows the lipoprotein levels of AGHD patients before (GH(-)) and after treatment with GH (GH(+))and the levels of the matched control subjects. Fasting plasma TG levels were significantly higher in the AGHD patients before and after rhGH treatment compared to control subjects (table 2). No differences were found in fasting plasma cholesterol and LDL-cholesterol between the AGHD patients after treatment and control subjects. The plasma LDL-cholesterol decreased significantly ($p=0.03$), while the plasma HDL-cholesterol tended to increase ($p=0.05$) during GH therapy. Fasting levels of RLP-C were higher in AGHD patients before and after GH therapy, compared to control subjects ($p<0.05$). Apo B levels were not significantly different between patients and control subjects. The plasma RLP-C levels in control subjects and in GH-substituted patients were positively associated with TG/apo B ratio ($r=0.57$; $p<0.01$) and VLDL-C ($r=0.53$; $p<0.05$).

Table 1: Characteristics of the AGHD patients before (GH(-)) and after treatment with GH (GH(+)) and of the matched control subjects

	AGHD GH(-)	AGHD GH(+)	Control Subjects
No.	9	9	13
Male/Female	5/4	5/4	7/6
Age (yr)	49.3 (7.8)	49.7 (7.6)	50.0(8.8)
BMI (kg/m^2)	26.9 (2.7)	26.9(4.0)	26.2 (2.8)
FM(kg)	21.2 (5.3)	22.5 (5.8)	20.0 (4.9)
FM (%)	25.9 (5.3)	25.4(6.9)	25.2 (6.7)
Waist/Hip ratio	0.92 (0.04)	0.94 (0.03)	0.83 (0.09)
Insulin (mU/L)	5.78 (1.01)	8.89 (4.20)^c	8.58 (3.18)
HOMA-index	1.15 (0.28)	1.97 (0.92)^c	1.88 (0.74)
IGF-1 (nmol/L)	105.22 (48.80)	215.30 (40.46)^c	162.80(26.93)^a

All values are expressed as mean ± SD. GH (-) indicates without GH treatment, and GH(+) indicates with GH treatment. AGHD vs. Controls:^{a} $P<0.05$; AGHD patients treated vs. untreated:^{c} $P<0.05.<0.01$

Cholesterol Precursors

Table 3 shows that the plasma levels of all measured cholesterol precursors (squalene, lanosterol, dihydrocholesterol (cholestanol), 7-dehydrocholesterol (7dhc) and lathosterol), were higher in the patient group compared to control subjects. No significant change in the plasma levels of the cholesterol precursors was found after GH therapy, although they tend to increase with the exception of squalene. The plasma lathosterol levels in controls were positively associated with the plasma RLP-C levels ($r=0.57$; $p<0.01$) and in all subjects ($r=0.42$; $p<0.05$). Plasma lathosterol levels were positively associated with TG/Apo B ratio in all subjects ($r=0.49$; $p<0.01$).

Table 2: Plasma lipoprotein levels of the AGHD patients before and after treatment with GH and of the matched control subjects

	AGHD GH(-)	AGHD GH(+)	Control Subjects
Cholesterol (mmol/L)	5.43(0.93)	4.98 (0.64)	5.23 (0.73)
LDL-cholesterol (mmol/L)	3.63(0.93)	3.05 (1.12)[c]	3.28 (0.73)
HDL-Cholesterol (mmol/L)	1.11 (0.20)	1.30(0.38)	1.52 (0.40)
VLDL-Cholesterol (mmol/L)	0.83 (0.38)[a]	0.80 (0.36)[a]	0.43 (0.17)
TG (mmol/L)	1.44(0.72)[a]	1.62 (0.41)[a]	0.96 (0.37)
RLP-C (mg/dL)	11.47(4.89)[a]	11.80(3.51)[a]	7.59 (2.16)
Apo-B (g/L)	1.06(0.29)	1.00(0.13)	0.98 (0.18)
Apo E Genotype			
E3/E3	7	7	10
E3/E4	1	1	1
E4/E4	1	1	2

All values are expressed as mean ± SD. GH (-) indicates without GH treatment, and GH(+) indicates with GH treatment. AGHD vs. Controls:[a] $P<0.05$; AGHD patients treated vs. untreated:[c] $P<0.05$.

Table 3: Cholesterol precursors in plasma of the AGHD patients before and after treatment with GH and in the matched control subjects

	AGHD GH(-)	AGHD GH(+)	Control Subjects
Squalene (μmol/L)	1.25 (1.02)[b]	1.02 (0.37)[b]	0.45 (0.22)
Lanosterol (μmol/L)	0.16(0.06)[a]	0.19(0.06)[a]	0.13(0.07)
Dihydrocholesterol (cholestanol) (μmol/L)	3.78(1.15)[b]	4.13(1.89)[b]	3.37 (0.98)
Lathosterol (μmol/L)	6.52(2.90)[b]	7.32(2.36)[b]	4.05(1.72)
Lathosterol/cholesterol ratio	1.21(0.52)+	1.31(0.40)[a]	0.78(0.34)
7-dehydrocholesterol (μmol/L)	0.41(0.15)[b]	0.52(0.18)[b]	0.22(0.09)

All values are expressed as mean ± SD. GH (-) indicates without GH treatment, and GH(+) indicates with GH treatment. AGHD vs. Controls:[a] $P<0.05$ and [b] $P<0.01$; AGHD patients treated vs. untreated:[c] $P<0.05$.

Discussion

The increased cardiovascular mortality in adult-onset GH deficiency is mostly explained by a pro-atherogenic lipid profile, that ameliorates during GH therapy. In this study, the elevated plasma LDL-cholesterol levels reduced significantly during GH therapy, but no effect was found on elevated plasma levels of TG, VLDL-cholesterol and RLP-C. In addition, the elevated plasma lathosterol levels and the elevated lathosterol/cholesterol ratio in GH deficient subjects, which reflects an elevated whole body cholesterol synthesis, were not decreased by GH therapy. The persistent elevated cholesterol synthesis and plasma levels of TG, VLDL-cholesterol and RLP-C in AGHD patients indicate that the effect of GH therapy is mostly mediated by an increased expression of hepatic LDL-receptors.

In general, plasma LDL-cholesterol levels in GH deficient subjects are mostly found to be in a moderate higher cholesterol range with a significant decrease during GH therapy (23; 24). In line with previous observations, we also found a 16% decrease after 6 months GH therapy. Reductions in plasma LDL-cholesterol levels in GH treated GH deficient subjects have been also reported in other intervention studies (1; 25; 26). The sustained elevated plasma TG and especially the RLP-C levels in GH treated AGHD patients is of clinical relevance (27). Cross-sectional studies found that the 90th percentiles of plasma RLP-C levels was 8.5 mg/dL in healthy subjects, and 10.9 mg/dL in coronary artery disease (CAD) patients (28). Moreover, a value above 75th percentile of distribution of RLP-C levels in CAD patients explored a higher mortality compared to those with a plasma RLP-C levels lower than 50th percentile of distribution of RLP-C levels in a three year follow-up trial (29). The RLP-C levels above the 90th percentile of distribution are a significant risk factor for the presence of CAD independent of LDL-cholesterol, HDL-cholesterol, TG and other traditional risk factors (30; 31). In this study, plasma levels of RLP-C in AGHD patients were above the high 90th percentile of distribution and remained elevated during GH therapy. From our results, we propose an additional lipid lowering strategy in individual GH deficient patients with elevated plasma RLP-C and TG levels. Gemfibrozil may be a candidate drug as it proved to prevent progression of coronary and vein graft atherosclerosis by lowering increased plasma RLP-C levels (32), in favour of statin intervention that had limited reducing effects (8).

In order to understand more the direction of the lipid pathways in AGHD that are influenced by GH therapy, the relationship between the L/C ratio (that reflects whole body cholesterol synthesis; CS) and plasma levels of both LDL-cholesterol and TRP were studied. The secretion of apo B100 VLDL is associated with CS. In this study, CS and estimated plasma VLDL-cholesterol levels in AGHD patients was increased compared to control subjects, and remained elevated during GH therapy. Our observation is in contrast with Russell-Jones et al (24)who found a decrease in plasma mevalonic acid (MVA) levels during one month of GH therapy. However, plasma MVA levels, as an indicator of whole body CS is less reliable than plasma lathosterol levels, due to significant diurnal intra-individual variations (33; 34). Our observation of an increased CS and estimated plasma VLDL-cholesterol level in AGHD is line with Kearney et al, who also found an increased VLDL synthesis (35). Notwithstanding the sustained increase in CS and plasma VLDL-cholesterol levels during GH therapy, the decrease in plasma LDL-cholesterol can be explained by a GH-induced increased expression of LDL-receptors on the surface of the hepatocytes (36)and our study confirms this possible mechanism in LDL-lowering as a cause of GH therapy. In addition, GH substitution in LDL-receptor deficient mice increases the activity of 7-alpha hydroxylase, which increases the flux of intracellular cholesterol

towards bile acids, and subsequently GH additionally lower plasma LDL-cholesterol levels (37). On the other hand, the effect of GH therapy on TRPs is less than on LDL-cholesterol. In a previous study, we already showed a limited effect of GH therapy on post heparin lipoprotein lipase and hepatic lipase activity, and therefore disturbances in lipolytic pathways cannot explain the persistent rise in plasma TRPs in AGHD. Probably, the hepatic receptors (such as scavenger receptors) in humans, that are responsible for the TRP removal are not influenced by GH therapy. This observation is line with results from cultured mesengial cells that also found no effect of IGF-I on the scavenger receptor activity, but only on the LDL-receptor expression and non-receptor mediated endocytosis (38). Plasma TRPs are only for a small part removed by the hepatic LDL-receptor. Plasma concentration of lathosterol were positively associated with plasma RLP-C levels, and this observation may indicate that the RLP fraction is a preferable transporter for lathosterol. Probably, GH modulates VLDL secretion by altering the amount of phospholipids- and/or apo B availability for formation of the VLDL particle (39). The decrease in insulin sensitivity with GH therapy, as determined by an increase in HOMA-index, may have contributed to the higher apoB VLDL levels in GH treated AGHD patients and consequently to the relatively higher plasma TRP levels (40).

In conclusion, the dyslipidemic profile in AGHD patients is characterized by increased plasma LDL-cholesterol levels and TRPs, such as RLP-C, which both are associated with an increased total body cholesterol synthesis. Plasma LDL-cholesterol levels decreased during GH therapy, indicating a significant effect of GH therapy on catabolic pathways of LDL-cholesterol. On the other hand, the increased whole body CS and elevated plasma levels of TG, RLP-C and VLDL-cholesterol persisted during GH therapy. Due to more evidence that TRPs play an important role in atherosclerotic disease, more research on the effect of GH therapy on TRP pathways in AGHD is needed, and additional lipid-lowering therapy besides GH therapy needs to be envisioned in those GH deficient subjects with persistent elevated plasma TG and RLP-C levels.

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1.8
Induction of postprandial inflammatory response in adult onset growth hormone deficiency is related to plasma remnant-like particle cholesterol (RLP-C) concentration

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Increased cardiovascular mortality due to premature atherosclerosis, is a clinical feature in the adult-onset growth hormone deficiency (AGHD) syndrome. Inflammation is a key feature in atherogenesis and may be triggered by postprandial lipoprotein remnants. We hypothesized that increased postprandial lipoprotein remnant levels in AGHD may be associated with an inflammatory response. In this case-control study, 10 AGHD patients (6 male, 4 female, age: 48 ± 9 y, BMI: 26.9 ± 2.6 kg/m2) and 10 healthy control subjects (matched for age, BMI, gender, baseline lipid levels and apo E genotype) were included. They all ingested an oral fat load. Fasting and postprandial levels of plasma RLP-C (0.31 ± 0.13 mmol/l and 4.14 ± 1.37 mmol/l*h in GHD; 0.18 ± 0.06 mmol/l and 2.56 ± 1.02 mmol/l*h in controls, respectively) were significantly increased in AGHD patients compared to control subjects. The median inflammatory cytokines, Interleukin-6 and TNF-a were higher in the fasting (3,9 (3.1 - 11.9) pg/ml and 6.8 (2.5 - 27.6) pg/ml) and postprandial state (151.7 (87.0 - 294.3) pg/ml*24h and 289.9 (87.5 - 617.6) pg/ml*24h) in AGHD than in controls (0.9 (0.2 - 5.2 pg/ml and 2.8 (2.5 - 5.7) pg/ml and postprandial: (54.5 (11.50- 126.5) and 118.3 (81.2 - 243.1) pg/ml*24h). In addition, postprandial profile of RLP-C and IL-6 in AGHD and in the total group were significantly associated (r2: 0.44; $p<0.05$ and r2: 0.38; $p<0.01$, respectively). In conclusion, the increased postprandial RLP-C level in GH deficiency is associated with an inflammatory response, that may result in increased susceptibility for premature atherosclerosis.

The syndrome of growth hormone deficiency is characterized by increased cardiovascular morbidity and mortality due to premature and progressive atherosclerosis (1-5). Endothelial dysfunction in GHD, that occurs early in atherosclerotic disease, is probably a consequence of low circulating levels of GH and IGF (that both are known to produce endothelial nitric oxide that cause vasodilation (6-8). Besides those direct effects of GH deficiency on the endothelial lining, disturbances in lipoprotein (9; 10) and lipoprotein remnant metabolism (11) are considered to be an indirect atherosclerotic process. Postprandially, lipoprotein remnants dominate (12)and they are atherogenic lipoproteins (13), that give rise to foam cell formation and inflammation in-vitro (14) Inflammation is an evident key factor in premature atherosclerosis (reviewed in ref (15-17)), but although its strong association the definite interaction of an inflammatory state and premature atherosclerosis is still under debate (18; 19). The pathophysiological explanation may be an excessive lipoprotein retention in the extracellular matrix with increased uptake of lipoproteins by macrophages (20-22). Consequently, atherogenic processes are initiated (23; 24). Reports in animal models support this retention hypothesis for TG-rich apo B lipoproteins, such as lipoprotein remnants. The plasma levels of these diet-derived-lipoproteins were associated with inflammatory components with increased intracellular nuclear factor κB (NF-κB) levels in stripped endothelium of rat aorta that subsequently activates pro-inflammatory genes and secretion of subsequent pro-inflammatory cytokines (25). Hitherto only an increase in postprandial hydroperoxides (26) has been reported.
In this study, we hypothesize that the presence of atherogenic lipoproteins in the postprandial period in GH deficiency may be associated with an inflammatory response.

Patients and Methods

Patients

Adult onset growth hormone deficient (AGHD) patients were recruited from the out patient clinic of the department of Internal Medicine and Endocrinology from the University Hospital Utrecht. All AGHD patients had an acquired AGHD in adult life due to recent (within one year) neurosurgery (pituitary adenoma) and or irradiation. Other deficient pituitary hormones were supplemented for at least six months and were at a stable level at the start of the study. GH deficiency was defined as a peak plasma growth hormone concentration < 5 μg/l after the argnine infusion test. Exclusion criteria were the presence of lipoprotein disorders (as familial hypercholesterolemia and familial combined hyperlipidemia), Body Mass Index (BMI) > 30, renal and/or liver disease, diabetes mellitus (DM), apo E genotype: E2/E2 and family history of premature atherosclerosis.

Normolipidemic controls.

Healthy control subjects, matched for age, gender, BMI and Apo E genotype, were selected for this study by advertisement. They had no diabetes, no hepatic, renal, thyroid or cardiac dysfunction and had a negative family history for cardiovascular disease. The protocol had been approved by the human investigation review committee of the University Hospital Utrecht and written informed consent was obtained from all participants

Oral fat loading test (OFLT)

Matched controls and AGHD patients underwent an OFLT. After a 12h overnight fast, participants were admitted to the metabolic ward at 7.30 h am. They ingested a test meal (consisting of 40% fat (w/v) with a P/S ratio of 0.06, 0.001% cholesterol (w/v) and 2.8% carbohydrates (w/v)) of 50 g fat per m^2 body surface area. Venous blood samples from the antecubital vein were obtained prior to the test meal and hourly after ingestion of the cream up to 24 hours. All blood samples were immediately put on ice. Only water or tea without sugar was allowed to drink during the OFLT. None of the subjects had gastrointestinal complaints after drinking the cream.

Baseline measurements.

Prior to the test meal blood samples were obtained for baseline values. Plasma was obtained by centrifugation at 3000 rpm for 15 min at 4°C. TG and cholesterol were measured with a colorimetric assay (Monotest cholesterol kit no. 237574 and GPO-PAP no. 701912, Boehringer Mannheim, Germany). Coefficient of variance for TG and Cholesterol was <5%. Cholesterol was determined in the HDL fraction isolated by the heparin-$MnCl_2$ dextran-sulphate precipitation method. Low-density lipoprotein (LDL) -cholesterol was calculated with the Friedewald Formula. Apolipoprotein (apo-) B concentrations were analysed automatically on a Cobas Mira autoanalyzer (Unimate 3 Apo B, Roche Diagnostics). The plasma Insulin and IGF-1 concentrations were determined with a radio-immuno assay (27). HOMA-index (fasting glucose*fasting insulin/22.5) was calculated to estimate the insulin sensitivity. Body composition was assessed with bioimpedance analysis. Apo E genotype was determined as described (28).

RLP-C analysis

The RLP fraction was prepared using an immunoseparation technique described by Nakajima et al (29; 30). Briefly, 5 μl of serum was added to 300 μl of mixed immunoaffinity gel suspension containing monoclonal anti-human apo A-I (H-12) and anti-human Apo-B-100 (JI-H) antibodies (Japan Immunoresearch Laboratories, Takasaki, Japan). The reaction mixture was gently shaken for 120 minutes at room temperature followed by standing for 15 minutes. Then 200μl of the supernatant was withdrawn for the assay of RLP-C. Cholesterol (CV% <3) in the RLP fraction were measured by an enzymatic

assay using a Cobas Mira S auto-analyzer (ABX Diagnostis, Montpellier, France).

Interleukin analysis

IL-6, IL-10 and TNF-α (in pg/ml) were analysed in fasting and in postprandial plasma samples with a commercial available ELISA-kit (CLB, Amsterdam, the Netherlands). Both the inter- and intra-assay coefficients of variation were below 10%.

Statistical analysis

Data are presented as means ± SD. In case of a skewed distribution, the median plus minimum and maximum value is presented. Total area under integrated curve (AUC) was calculated for postprandial plasma RLP-C, postprandial IL-6,TNF-α and IL-10 using GraphPad Prism software (version 3.1, San Diego, California, USA). In case of significant difference in baseline levels, the incremental (with correction for baseline levels) AUC was calculated. Differences between AGHD and controls were analyzed by unpaired t-test. Pearson's correlation or Spearman's rank correlation was calculated to assess the relationships between different variables. $P<0.05$ (two-tailed) was considered to be significant. Statistical analysis was performed with Graphpad InStat version 3.00 for Windows 95, Graphpad Software, San Diego, California, USA.

Results

Subjects

Table 1 shows characteristics of the subjects. The AGHD patients were strictly matched with the control subjects for age, gender and BMI and apo E genotype. Average duration of hypopituitarism was 28 ± 8 months. All 10 patients were substituted with T_4, 8 patients with hydrocortison, 2 patients with cortisone acetate, 9 patients with sex hormones (males, testosterone esters; females, cyclic estrogen and progesterone) and 8 patients with desmopressin. The distribution of the Apo E genotype in AGHD was as followed: E3/E3 (n=8), E2/E3 (n=1) and E3/E4 (n=1).
Fasting plasma TG levels were significantly higher in AGHD patients (1.44 ± 0.68 mmol/l) than in control subjects (0.88 ± 0.26 mmol/l; $p<0.05$). No differences were found in baseline plasma cholesterol, Apo B and LDL-cholesterol (table 1). Baseline plasma IGF-1 levels were lower in AGHD than in controls. No difference was found in free T_3 levels.

Postprandial responses

Postprandial RLP-C response

After the oral fat load, the time course of postprandial plasma RLP-cholesterol (RLP-C) concentrations is shown in figure 1. Fasting levels of RLP-C were significantly higher in AGHD patients (0.31 ± 0.13 mmol/l) than in control subjects (0.18 ± 0.06 mmol/l;

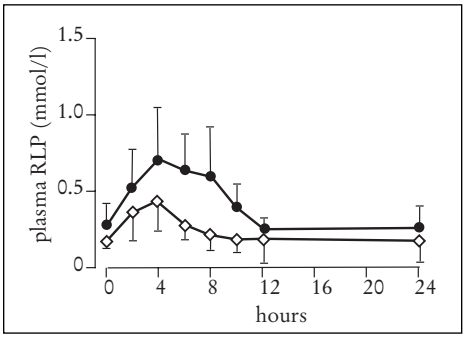

Figure 1. Postprandial plasma RLP-C response for the GH deficient patients (●) and control subjects (◇) Values are presented as mean (SD).

Table 1: Baseline characteristics

	Control (n=10)	AGHD (n=10)
Male/Female	6/4	6/4
Age (yr)	48,60 (7,73)	47,90(8,66)
BMI (kg/m^2)	25,43 (1,62)	26,87(2,59)
WH ratio	0,86 (0,06)	0,93 (0,04)[a]
FM (kg)	19,70 (4,55)	20,58 (4,19)
FM (%)	24,9 (6,44)	25,35 (5,63)
Cholesterol (mmol/L)	4,99 (0,64)	5,30 (0,97)
LDL-Chol (mmol/L)	3,04 (0,61)	3,49 (0,97)
HDL-chol (mmol/L)	1,49 (0,33)	1,28 (0,43)
TG (mmol/L)	0,88 (0,26)	1,44 (0,68)[a]
ApoB	0,88 (0,19)	0,97 (0,29)
IGF-1 (nmol/L)	161,78 (31,18)	106,7 (46,28)[a]
Free T3 (nmol/L)	1,53 (0,27)	1,32 (0,43)

All values are expressed as means (SD). WH, waist to hip; FM, fat mass; LDL, low-density-lipoprotein; HDL, high-density-lipoprotein; TG; triglyceride; IGF-I, insulin-like growth factor-I. AGHD vs. controls: [a] P < 0.05 and b P <0.01

Table 2: Postprandial data for RLP-C, IL-6, TNF-a and IL-10

	Control	AGHD
RLP-C (mmol/l)	0.18 (0.06)	0.31 (0.13)[a]
AUC RLP-C (mmol/l*h)	2.56 (1.02)	4.14 (1.37)[a]
dAUC RLP-C (mg/dl*24h)	0.90(0.35)	1.86 (0.92)[a]
Il-6 (pg/mL)	0.9 (0.2 - 5.2)	3.9 (3.1 - 11.9)[b]
AUC Il-6 (pg/mL*24 h)	54.5 (11.5 - 126.5)	151.7 (87.0 - 294.3)[b]
dAUC-IL-6 (pg/mL*24 h)	8.0 (0.3 - 45.8)	34.9 (18.6 - 169.8)[a]
TNF-a (pg/mL)	2.8 (2. - 5.7)	6.8 (2.54-27.6)
AUC TNF-a (pg/mL*24 h)	118.3 (81.2 - 243.1)	289.9 (87.5 - 617.6)[a]
Il-10 (pg/mL)	6.1 (5.0-15.1)	16.7 (5.3-24.2)[a]

All values are expressed as means (SD). RLP-C, remnant-like particle-cholesterol, AUC, integrated Area under the Curve; dAUC, incremental AUC; IL-6, interleukin-6; IL-10, interleukin-10. AGHD vs. controls: [a] P < 0.05 and [b] P <0.01

p<0.05). The postprandial peak time of RLP-C was between 2 and 4 h in control subjects and between 4 and 6 h in AGHD patients, with a maximum level of 0.43 ± 0.2 mmol/l, in controls, and 0.70 ± 0.34 mmol/l in AGHD patients (p<0.05). The AUC RLP-C (table 2) was enhanced in AGHD (4.14 ± 1.37 mmol*h/l) compared to control subjects (2.56 ± 1.02 mmol*h/l; p<0.05).

Postprandial cytokines

After the oral fat load, the time course of postprandial plasma IL-6 concentration is shown in figure 2. Median fasting levels of Il-6 were higher in AGHD patients (3.9 pg/ml (range: 3.1 - 11.9) than in control subjects (0.9 pg/ml; range: 0.2 - 0.5; p<0.01). In controls, the postprandial IL-6 response remained low with maximum levels of 3.4 ± 2.3 pg/ml at 10 hr. In GH deficiency, a distinctive peak level was reached at 10 hr of 13.1 ± 4.6 pg/ml (p<0.01). The AUC IL-6 (table 2) was enhanced in AGHD (151.2 pg/ml *24h, range: 87.0 - 294.3) compared to control subjects (54.5 pg /ml*24h; range: 11.5 - 126.5; p<0.05).

After the oral fat load, the time course of postprandial plasma TNF-α concentration is shown in figure 2. Fasting levels of TNF-_ were not significantly higher in AGHD patients than in control subjects. The postprandial peak time of TNF-α was between 4 and 8 hr in control subjects and at 10 hr in AGHD patients with a maximum level of 4.7 ± 3.4 pg/ml, respectively, 20.4 ± 7.2 pg/ml; p<0.01. The AUC TNF-α (table 2) was enhanced in AGHD (289.9 pg/ml* 24h; range: 87.5 - 617.6) compared to control subjects (118.3 pg/ml *24h, range: 81.2 - 243.1; p<0.05).

After the oral fat load, a decrease in postprandial IL-10 was found in AGHD while a significant increase in postprandial IL-10 was found in the control subjects (figure 2). Fasting levels of IL-10 were higher in AGHD patients (16,7 pg/ml, range: 5.3 - 24.2) than in control subjects (6.1 pg/ml, range: 5.0 - 15.1; p<0.05). The postprandial peak time of IL-10 was at 10 h in control subjects (14.6 ± 8.7 pg/ml) and the lowest level of postprandial IL-10 was reached at 12 h in AGHD patients (10.5 ± 4.8 pg/ml).

Correlations

The integrated area under the IL-6 and RLP-C curves (AUC IL-6 vs AUC RLP-C) were significantly associated in all the subjects together (r^2 = 0.38; p<0.01), and in only the AGHD patients (r^2 = 0.44; p<0.05; Figure 3).Also, the incremental postprandial

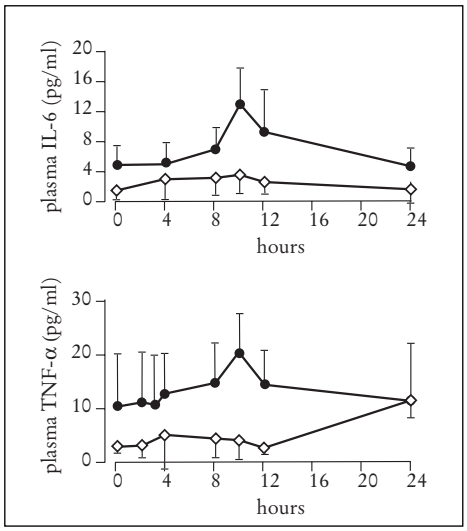

Figure 2. Postprandial plasma cytokine response for the GH deficient patients (▯) and the control subjects (▯). Interleukin-6 (IL-6), TNF-alpha and IL-10. Values are presented as mean (SD).

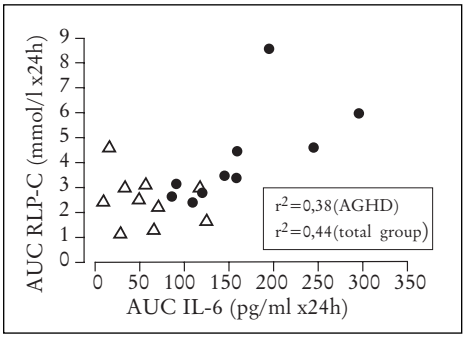

Figure 3. Scatter plot for the relationship between the postprandial plasma RLP-C response (AUC RLP-C) and postprandial IL-6 response (AUC IL-6). GH deficient patients (▯) and control subjects (▯). Correlations were calculated with the use of SPSS as indicated in the method section.

response of IL-6 (dAUC IL-6) and RLP-C (dAUC RLP-C) were positive associated in the GHD patients ($r^2 = 0.24$; $p<0.05$) and all the subjects together ($r^2 = 0.34$; $p<0.01$). No correlations between AUIC TG and AUIC IL6 or AUIC TNF-α could be demonstrated (data not shown).The AUC TNF-α was significantly associated to waist to hip ratio ($r^2 = 0.21$; $p<0.05$) and plasma IGF-1 ($r = -0,57$; $p<0.05$) in all subjects together and in AGHD patients (for both waist to hip ratio and plasma IGF-1 $r^2 = 0.30$; $p<0.05$). Baseline plasma IL-6 or IL-10 levels were not associated with baseline IGF-1 levels, waist to hip ratio or RLP-C levels.

Discussion

In this report, we found evidence that the presence of atherogenic lipoproteins in the postprandial period in GH deficiency may be responsible for the induction of an inflammatory response. Recent observations stress the importance of a pro-inflammatory profile in the development of progressive atherosclerosis (31), but the origin of inflammation is still under debate. These observations suggest that the postprandial period in GH deficiency may be an atherogenic interval that needs a supplemental approach in treatment. We realize that deficiencies in other pituitary hormones, such as TSH and ACTH, may have an effect on the postprandial inflammatory response. However, currently no support is available for such an influence in human, in contrast to reported effects in animal models and *in-vitro* cell culture systems. Moreover, the patients were adequately substituted for all end-organ hormone deficiencies. Substitution with end-organ hormones, albeit adequate, does not mimic completely the diurnal variations of these hormones.

In previous studies, atherosclerotic disease in GH deficiency present as endothelial dysfunction (post-ischemic dilation of brachial artery) or increased intima media thickness (IMT) of femoral or carotid artery (32). During rhGH treatment, initial increased IMT decreased with a marked progress. Parallel results in IMT decrease were estimated to be equal to 3 to 4 years' aggressive lipid lowering treatment in dyslipidemic patients (33-35). Therefore, it is of general importance to get more insight into the pathways that give rise to this accelerated atherogenesis and that could be reversed so adequately by GH therapy.

Negative influence by the GH deficient state on metabolic pathways (such as the lipoprotein remnant) with a high atherogenetic potential is an example of an indirect implication in atherosclerosis. In line with earlier observations, elevated postprandial lipoprotein remnant levels are counteracted by rhGH therapy with additionally an improvement in flow mediated dilation (FMD) of the brachial artery after 6 months' therapy. Furthermore, IMT of carotid arteries and the angiographically verified progression of focal coronary atherosclerosis were positively associated with the plasma RLP-C levels, even independently from plasma LDL-cholesterol and TG levels (36; 37). In a statin-fibrate cross-over study in healthy subjects, fluctuations of postprandial levels of plasma lipoprotein remnants are closely related to post-ischemic changes in diameter of the brachial artery, a marker for early atherosclerosis (38). Additionally, in a model with rat aortic rings, incubation with RLP-C results in endothelial dysfunction that was mediated through the NF-κB upregulation, resulting in an enhanced endothelial response (39-41). The intracellular NF-κB pathway is part of the inflammatory response. Moreover, incubation of beta-VLDL with endothelial cells indeed increases the expression of endothelial TNF-α. This observation is in line with the presence of elevated fasting plasma RLP-C levels in AGHD, and increased fasting plasma levels of TNF-α and IL-6. Moreover, in the present study, we show in humans, that plasma levels of pro-inflammatory cytokines (such as IL-6 and TNF-α) are increased during the postprandial period and

are related to the presence of elevated levels of lipoprotein remnants. The plasma levels of these inflammatory cytokines are a result of spilling into circulation. Endothelial cells and monocyte/macrophages are secretors of cytokines, whereas TG-rich lipoproteins, of which lipoprotein remnants are a subset from, are able to induce an inflammatory response in endothelial cells and macrophages through specific receptors on their surface (42; 43). The postprandial response of RLP-C was closely associated with the postprandial IL-6 response, which suggests that lipoprotein remnants may induce an inflammatory response. Inflammation is a key feature in atherogenesis (44). In several clinical studies, strong correlations between mortality from coronary artery disease (CAD) with other inflammatory markers, such as fibrinogen and C-reactive protein (CRP), was found (45; 46). Moreover, TNF induce apoptosis of endothelial cells, blockade of TNF-α accelerates functional endothelial recovery after balloon angioplasty and plasma TNF-α levels are associated with the carotid IMT in humans (47-49). As a consequence, three meals a day, as occurs in Western diet, result in several postprandial inflammatory responses during the day. The inflammatory response consists of a pro-inflammatory and a anti-inflammatory pathway that are both fine tuned (50). An exaggerated pro-inflammatory response is also found in animal models and in patients with a defect in the anti-inflammatory response, for example a deficiency in the IL-10 secretion. In rat models with IL-10 deficiency, the area of the atheromatous plaque is more extensive and more suspicious to rupture and restoration of plasma IL-10 levels decreased the atherosclerosis (51; 52). In the present study, baseline plasma IL-10 levels were elevated in AGHD patients as compared to controls, revealing an induction of anti-inflammatory factors during an ongoing inflammatory condition in AGHD. This explained the observation that the postprandial IL-10 response in the patients is absent. Currently we are conducting in vitro experiments to elucidate further these observations.

In conclusion, we observed a pronounced postprandial inflammatory response in GH deficiency in relation to the presence of elevated plasma levels of postprandial RLP-C. This observation offer an additional approach for studying the importance of increased susceptibility of premature atherosclerosis in AGHD patients.

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1.9
Elevated remnant-like particles in heterozygous familial hypercholesterolemia and response to statin therapy

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Remnant lipoproteins (RLP-C) are considered important in atherogenesis. Hence, this study was designed to assess RLP-C levels and the effect of statin therapy in patients with familial hypercholesterolemia (FH). Elevated RLP-C levels have been associated with the presence and progression of atherosclerotic disease and their presence in FH patients has been proposed but never established in a large cohort, or their response to statin therapy. FH patients were recruited from 36 Lipid Clinics. After a washout period of 6 weeks, all patients were started on monotherapy with 80 mg simvastatin for two years. RLP-C levels were assessed by an immune-separation assay. In 327 FH patients RLP-C measurements could be performed before and after treatment. Mean total (10.55 ± 2.17 mmol/L), mean LDL cholesterol (8.40 ± 2.13 mmol/L) and median RLP-C (0.47 mmol/L) levels were all severely elevated at baseline. After treatment, RLP-C levels were reduced by 49% (0.24 mmol/L; p<0.0001). Even patients with normal TG levels had elevated RLP-C levels at baseline and those with high RLP-C levels were generally characterized by a very atherogenic lipoprotein profile.Baseline RLP-C levels are severely elevated in FH patients, are reduced by simvastatin, but do not return to normal. These elevated RLP-C levels could be the consequence of impaired function of the LDL-receptor in FH. RLP-C levels in FH contribute to an atherogenic lipoprotein profile and could identify patients who require additional treatment.

LDL-cholesterol and atherogenesis

Familial Hypercholesterolemia (FH) is an autosomal dominant disorder of lipoprotein metabolism (1) Mutations in the LDL-receptor gene are the cause of this disease and lead to a reduction in the clearance of LDL-cholesterol (LDL-C), which consequently causes a rise in LDL-C levels and predisposes to the development of atherosclerosis (2) Therefore, FH patients are at great risk of developing premature coronary artery disease (CAD). However, there is a wide variation of coronary risk among FH patients (3)

Remnant lipoproteins and atherogenesis

Increasing experimental and clinical evidence suggests that triglyceride-rich lipoproteins (TRL) and in particular remnant-like particles (RLP) contribute to atherogenesis and consequently to cardiovascular disease progression. High levels of remnant lipoproteins of both hepatic (very-low-density lipoproteins (VLDL)) and intestinal (chylomicron) origin are associated with the progression of coronary atherosclerosis.(4-7) In a study of Phillips et al, it was found that neither LDL-C nor triglyceride (TG) levels correlate well with lesion progression or clinical events.(7) TRL remnant levels, however, did correlate with both lesion progression and cardiac events. Recently, Nakajima et al developed a simple technique to analyze remnant-like particle cholesterol (RLP-C) using an immune-affinity gel containing anti-apolipoprotein (apo) A-I and anti-apoB100 monoclonal antibodies.(8-10) This unique anti-apoB100 monoclonal antibody was shown to recognize apoB100 in LDL and most VLDL but not in apoE-enriched VLDL, whereas anti-apoA-I recognizes and binds all HDL particles. This technique isolates apoE-rich VLDL particles containing apoB100 together with chylomicron remnants containing apoB48, neither of which binds to the immunoaffinity gel. Increased levels of these remnant particles have already been associated with the presence and progression of cardiovascular disease (CVD) and with endothelial dysfunction.(11-18) However, it should be stressed that these previous results are examined in non-FH patients. Evidently, these patients have a different lipid profile compared to the extreme-

ly elevated LDL-C levels seen in FH patients. Therefore, this association between elevated levels of RLP-C and CVD cannot be extrapolated to FH patients as such.
Levels of remnant particles could be relevant for the understanding of the heterogeneity in coronary risk among FH patients. RLP-C levels have so far not been assessed in a large FH population. Up to now only two small studies (in 15 and 7 FH patients, respectively) reported RLP-C levels of FH patients and did indeed show increased RLP-C levels.(19;20) In the current study we aimed to assess accumulation of RLP-C and also to evaluate whether RLP-C levels will be lowered by statin therapy in a large and well-defined cohort of FH patients.

Methods

Subjects
The present study is a substudy of the ExPRESS FH (Examination of Probands and Relatives in Statin Studies with Familial Hypercholesterolemia) study, in which efficacy, safety and pharmacogenomics of simvastatin 80 mg was assessed in 526 heterozygous FH patients. For this open label multicenter study FH patients were recruited from 36 Lipid Clinics in the Netherlands. Patients were included if they met the following criteria: all patients had to have either a molecular diagnosis for FH or were diagnosed with definite FH and had to have 6 or more points, according to an algorithm (to allow standardization of the diagnosis of FH based on clinical findings, personal and familial clinical history and biochemical parameters)(21); at least 18 years of age; and patients with a history of myocardial infarction (MI), coronary artery bypass graft (CABG) or percutaneous transluminal coronary angiography (PTCA) could be included if the physician thought it was medically allowed for the patient to have a washout period. Patients were excluded if they: were pregnant or nursing women, or premenopausal women not using adequate contraceptives; had acute liver disease, hepatic dysfunction, or persistent elevations of serum transaminases; had hypersensitivity or intolerance to simvastatin or any of its components; had hyperlipidemia Type I, III, IV or V or homozygous FH; had a recent history of alcohol or drug abuse; had secondary hypercholesterolemia due to any cause; had inadequately controlled diabetes, unstable angina or intermediate coronary syndrome or clinically significant ventricular arrhythmia at study entry or MI within the past 3 months; were on concurrent use of erythromycin and similar drugs affecting the cytochrome P450 enzyme; had a history of cancer.

Controls
Controls for the 327 FH patients, in whom RLP-C levels were measured, were recruited post-hoc from their families and matched for age and sex. In these 77 individuals we obtained demographic characteristics, lipids and lipoproteins.

Study design
After a washout period of six weeks, patients were started on monotherapy with simvastatin 80 mg. No other lipid lowering medication was allowed. Medical history, physical examination and additional risk factors for cardiovascular disease as well as laboratory analysis of lipid and lipoprotein levels and routine safety parameters were obtained in all patients. The biochemical analyses of lipid levels and safety parameters were performed in the hospitals themselves and were standardized by a virtual central laboratory. The apolipoprotein determinations were performed in the Academic Medical Center in Amsterdam and the RLP determinations in the University Medical Center in Utrecht. The Ethics Committees of all the 36 centers approved the protocol and written informed consent was obtained from all participants.

Biochemical analysis
Blood samples were taken in the morning after an overnight fast. Total cholesterol (TC), HDL-cholesterol (HDL-C) and

triglycerides (TG) were routinely determined in the different laboratories and standardized by a virtual central laboratory. LDL-C was calculated using the Friedewald formula.(22) Apolipoprotein A-I (apoA-I) and apolipoprotein B (apoB) were determined by an immunological rate-nephelometric procedure using a polyclonal goat anti-human antibody (Array protein system, Beckman Coulter, Netherlands).(23)

The RLP fraction was prepared by use of an immune-separation technique described by Campos, Nakajima and colleagues.(24;25) Briefly, 5 μL of serum was added to 300 μL of mixed immunoaffinity gel suspension containing monoclonal anti-human apoA-I (H-12) and anti-human apoB100 (JI-H) antibodies (Japan, Immunoresearch Laboratories, Takasaki, Japan). The reaction mixture was gently shaken for 120 minutes at room temperature. After the supernatant was left standing for 15 minutes, 200 μL was withdrawn for the assay of RLP-C. Cholesterol in the RLP fraction (coefficient of variation <3%) was measured by an enzymatic assay on a Cobas Mira S auto analyzer (ABX Diagnostics, Montpellier, France). Apo E genotyping was performed as described by Reymer et al.(24)

Statistical analysis

Data were expressed as mean ± standard deviation. Skewed data distributions were presented as median and the interquartile range. Mean and median cholesterol levels in FH patients compared to controls were tested by the independent sample t-test and the Mann-Whitney test, respectively. Mean values in lipids, lipoproteins before and after treatment were compared using the paired sample t-test. TG and RLP-C were compared by the non-parametric Wilcoxon test, because they had a skewed distribution. Mean values in baseline characteristics and lipids for the different groups were compared using the one-way ANOVA test. Parameters with a skewed distribution (TG, RLP-C and RLP reduction) were compared using the Kruskal-Wallis test. Chi-square tests were applied for comparing distributions of dichotomous data (gender, diabetes and apoE2). All statistical analyses were performed using the SPSS package (version 10.1, Chicago; Illinois). A p-value of less than 0.05 was considered to be statistically significant.

Results

From the 526 patients, participating in the ExPRESS FH study blood samples were stored in 327 patients. Consequently, these patients were available for RLP-C analyses and comprised our study group. This group did not differ from the source population with regard to baseline characteristics and lipid parameters. Ages ranged from 18 to 80 years. Mean age was 47.4 years (standard deviation (SD) ± 13.2). Slightly more males (54%) than females (46%) were included. Mean body mass index (BMI) was 25.8 kg/m^2 (SD ± 3.6). Lifestyle and physical characteristics such as dietary adherence, BMI, alcohol intake and physical exercise did not change over the course of the trial.

Mean lipid, lipoprotein and RLP-C levels at baseline and one year after therapy with simvastatin 80 mg are shown in table 1. As expected, mean TC (10.55 ± 2.17 mmol/L) levels were severely elevated in FH patients compared to their family controls (4.58 ± 0.77 mmol/L; $p<0.0001$) and this could largely be attributed to elevated LDL-C levels (8.40 ± 2.13 mmol/L). Mean HDL-C levels were lower in FH patients compared to the controls (1.25 ± 0.35 mmol/L vs. 1.39 ± 0.38 mmol/L; $p=0.04$), whereas median TG levels were elevated compared to the controls (1.80 mmol/L vs. 0.97 mmol/L; $p<0.0001$). Median RLP-C levels (0.47 mmol/L) were severely elevated in FH patients. Median RLP-C levels in controls were 0.20 mmol/L, which indicates that FH patients have a twofold elevation of RLP-C levels ($p<0.0001$). After simvastatin treatment, plasma cholesterol, LDL-C, TG and apoB decreased, and HDL-C and apoA-I increased, all significantly. RLP-C levels

decreased significantly from a median of 0.47 mmol/L to 0.24 mmol/L ($p<0.0001$), which is consistent with a 49 % median reduction.

At baseline, only 5 FH patients (1.5%) had normal RLP-C levels (≤ 0.20 mmol/L); in contrast after simvastatin therapy 84 patients

Table 1: Lipid, Lipoprotein and RLP-C Levels in controls and in FH patients at baseline and after one year of therapy

Variable	Baseline (n = 327)	Simvastatin 80 mg (n = 327)	p-value
TC (mmol/L)	10.55 ± 2.17	6.36 ± 1.37	<0.0001
LDL-C (mmol/L)	8.40 ± 2.13	4.32 ± 1.30	<0.0001
HDL-C (mmol/L)	1.25 ± 0.35	1.39 ± 0.39	<0.0001
TG (mmol/L)	1.80 (1.20-2.40)	1.20 (0.90-1.73)	<0.0001
ApoA-I (g/L)	1.24 ± 0.22	1.36 ± 0.24	<0.0001
ApoB (g/L)	1.97 ± 0.45	1.19 ± 0.31	<0.0001
RLP-C (mmol/L)	0.47 (0.34-0.80)	0.24 (0.20-0.31)	<0.0001

RLP-C, remnant-like particle cholesterol; FH, familial hypercholesterolemia; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; Apo, apolipoprotein.
All values are given as mean with SD except that TG and RLP-C are given as median with interquartile range.

Table 2: Lipids, RLP, ApoE2 and clinical features in FH patients with baseline RLP-C levels divided in 3 equal groups (all in mmol/l)

Variable	RLP-C <0.39 n = 109	0.3≤ RLP-C <0.65 n = 109	RLP-C ≥ 0.65 n = 109	p-value
Age (years)	44.0 ± 14.2	46.6 ± 13.2	51.5 ± 11.0	<0.0001
Male gender (N)	41 (45.0%)	47 (51.4%)	60 (65.1%)	0.009
BMI (kg/m^2)	24.6 ± 3.7	25.6 ± 3.2	27.3 ± 3.5	<0.0001
Diabetes (N)	0 (0%)	1 (0.9%)	5 (4.6%)	0.029
TC (mmol/L)	9.50 ± 1.70	10.68 ± 2.08	11.47 ± 2.23	<0.0001
LDL-C (mmol/L)	7.58 ± 1.63	8.64 ± 2.13	8.99 ± 2.32	<0.0001
HDL-C (mmol/L)	1.37 ± 0.36	1.29 ± 0.35	1.09 ± 0.27	<0.0001
TG (mmol/L)	1.10 (0.90-1.50)	1.80 (1.30-2.05)	2.80 (2.10-3.60)	<0.0001
ApoA-I (g/L)	1.28 ± 0.21	1.25 ± 0.24	1.18 ± 0.19	0.004
ApoB (g/L)	1.74 ± 0.35	2.00 ± 0.39	2.17 ± 0.49	<0.0001
RLP-C (mmol/L)	0.32 (0.26-0.34)	0.47 (0.43-0.53)	1.06 (0.79-1.62)	<0.0001
RLP reduction (%)	29.0 (14.9-41.0)	50.5 (39.8-57.7)	67.5 (57.2-76.8)	<0.0001
ApoE2 allele (N)	5 (4.6%)	4 (3.7%)	17 (15.6%)	0.001

FH, familial hypercholesterolemia; RLP-C, remnant-like particle cholesterol; BMI, body mass index; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; Apo, apolipoprotein. All values are given as mean with SD or (%), except that TG, RLP-C and RLP-C reduction are given as median with interquartile range.

(25.7%) were below this RLP-C level.
In table 2 baseline RLP-C levels are divided into three equal groups using the 33rd and 66th percentiles. FH patients in the highest third were older, more often male, had a higher BMI and had more often diabetes. To address whether FH patients with type 2 diabetes were outliers in terms of remnant accumulation and caused significant shifts of the medians in the different 3 groups we calculated median RLP-C levels with these individuals included or excluded. However, results indicated no significant changes in the medians of these three groups (data not shown). In addition, the statistically significant increase in total cholesterol, LDL-C and TG levels and the decrease in HDL-C, illustrates the association between plasma RLP-C and the presence of a severe atherogenic lipoprotein profile. RLP-C levels were also correlated to HDL-C levels, the Spearman's rank correlation coefficient was r = -0.37 at $p<0.0001$. In the lowest third of baseline RLP-C, median TG levels were normal (1.10 mmol/L) whereas in that third median RLP-C levels were still above normal (0.32 mmol/L). Moreover, significantly more patients in the highest RLP-C third had an apoE2 allele compared to the two lower thirds ($p=0.001$). Lastly, RLP-C levels were more reduced in the highest third compared to the lower thirds. Since pre- and post treatment measurements on the variable of interest are, in general, not perfectly correlated, the evaluation of treatment effects must be adjusted for regression to the mean. Chen et al. proposed 4 models, which included either or both additive and multiplicative effects.(25) We have applied this model to evaluate RLP-C changes. The model, which included both additive and multiplicative effects fit better than that with only additive effects (regression to the mean) for RLP-C change ($p<0.0001$). Therefore, changes in RLP-C could not be attributed to regression to the mean only, but indeed exhibit a relationship with baseline levels. FH patients in the highest versus the lowest third of RLP-C suffered from CVD in 53 (48.6%) versus 30 (27.5%) of cases ($\chi^2=10.5$, $p=0.005$). However, upon multiple logistic regression analysis with age, gender, BMI and major lipids in the model this relation was no longer statistically significant ($p=0.32$).
In table 3 the data were stratified according to baseline TG levels in quartiles. Patients in the lowest quartile had completely normal median baseline TG levels (0.90 mmol/L), however the corresponding RLP-C levels were already strongly elevated (0.32 mmol/L), whereas plasma LDL-C levels were similar in all groups ($p=0.09$).

Discussion

RLP-C increase at baseline

In this study we observed that median RLP-C levels in FH patients are severely elevated compared to their siblings (0.47 mmol/L vs. 0.20 mmol/L; $p<0.0001$). Elevated RLP-C levels were reported before in FH patients,

Table 3: Lipids and RLP-C Levels in FH patients with baseline TG levels divided in quartiles (all in mmol/L)

	TG≤ 1.10 (n=80)	1.10<TG≤ 1.80 (n=91)	1.80<TG≤ 2.40 (n=76)	TG>2.40 (n=80)	p-value
LDL-C	8.03 ± 1.90	8.81 ± 2.17	8.48 ± 2.30	8.23 ± 2.07	0.09
TG	0.90 (0.80-1.00)	1.50 (1.30-1.70)	2.10 (1.90-2.20)	3.25 (2.73-4.10)	<0.0001
RLP-C	0.32 (0.25-0.41)	0.42 (0.33-0.49)	0.52 (0.42-0.75)	1.16 (0.79-1.80)	<0.0001

RLP-C, remnant-like particle cholesterol; FH, familial hypercholesterolemia; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol. LDL-C is given as mean with SD; TG and RLP-C are given as median with interquartile range.

albeit in very small cohorts. Twickler et al measured RLP-C levels in 7 FH patients and found significantly elevated mean RLP-C levels compared with 7 controls (42.10 mg/dl (1.09 mmol/L) vs. 7.49 mg/dl (0.19 mmol/L); $p<0.01$). Dane-Stewart et al also found elevated median RLP-C levels in 15 FH patients compared with 15 controls (16.2 mg/dl (0.42 mmol/L) vs. 8.5 mg/dl (0.22 mmol/L); $p=0.003$).(19) In our cohort, mean age and BMI increase from the lowest to the highest RLP-C thirds. Why RLP levels rise with age is unknown, but older people have higher BMI 's often combined with higher TG levels. This association points to increased synthesis of VLDL, which could consequently lead to higher RLP levels.(26) We also found a higher prevalence of apoE2 allele carriers in the highest RLP-C third. ApoE is the major ligand for hepatic lipoprotein receptors and mediates chylomicron and VLDL remnant uptake.(27) ApoE2 is one of the three E isoforms and exhibits a very low affinity to the LDL-receptor.(28) It is therefore not unexpected to find more apoE2 alleles in the highest third of RLP-C levels. The apoE allele distribution was in Hardy Weinberg equilibrium ($\chi^2 = 3.59$, $p>0.1$) in our FH sample but the frequency of the (2 allele was lower compared to the general Dutch population as previously reported (0.047 and 0.082, respectively).(29) It is therefore unlikely that an overrepresentation of the $\varepsilon 2$ allele of the apoE gene will have contributed to our findings.

These elevated RLP-C levels might play an important role, in addition to elevated LDL-C levels, in the acceleration of atherosclerosis in FH. This idea is supported by the findings of Karpe et al who did not find an association between LDL-C and new lesions in vein grafts in 395 patients with coronary artery disease (CAD), but did find a strong trend for higher RLP-C concentrations.(17)

RLP-C reduction after statin intervention

RLP-C levels are significantly decreased by simvastatin. Median RLP-C levels almost returned to normal, but remained slightly elevated. This observation was previously reported in 7 FH patients.(20) Recently, two studies in patients with combined dyslipidemia showed similar results.(30;31) Stein et al found that median elevated RLP-C levels of 0.34 mmol/L were reduced by simvastatin 20 mg (6.0%) and by atorvastatin 10 mg (25.9%), but not by pravastatin 40 mg in 22 patients in a crossover study.(30) In addition, Sasaki et al found that atorvastatin 10 to 20 mg reduced RLP-C levels from 0.31 mmol/L to 0.16 mmol/L.(31) These studies also illustrate a treatment effect of statins on the RLP-C fraction.

Accumulation as the proposed mechanism

The central abnormality in heterozygous FH is an impaired function of the LDL receptor. As a consequence, plasma levels of LDL-C are severely elevated, and levels of RLP-C as well. Moreover, these elevated RLP-C levels are significantly reduced by simvastatin treatment. Statins likely improve RLP clearance by upregulating LDL-receptors and by decreasing hepatic VLDL synthesis.(32;33) Both mechanisms lead to less competition for the clearance mechanisms shared by chylomicrons and VLDL.(34) The reductions in apoB and LDL-C were very consistent with those of RLP-C, as is anticipated since by upregulating the LDL (apoB, E) receptor all apoB (LDL) as well as apoE (RLP-C) containing lipoproteins will be reduced in essentially similar proportions.

The raised TG levels in the higher RLP-C thirds support the accumulation of TG-rich lipoproteins. The conclusion could therefore be drawn that TG levels measured in FH patients primarily reflect atherogenic remnant lipoproteins. However, even patients with normal TG levels had elevated RLP-C levels. This observation was also made previously.(20) All these data indicate the presence of remnant particle accumulation in FH patients, irrespective of concomitant TG elevation. Likewise, stratification of the data according to baseline TG into quartiles resulted in an association between TG and RLP-C levels, but LDL-C levels were equally

elevated in all quartiles. This suggests that despite the fact that plasma LDL-C levels are strongly elevated in FH patients, RLP-C levels could further contribute to the atherogenic lipoprotein profile over and above LDL-C measurements. We, therefore, hypothesize, that remnant accumulation in FH might be explained by a combination of factors such as the LDL-receptor mutation, VLDL production associated with advancing age, central obesity and glucose intolerance, carriership of an $\varepsilon 2$ allele of the apo E gene and possibly by defective LDL-receptor related protein function. These findings may raise the need to prescribe combination therapy with simvastatin 80 mg and either nicotinic acid or fenofibrate or with more powerful statins to lower the risk associated with the residual remnant increase.

In conclusion, baseline RLP-C levels are severely elevated in FH patients. Treatment with high dose simvastatin resulted in a strong reduction of RLP-C, but in the majority of the patients RLP-C levels remained elevated. RLP-C levels in FH contribute to an atherogenic lipoprotein profile and could identify patients who require additional treatment.

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1.10
High dose of Simvastatin normalizes post-prandial remnant-like particle response in patients with heterozygous Familial Hypercholesterolemia

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Both Familial Hypercholesterolemia (FH) and disturbances in postprandial lipoprotein metabolism are both associated with premature atherosclerosis. The effect of HMG-CoA reductase inhibitors on plasma cholesterol levels in patients with Familial Hypercholesterolemia is well established, however, it is not known whether postprandial lipoproteins are also influenced. In this case-controlled intervention study, we investigated the effects of high dose Simvastatin on postprandial lipoproteins. We used a new method to analyze remnant lipoproteins based on immunoseparation principle (RLP-Cholesterol assay), as well as the well established measurement of retinyl ester (RE) analysis in plasma and in the Sf<1000 fraction. Seven heterozygous FH patients and seven controls matched for gender, age, BMI, TG and apo E genotype were enrolled in the study. Oral vitamin A (retinyl ester, RE) fat loading test was performed at baseline in both groups and after 3 month of high dose Simvastatin (80 mg/day) treatment in the FH patients. Before treatment, FH patients had significantly higher fasting and postprandial concentrations of lipoprotein remnants (plasma RLP-C: 42 ± 19 mg/dL and AUC-RLPC: 415 ± 82 mg. $L^{-1}.h^{-1}$, respectively) than in controls (7 ± 3 mg/dL and 101 ± 35 mg. $L^{-1}.h^{-1}$; respectively, $p<0.05$), suggesting a delayed clearance of chylomicron remnant particles in the FH patients. Treatment with Simvastatin significantly reduced both fasting and postprandial remnant lipoprotein cholesterol concentrations (13 ± 3 mg/dL and 136 ± 53 mg. $L^{-1}.h^{-1}$; respectively, $p<0.05$ for both). Postprandial RE in the Sf<1000 fraction, not total RE in plasma, were also significantly higher in FH patients than in controls (24 ± 10 mg. $L^{-1}.h^{-1}$ vs. 6.3 ± 5.9 mg. $L^{-1}.h^{-1}$ $P<0.05$), but treatment with Simvastatin did not result in improvement of the postprandial RE response, either in the Sf<1000 fraction or in plasma. It is concluded that heterozygous FH patients have increased fasting and postprandial remnant lipoprotein concentrations. Treatment with Simvastin significantly reduced the fasting and postprandial remnant-like particle cholesterol concentrations but did not result in improved postprandial RE response.

Growing evidence exists that a disturbed plasma triglyceride (TG) metabolism plays an important role in the development of premature atherosclerosis (1; 2). Disturbances in triglyceride metabolism are characterized by postprandial accumulation of lipoprotein remnants and were observed in various populations with elevated cardiovascular risk (3-7). Patients with Familial Hypercholesterolemia (FH) and disturbances in postprandial lipoprotein metabolism have higher risks for coronary artery disease (8). Animal studies have shown that a deficiency in the LDL receptor (LDL-R) is associated with a delayed chylomicron remnant removal (9-11). Castro Cabezas et al (12) have demonstrated a small but significant postprandial increase in retinyl palmitate concentration in the chylomicron remnant fraction (Sf<1000) in heterozygous FH patients compared to normolipidemic control subjects. However Rubinsztein et al (13) did not observe any accumulation of vitamin A labeled chylomicrons, IDL-sized remnants or chylomicron remnants in homozygous FH patients. Additionally Kowal et al.(14) reported that only 5% of the binding capacity of the LDL receptor was needed to remove chylomicron particles, suggesting only a minor role for the LDL receptor in the removal process of lipoprotein remnants.

Several laborious methods, i.e. incorporation of vitamin A as core label and HPLC analyses of retinyl esters (RE) in isolated lipoprotein fractions or measurements of apo B48

and apo B100 concentration in the different lipoprotein fractions using SDS-PAGE, have been used to study postprandial lipoprotein remnant metabolism. The suitability of vitamin A as a marker for chylomicrons and its remnants has been criticized (15; 16). Incorporation of vitamin A by the enterocyte occurs mostly in the larger sized chylomicron particles in the late postprandial period (17) as reflected by the delayed postprandial RE response compared with the Apo-B48 in the VLDL/chylomicron density fraction. A new remnant lipoprotein method based on immunoseparation principle (RLP-Cholesterol assay) offers the possibility to separate lipoprotein remnant particles using an immunoaffinity gel with coupled monoclonal antibodies against Apo B100 and Apo AI (18; 19). In the unbound fraction cholesterol and triglyceride concentrations in apo B48 particles (chylomicron-remnants) and apo E enriched apoB100 (β-VLDL and IDL) particles are detected (20).

HMG-CoA reductase inhibitors (statins) upregulate the LDL receptor and partly inhibit hepatic apoB secretion (21) resulting in an increased removal of LDL particles from the circulation thereby improving the atherogenic lipid profile. Their effects on postprandial remnant metabolism have not been completely established. Most studies showed a tendency to decrease postprandial triglyceride concentrations that appear to correlate with the degree of fasting plasma triglyceride reduction (22). Therefore we investigated postprandial lipoprotein remnant metabolism in heterozygous FH patients and the effect of Simvastatin treatment using RE analysis and the RLP-C assay for remnant characterization.

Methods

Subjects

This study was a substudy from the Express multi-center study in which the safety and efficacy was assessed of a high dose Simvastatin (80 mg once a day) in heterozygous patients with Familial Hypercholesterolemia (FH). Patients were recruited from the lipid clinic of the University Hospital Utrecht. Diagnosis of heterozygous FH was made on the basis of existing hypercholesterolemia (> 6.50 mmol/L), presence of tendon xanthomas and hypercholesterolemia in at least one first-degree relative (23). These patients were asked to stop all lipid lowering drugs at least 8 weeks before the study (washout period). At the end of the washout period, patients were given intravenous injection of heparin and then blood samples were collected for lipoprotein lipase (LPL) and hepatic lipase (HL) activity measurements. On a separate day, patients were given an oral fat loading test followed by a 4-month treatment with Simvastatin in a dose of 80 mg a day. Oral fat loading test was repeated at the end of the treatment period. Healthy, normolipidemic subjects with fasting plasma cholesterol < 6.0 mmol/L and TG < 2.0 mmol/L were recruited by advertisement to match the FH patients in their age, gender, BMI, apoE genotype and fasting plasma TG concentration. Exclusion criteria included diabetes, hepatic, renal or thyroid diseases and a positive family history for cardiovascular diseases and type 2 diabetes mellitus. Control subjects also had an oral fat loading test. The human investigation review committee from University Hospital Utrecht and a national committee representing the Express multicenter study approved this study protocol and written informed consent was obtained from all participants.

Oral fat loading test

After an overnight fast (12 hrs), participants were admitted to the metabolic ward at 7.30 h am. Cream (consisting of 40% fat (w/v) with a P/S ratio of 0.06, 0.001% cholesterol (w/v) and 2.8% carbohydrates (w/v)) was given as a single fat load in a dose of 50 g fat per m^2 body surface area. After ingestion of the cream supplemented with 120. 000 IU aqueous vitamin A, 10 hourly venous blood samples were collected from an indwelling catheter in the antecubital vein into EDTA containing tubes. All blood samples, protect-

ed from light, were immediately put on ice, centrifuged and analyzed. During the postprandial period, the subjects were allowed to drink only water or tea without sugar. None of the subjects experienced gastrointestinal complaints after drinking the cream.

Laboratory measurements

Plasma was obtained by centrifugation at 3000 rpm for 15 min at 4°C. Plasma TG and cholesterol were analyzed in duplicate and measured with an enzymatic colorimetric assay (Monotest cholesterol kit no. 237574 and GPO-PAP no. 701912, Boehringer Mannheim, Germany). LDL cholesterol was calculated with the Friedewald formula (24) in the control subjects and determined with ultracentrifugation as described by Redgrave et al (25) in FH patients. Cholesterol was analyzed in the HDL fraction isolated by the heparin-$MnCl_2$ dextran-sulphate precipitation method (26). Plasma apo E concentrations (mg/L) and plasma apo CIII concentrations (mg/L) were determined with a commercial test kit using the electroimmunodiffusion technique (CV < 7.5%) (Hydragel LP E Ref. 4058 and LP CIII, Sebia Inc. USA). Apo E genotype was determined as described by Dallinga-Thie et al (27). Plasma for LPL and HL was obtained 20 minutes after intravenous injection of 50 IU/kg of heparin. Postheparin Lipoprotein lipase activity and hepatic lipase activity were assayed as described previously (28; 29). Non-esterified fatty acids (expressed as nmol free fatty acids (FFA) min^{-1} (mU) per mL) were measured with an enzymatic assay (WAKO chemicals, Neuss, Germany).

Assessment of lipoprotein remnants

Lipoproteins were separated in a single ultracentrifugation step by flotation in a Sf>1000 fraction which contains chylomicrons, large chylomicron remnants and large hepatic triglyceride rich lipoproteins, and a remaining infranatant fraction (Sf<1000) containing small chylomicron remnants and all the other lipoproteins (30; 31). In both fractions retinyl-ester concentrations were determined using high-performance liquid chromatography (HPLC) as described by Ruotolo et al (32).

The RLP fraction was prepared using an immunoseparation technique described by Nakajima et al (18; 19). Briefly, 5 μl of serum was added to 300 μl of mixed immunoaffinity gel suspension containing monoclonal anti-human apo A-I (H-12) and anti-human apo B100 (JI-H) antibodies (Japan Immunoresearch Laboratories, Takasaki, Japan). The reaction mixture was gently shaken for 120 minutes at room temperature. After standing for 15 minutes 200 μl of the supernatant was withdrawn for the assay of RLP-C. Cholesterol in the RLP fraction (CV% <3) was measured by an enzymatic assay using an automatic chemistry analyzer (Cobas Mira autoanalyzer, ABX, USA).

Statistical Analysis

Data are presented as means ± SD, unless stated otherwise. Area under the integrated curve was calculated using data from the first 8 hours after start of the oral fat loading test for postprandial TG, RE and RLP-C using GraphPad Prism software (version 3.1, San Diego, California, USA). Normality was tested with the Kolmogorov-Smirnov test. If non-Normality occurred, data were normalized by log-transformation. The effects of Simvastatin treatment was tested by a paired Student's t-test. Comparisons between FH patients and controls were tested by two-tailed unpaired Student's t-test. Pearson's correlation or Spearman's rank correlations were applied to evaluate relationships between parameters. A two-sided p-value < 0.05 was considered to be significant. Statistical analysis was performed with Graphpad InStat version 3.00 for Windows 95, Graphpad Software, San Diego, California, USA.

Results

Study Population

Characteristics of the subjects were summarized in Table 1. Both FH patients and con-

trol subjects were normotriglyceridemic. FH patients have significantly increased fasting plasma cholesterol, LDL cholesterol, apo B and apo E concentrations and decreased HDL cholesterol concentrations compared to control subjects. After Simvastatin treatment plasma cholesterol, LDL cholesterol and apoB concentrations significantly decreased, whereas plasma apo E levels remained significantly elevated. Due to subject selection criteria, fasting TG concentrations did not differ significantly between the FH and control groups. No significant differences in postheparin LPL and HL activities were found in FH patients and control subjects. Simvastatin treatment did not change the LPL and HL activities.

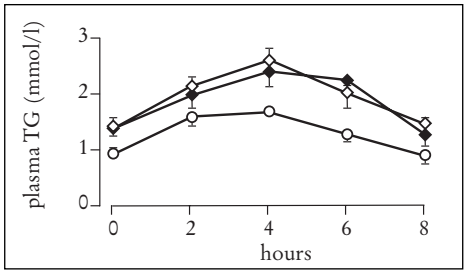

Figure 1. postprandial TG responses in patients with FH without Simvastatin (†), with Simvastatin (∪) and the matched control subjects (m). Values are expressed as mean ± SEM.

Postprandial responses

Postprandial TG responses

After the fat load, maximal postprandial plasma TG concentrations were reached at 4 hours and were higher in FH patients than in matched control subjects, 2.61 * 0.50 mmol/L vs. 1.66 * 0.53 mmol/L (P=0.02). (Figure 1). Area under the TG curve (AUC-TG) was also significantly higher in FH patients, after correction for baseline plasma TG concentrations the ΔAUC-TG in FH patients was not significantly different from that in control subjects (Table 2). Simvastatin treatment did not result in improvement of postprandial plasma TG concentration, AUC-TG and ΔAUC-TG.

Postprandial RLP-C responses

Fasting plasma RLP-cholesterol concentrations (RLP-C) were significantly higher in FH patients than in control subjects (P<0.05, Figure 2). After Simvastatin treat-

Table 1: Characteristics of patients with Familial Hypercholesterolemia and control subjects.

	FH Simva (-)	FH Simva (+)	Controls
n	7	7	7
Male	4	4	4
Age (y)	46.7 (6.9)	-	47.7 (6.9)
BMI	25.5 (1.5)	25.8 (1.7)	25.23 (1.63)
Cholesterol (mmol/L)	12.12 (1.86)$^{\#\#\ \$\$}$	6.34 (1.11)$^{\#}$	5.11 (0.640
TG (mmol/L)	1.39 (0.4)	1.36 (0.4)	0.91 (0.3)
HDL-cholesterol (mmol/L)	0.94 (0.22)$^{\#\ \$\$}$	1.03 (0.32)	1.51 (0.47)
LDL-cholesterol (mmol/L)	10.28 (1.6)$^{\#\#\ \$\$}$	4.78 (0.91)$^{\#\#}$	3.18 (0.64)
Apo B (mg/L)	210 (21) $^{\#\#\ \$\$}$	140 (15)$^{\#\#}$	92 (14)
Apo CIII (mg/L)	25.82 (7.56)	21.36 (7.44)	27.44 (9.88)
Apo E (mg/L)	62.29 (8.88)$^{\#}$	60.30 (12.63)$^{\#}$	43.48 (10.10)
LPL activity (mU/mL)	171 (50)	164 (48)	158 (33)
HL activity (mU/mL)	543 (269)	398 (146)	343 (174)

All values are expressed as mean ± SD. Simva (-) indicates without Simvastatin treatment and Simva(+) indicates with Simvastatin treatment. FH vs controls: $^{\#}$ P<0.05 and $^{\#\#}$ P<0.01; treated vs. untreated: $^{\$}$ P<0.05 and $^{\$\$}$ P<0.01.

ment fasting plasma RLP-C concentrations normalized and were similar to control subjects. Fasting RLP-C correlated positively with baseline plasma cholesterol (r=0.80; p<0.01), TG (r=0.52; p<0.05), LDL-cholesterol (r=0.79; p<0.01), apo B (r=0.84; p<0.01) and apo E concentrations (r=0.52; p<0.05). The maximal postprandial plasma RLP-C concentration was reached between 2 and 4 hours and was significantly higher than in control subjects (72.15 * 8.77 mmol/L vs. 18.00 * 2.63 mmol/L; P=0.004). So were the

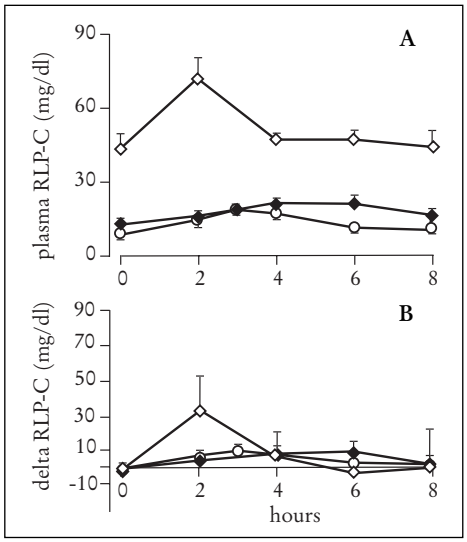

Figure 2. Postprandial remnant-like particle (RLP) responses in FH patients without Simvastatin (†), with Simvastatin (u) and the matched control subjects (m). Total plasma RLP-cholesterol (panel A) and the incremental plasma RLP-C (panel B) are presented. Values are expressed as mean ± SEM.

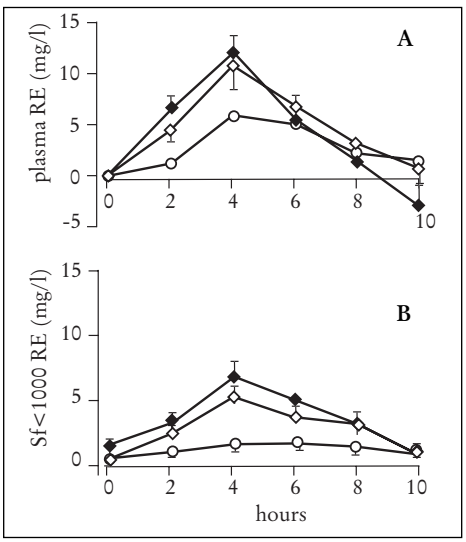

Figure 3. Postprandial retinyl esters (RE) response of patients with FH without Simvastatin (†) and with Simvastatin (u) and the matched controls (m). The total plasma RE (Panel A)and the Sf<1000 RE (panel B) are presented. Data are expressed as mean ± SEM.

Table 2: Postprandial data for TG, retinyl esters and RLP-C

	FH Simva (-)	FH Simva (+)	Controls
Fasting plasma TG	1.39 (0.4)	1.36 (0.4)	0.91 (0.3)
AUC-TG 0-8h	16.4 (2.6)##	15.7 (4.8)	10.06 (3.3)
DAUC-TG 0-8h	5.35 (1.6)	4.96 (2.2)	3.62 (1.4)
Fasting RLP-C	42.10 (19.2)##	12.48 (10.8)$	7.49 (7.8)
AUC-RLPC	414.82 (81.7)##	135.64 (52.9)$$	101.22 (35.2)
DAUC-RLPC	120.77 (67.2)#	40.83 (29.1)$	42.21 (22.2)
RE-AUC	51.35 (25.9)	50.48 (21.0)	28.00 (21.8)
Sf>1000 RE-AUC	19.07 (12.0)	27.60 (10.8)	17.29 (7.8)
Sf<1000 RE-AUC	24.05 (9.5)#	22.65 (11.4)#	6.34 (5.9)

All values are expressed as mean ± SD. Simva (-) indicates without Simvastatin treatment and Simva(+) indicates with Simvastatin treatment. FH vs controls: # P<0.05 and ## P<0.01; FH patients treated vs. not treated: $ P<0.05 and $$ P<0.01. † DAUC indicates area under the incremental curve, after correction for baseline concentrations, calculated over the first 8 hours..

area under the RLP-C curve (AUC-RLP-C) and DAUC-RLP-C (Table 2). Simvastatin treatment resulted in a significant decrease in maximum postprandial RLP-C concentration, AUC-RLPC ($P<0.01$) and DAUC-RLP-C ($P<0.05$) in the FH patients.

Postprandial retinyl ester response

Maximal postprandial plasma retinyl ester concentrations were reached at 4 hours and were higher, albeit not statistically significant, in FH patients than in controls (10.97 * 2.22 mmol/L vs. 6.18 * 1.86 mmol/L; (Figure 3A)). There was no statistical difference in area under the retinyl ester curve (AUC-RE) between the FH and control subjects (Table 2). Treatment with simvastatin did not decrease the maximal postprandial plasma retinyl ester concentrations, nor AUC-RE in the FH patients. AUC-RE in the Sf<1000 fraction was correlated with baseline plasma TG ($r=0.61$; $p<0.05$), Apo B ($r=0.49$; $p<0.05$) and Apo E concentrations ($r=0.53$; $p<0.05$). Negative correlation between AUC-RE in the Sf<1000 fraction and plasma HDL cholesterol concentrations was found ($r= -0.54$; $p<0.05$). Unlike total retinyl esters, maximum postprandial plasma retinyl ester concentrations and AUC-RE in the Sf<1000 fraction were significantly higher in FH patients than in control subjects (Figure 3B). However, like total retinyl esters, Simvastatin treatment did not result in improvement of the postprandial retinyl ester in the Sf<1000 fraction.

Discussion

In the present study, specific monoclonal antibodies for apo B100 and for apo AI have been used to isolate remnant like-particles (RLP). We observed higher fasting RLP-C concentrations and an increased postprandial RLP-C response in heterozygous FH patients compared with matched control subjects. Treatment with high dose Simvastatin resulted in a significantly decreased postprandial lipoprotein remnant concentration. The importance of detecting a disturbed postprandial lipoprotein metabolism in FH patients was recently stressed, because these patients had significantly increased risks for coronary artery disease (8; 33-37).

Hitherto, contradictory results about possible abnormalities in postprandial lipoprotein remnant metabolism in FH patients were reported. Different methodologies were used to isolate lipoprotein remnants. Most studies on postprandial lipoprotein remnant metabolism in FH used vitamin A (retinyl esters) as a core label for chylomicron particles. Studies on postprandial lipoprotein remnants with retinyl esters as a marker in homo- or heterzygous FH patients revealed either abnormalities (12; 38) or a normal removal of chylomicron remnants (13; 31). We confirmed an earlier report by Castro Cabezas et al (12) that postprandial chylomicron remnants reflected by retinyl esters in Sf<1000 fraction were elevated in heterozygous FH patients compared to matched control subjects. In the fasting state and the early postprandial period (first three hours after a meal) smaller sized chylomicrons (considered to be atherogenic) are secreted. Later in the postprandial period, *de-novo* formed larger chylomicrons are secreted. It has been shown *In-vivo* that conversion of larger chylomicron particles into smaller sized remnant particles is a phenomenon that is not occurring very frequently (39). Retinyl esters are mostly incorporated in larger-sized chylomicron particles. This could be observed in our study by the delay of retinyl ester appearance in the blood. A similar observations was reported for apo B48 and RE (40). We hypothesize that apo B48 and RLP-C reflects particles with identical behavior, whereas RE marks the properties of intestinal postprandial lipoprotein particles with a different metabolic behavior.

The results suggest that secretion of larger particles continued to be abnormal in FH patients even after Simvastatin treatment. An *in vivo* study in rats (41) supported the concept that larger chylomicron particles were removed by the liver via alternative pathways

involving the LDL receptor related protein (LRP) and proteoglycans. This process was not influenced by LDL receptor modulation (42). Therefore, even after high dose Simvastatin treatment with its positive effects on plasma cholesterol homeostasis, the peripheral pathway through which Sf <1000 retinyl esters were removed was still saturated. In contrast the removal of the smaller postprandial plasma RLP-C levels decreased after high dose Simvastatin therapy.

Our results showed for the first time increased fasting and postprandial RLP-C concentrations in heterozygous FH patients despite normal TG concentrations in these patients. It has been recognized that there is a strong correlation between RLP-C and TG concentrations. Therefore it has been argued that TG measurements are sufficient to estimate remnant concentrations. However, several clinical studies (43; 44) demonstrated that RLP-C offered independent assessment for CHD risk in addition to TG. In the present study we show that RLP-C and TG clearly had different postprandial responses to Simvastatin treatment therefore they are not interchangeable (Figure 1 and 2). Secretion of VLDL apo B100 was increased and hepatic removal of VLDL/IDL particles by the liver was decreased in untreated FH patients. As a result plasma IDL and LDL concentrations are increased in untreated FH. Therefore, the increase in fasting RLP-C levels reflects increasing levels of circulating IDL-like apo B100/apo E remnant particles. Since removal pathways are shared by RLP and IDL, accumulation of IDL could be expected when influx of RLP increased after a fatty meal. Our observed postprandial RLP-C peak is a reflection of this process and is the result of accumulation of apo B48 remnant particles and apo B100 / apo E enriched remnants. The strong association of AUC-RLP-C with baseline plasma apo B is suggestive for this concept. After treatment with Simvastatin, postprandial RLP-C concentrations in FH patients was comparable to that observed in control subjects. As a result of Simvastatin intervention, hepatic secretion of precursor lipoproteins, that eventually will become IDL/LDL-density-like particles, decreased whereas catabolism of apo B containing particles increased leading to less accumulation. The role of apo E in this process is less clear. Elevation of apo E levels in patients with FH have been reported earlier (45). More extensive studies are required to analyze the effect of Simvastatin treatment on apo E in FH.
In conclusion, heterozygous FH patients have a disturbed postprandial lipoprotein metabolism. After Simvastatin treatment the postprandial RLPC response was decreased towards that of matched control subjects. No differences were observed in postprandial plasma retinyl ester response after treatment. This observation stresses the importance of the different approaches for analysis of postprandial lipoprotein remnant metabolism. Additionally, response of lipoprotein remnants that dominate the early postprandial period can be modulated by Simvastatin treatment, whereas the “later” and larger plasma chylomicron particle concentrations continued to be elevated. Improvement of RLP-C response after Simvastatin treatment in FH reduces the postprandial atherogenicity of plasma in addition to lowering LDL-cholesterol.

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1.11
Remnant Lipoprotein levels and Carotid Intima MediaThickness in patients with Heterozygous Familial Hypercholesterolemia (FH); the effect of one-year Simvastatin treatment.

Brief Rapid Communications

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Triglyceride (TG)-rich lipoproteins, such as remnant-like particles (RLP), are related to an increased cardiovascular mortality in patients after a first ischemic event. In patients with established heterozygous familial hypercholesterolemia (FH) and elevated plasma triglyceride levels, plasma RLP-cholesterol (RLP-C) concentration is increased, independent from plasma LDL-cholesterol levels. In the present study plasma RLP-C concentrations were associated with the combined carotid and femoral artery intima-media thickness. The effect of one-year simvastatin treatment was evaluated.

Methods and Results From the FH Express study, a subpopulation of 100 heterozygous FH patients was investigated. Plasma lipid profile (including total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), TG and RLP-C) and IMT were analysed at baseline and after one year simvasatin treatment (80 mg once daily). Plasma levels of TG, Cholesterol LDL-C and RLP-C decreased with 35%, 37%, 46%, and 50% respectively (all $p<0.001$) and HDL-C increased with 11% ($p=0.001$). Baseline plasma RLP-C ($r=0.29$), TG ($r=0.28$), and LDL-C ($r=0.23$) were positively associated with IMT (all $p<0.001$), and HDL-C and the RLP-C/TG ratio did not show a significant correlation with IMT. In a multivariate analyses both LDL-C and RLP-C were associated with IMT.

Conclusions Plasma RLP-C concentrations are increased in FH patients, and are positively associated with IMT, but not independent from LDL-cholesterol. Consequently, an increase in plasma RLP-C will extent the atherogenic lipoprotein phenotype in FH patients and may play a role in the progression of atherosclerotic disease in addition to LDL. Simvastatin reduces cardiovascular risk by decreasing plasma lipid parameters including LDL-C and RLP-C.

Familial hyperchylesterolemia (FH) is a disorder of lipoprotein metabolism with an autosomal dominant mode of inheritance. The underlying cause of FH are mutations in the gene for the LDL-receptor located on chromosome 19 (1). The atherogenic lipoprotein phenotype in heterozygous FH patients is defined by elevated plasma LDL-cholesterol concentrations (2; 3). The expression of the clinical phenotype varies widely even in subjects with the same mutation in the LDL-receptor gene. Recently, evidence has accumulated that triglyceride rich particles (TRP), such as very low density lipoproteins (VLDL), VLDL remnants, chylomicrons, chylomicron remnants, and intermediate density lipoproteins (IDL) share atherogenic properties (4-7). In addition, it has been shown that plasma triglyceride-rich lipoproteins remnants concentrations are elevated in FH (8-10). A specific subspecies of these lipoprotein remnants can be measured with the use of an immunoseparation assay that consist of an immuno-affinity mixed gel containing anti-apolipoprotein (apo) A-I and anti-apoB-100 monoclonal antibodies (11; 12). Plasma levels of RLP-C are elevated in heterozygous FH patients independent from plasma LDL-cholesterol (13; 14). The relationship between elevated plasma RLP-C levels and atherosclerotic disease is supported by in vitro studies using rat aorta rings showing a direct dose dependent effect of RLP on endothelial function (15). In addition, plasma RLP-C levels are positively associated with the extent of carotid arterial wall thickening as measured by intima-media thickness (IMT), a surrogate marker of atherosclerosis, in a cohort of healthy 50-year old men (16).

In the present study, we investigate the relationship between parameters of the atherogenic lipoprotein phenotype, including plasma RLP-C, LDL-C and TG, and markers representing atherosclerotic burden, such as carotid and femoral intima-media thickness IMT in patients with heterozygous FH.

Material and Methods

Patients

The present study is a sub-study (n=100 patients) of the FH ExPRESS study (Examination of Probands and Relatives in Simvastatin Studies with Familial Hypercholesterolemia). This is an open label, multicenter study, in which efficacy, safety, and pharmacogenetics of simvastatin 80 mg were assessed in 526 heterozygous FH patients (10; 17; 18). In short, patients were included if they met the following criteria: all patients had to have either a molecular diagnosis for FH or the clinical diagnosis of FH. All participating FH patients were informed thoroughly about the content of the study and had to sign an informed consent. The study protocol was approved by the ethical Review Boards of the participating centres.

Measurements of Lipoproteins

Fasting venous blood samples were obtained at baseline and after one year simvastatin therapy. Plasma was obtained by centrifugation at 3000 rpm for 15 min at 4∞C and stored at -800C. TC, HDL-C, and TG were routinely determined in the different laboratories and standardized by a virtual central laboratory. LDL-C was calculated using the Friedewald formula (19). Apolipoprotein A-I (apo A-I) and apo B were determined by an immuno rate-nephelometric procedure using a polyclonal goat anti-human antibody (Array protein system, Beckman Coulter, Netherlands).

Measurements of RLP-C

The RLP fraction was prepared using an immuno-affinity separation technique described by Nakajima et al (11; 12). Briefly, 5 μl of serum was added to 300μl of mixed immunoaffinity gel suspension containing monoclonal anti-human apo A-I (H-12) and anti-human apo B-100 (JI-H) antibodies (Japan Immunoresearch Laboratories, Takasaki, Japan). The reaction mixture was gently shaken for 120 minutes at room temperature followed by standing for 15 minutes. Then 200μl of the supernatant was withdrawn for the assay of RLP-C. Cholesterol (CV% <6%) in the RLP fraction were measured by an enzymatic assay using a Cobas Mira S auto-analyzer (ABX Diagnostics, Montpellier, France).

Assessment of IMT

The ultrasound scanning protocol was similar to that used in the ASAP study (20; 21). In summary, all examinations were performed by the same sonographer. A Biosound phase-II ultrasound instrument equipped with a 10 mHz transducer (Biosound Esaote, USA) was used. Scans were done at baseline and after one year of statin therapy. Carotid arteries were scanned bilaterally. Three 10 mm segments were scanned: the distal portion of the common carotid artery, the carotid bifurcation, and the proximal portion of the internal carotid artery. The 10 mm proximal to the femoral dilatation, the common femoral artery segment, was scanned in the right femoral artery. Images of segments were stored on optical disk. IMT measurements of far walls were performed. The image analysis was done with Eurequa (Eurequa; TSA Company, Meudon, France). Ultrasound images were analysed by one reader blinded to any patient information. Repeated scans were performed regularly by the sonographer. The repeatability had a coefficient of variation less than 5%.

Statistical Analysis

Data are presented as mean ± SD, unless shown otherwise. Treatment effect of simvastatin on plasma RLP-C, other lipid parameters, and IMT was assessed using Wilcoxon's matched-pairs signed-rank test. Association between IMT on the one hand and baseline or follow-up levels of plasma RLP-C and other lipid parameters on the other was quantified using Spearman's rank correlation coefficient. Multivariant analysis of IMT on plasma RLP-C and other plasma lipid parameters jointly was done after rank transformation.

Results

Only patients from the FH ExPRESS cohort with available data on plasma RLP-C levels and IMT data were included in the present analysis. This subset of patients was not significant different from the complete cohort with regards to all measured parameters. Characteristics and plasma lipid levels at baseline and after one year high dose simvastatin treatment, are given in table 1. Baseline plasma LDL-cholesterol and total cholesterol were significantly elevated. Plasma triglyceride levels ranged from 0.5 – 7.9 mmol/L. Median plasma RLP-C levels were 0.40 mmol/L (range: 0.2-0.8). Upon simvastatin 80 mg, plasma TG (35%), TC (37%), LDL-C (46%) levels decreased significantly , whereas plasma HDL-C and apo A-I levels increased after treatment. Plasma RLP-C levels decreased significantly to 0.2 mmol/L (range 0.1-1.0 mmol/L), but only 34 patients (34/100 patients) reached plasma RLP-C levels within the normal range in our laboratory (<0.2 mmol/l).

Baseline mean combined IMT of the carotid and femoral arteries was increased (1.05 ± 0.25 mm, Table 1). The femoral far wall accounted for most of this increase. No statistically significant decrease in carotid and femoral IMT was observed after one year simvastatin treatment. A positive relationship between baseline plasma RLP-C levels and both IMT parameters was found (Figure 1). A linear association between plasma RLP-C levels and far wall IMT was observed for plasma RLP-C levels in a range from 0.2 to 0.6 mmol/l (table 2). Positive associations were also found for plasma LDL-C and TG. In a multivariate analysis (including plasma TG, LDL-C and HDL-C), the significant association between RLP-C and IMT was lost when LDL-C was included into the analyses. On the other hand, the association with LDL-C was lost when RLP-C was added to the equation.

After therapy, no association was found between the decrease in plasma RLP-C levels and change in IMT (p>0.20). However, baseline and far wall IMT 1.02 (SD 0.22 mm, p=0.03) was found to be lower in 34/100 patients who respond upon treatment with a

Table 1: Characteristics of 100 patients with baseline RLP-measurement and IMT measurements.

	Baseline	**after treatment**	**P-value***
Male gender: n (%)	53 (53%)	-	-
Age (year): mean (SD)	45.4 (13.6)	-	-
BMI (kg/m^2): mean (SD)	24.7 (3.1)	-	-
TC (mmol/L): mean (SD)	10.2 (2.0)	6.4 (1.6)	<.0001
TG (mmol/L): median (min-max)	1.7 (0.5 – 7.9)	1.1 (0.4 – 7.1)	<.0001
HDL-C (mmol/L): mean (SD)	1.4 (0.3)	1.5 (0.4)	
LDL-C (mmol/L): mean (SD)	8.0 (1.9)	4.3 (1.5)	<.0001
ApoA-I (g/L): mean (SD)	1.3 (0.2)	1.4 (0.3)	
ApoB (g/L): mean (SD)	2.0 (0.4)	1.2 (0.4)	<.0001
RLP-C (mmol/L): median (min-max)	0.4 (0.2 – 3.2)	0.2 (0.1 – 2.7)	<.0001
IMT total (mm): mean (SD)	0.99 (0.21)	0.98 (0.20)	0.11
Far wall IMT (mm): mean (SD)	1.05 (0.25)	1.03 (0.23)	0.09
Near wall IMT (mm): mean (SD)	0.87 (0.20)	0.87 (0.21)	0.80
CFA IMT (mm): mean (SD)	1.82 (0.75)	1.85 (0.62)	0.94

* P-value of paired t-test, or Wilcoxon matched-pairs signed-ranks test; CFA = common femoral artery.

decrease in plasma RLP-C below normal levels (<0.20 mol/L) as compared to 66/100 patients who responded upon therapy with a decrease in plasma RLP-C levels which did not reach the normal value (plasma RLP-C > 0.20 mmol/L). Similar results were found for CFA IMT (p=0.04).

Discussion

In this study, we showed that baseline plasma RLP-C levels in patients with FH are associated with IMT as a validated marker for atherosclerosis. However, in multivariate analysis, both LDL-C and RLP-C were correlated with endothelial function, but either one or the other sufficed for predicting IM level. Univariately RLP-C correlated stronger with IMT than LDL-C, but the difference was not statistically significant. A one-year treatment period with simvastatin (80-mg od) decreased plasma RLP-C levels significantly, as has been shown before (13; 22).

Lipoprotein remnants are involved in the progression of atherosclerotic disease and are related to later occurring cardiovascular ischemic events (23). Inclusion of triglyceride-rich particles as an additional risk factor in the atherogenic lipoprotein profile has

Table 2: Mean (SD) of IMT data in quartiles of baseline RLP-C

Plasma RLP-C	IMT total	Far wall IMT	Near wall IMT	CFA IMT
<0.34 mmol/L	0.93 (0.23)	0.98 (0.25)	0.85 (0.22)	1.66 (0.74)
0.34 – 0.47 mmol/L	0.94 (0.17)	1.01 (0.20)	0.81 (0.14)	1.78 (0.72)
0.47 – 0.80 mmol/L	1.07 (0.20)	1.14 (0.24)	0.93 (0.20)	2.07 (0.78)
> 0.80 mmol/L	1.06 (0.22)	1.12 (0.27)	0.92 (0.22)	1.85 (0.77)
P-value*	0.03	0.04	0.11	0.31

* P-value of one way ANOVA; CFA = common femoral artery.

Table 3: Spearman correlation coefficients of baseline IMT with baseline RLP-C, TG, LDL-C, HDL-C, and RLPC/TG ratio

Plasma RLP-C	IMT total	Far wall IMT	Near wall IMT	CFA IMT
RLP-C	0.31 **	0.29 **	0.24 *	0.20 *
TG	0.27 **	0.28 **	0.11	0.22 *
LDL-C	0.28 **	0.23 *	0.34 **	0.18
HDL-C	-0.08	-0.07	-0.02	-0.21 *
RLP-C/TG	0.04	-0.01	0.18	-0.09

* p<0.05; ** p<0.001.

proven to be a better indicator for cardiovascular risk in patients with CVD or type II diabetes mellitus than plasma LDL-C alone (24). Elevated baseline plasma RLP-C levels in patients with coronary artery disease (CAD) predict future coronary events, independently of other risk factors (including plasma LDL-C). This observation is in line with Karpe et al (16), who also showed a relationship between plasma lipoprotein remnants in CAD patients and carotid IMT, independent of plasma TG and LDL-C.

In this study, a positive association between plasma RLP-C levels and IMT was found, that was linear up to a plasma RLP-C concentration of 0.6 mmol/l after which this linearity disappeared. A saturation of the pro-atherogenic pathways (such as a maximal amount of subendothelial retention of lipoprotein remnants, and/or a limited possibility to interact with other pro-atherogenic components, i.e. smooth muscle cells) is suggested as a possibility to explain this relationship. In vitro, RLP induce a dose dependent positive response in endothelial cells on the expression of inflammatory markers (25) and a negative effect on the expression of NO synthesis. Moreover, lipoprotein remnants were able to induce foam cells (26) .

In a cohort with patients with CAD (16) , the association of plasma RLP-C with IMT was independent of plasma LDL-C. In the present study we were not able to find an association of RLP-C with IMT independent from plasma LDL-C. In multivariate analyses both contributed to a similar extend but not independent from each other. It is therefore interesting, that a twofold decrease in plasma LDL-C levels after 1 year of treatment was not beneficial for to both carotid and femoral IMT. Significant IMT decrease was observed after two years of treatment, but plasma RLP-C levels were not determined at this time point. IMT at two years was related to baseline plasma RLP-C, but IMT change not. In the ASAP study (21) a lower dose simvastatin treatment (40 mg once a day) in heterozygous FH patients on the IMT was observed, despite a reduction of 41 % in plasma LDL-C levels. On the other hand, atorvastatin treatment (80-mg od) result in a decrease in LDL-C 50% together with a decrease in IMT. This small difference in LDL-C reduction may not account for the beneficial effect of atorvastatin on IMT. In the ASAP study, baseline plasma TG concentrations were positively associated with IMT and may explain part of the observed differences.

In conclusion, we observed an association between plasma RLP-C levels and combined carotid IMT in heterozygous FH patients. This observation further supports the hypothesis that a complete atherogenic lipid phenotype in FH patients should include analysis of plasma RLP-C levels. Simvastatin lowered significantly plasma TC, TG, RLP-C and LDL-C. The decrease in cardiovascular disease risk may therefore be augmented by statin induced RLP-C reduction.

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1.12
The atherogenic plasma remnant-like particle cholesterol (RLP-C) concentration is increased in the fasting and postprandial state in active acromegalic patients

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Premature atherosclerosis is a clinical feature in untreated acromegaly. Increased postprandial lipoprotein remnant levels are associated with premature atherosclerosis. In most studies, remnants were measured indirectly using retinyl esters (RE) as a chylomicron core label. Remnants can also be directly quantified by immunoseparation using monoclonal antibodies to apo AI and apo B100 to remove non-remnant lipoproteins. Cholesterol is quantified in the remaining apo E-rich remnant fraction (RLP-C). The outline of the present study is to investigate the role of postprandial lipemia in patients with acromegaly to further define abnormalities leading to increased susceptibility for atherosclerosis. In a case-controlled study, the plasma postprandial lipoprotein remnant fraction (RLP-C and RE) were analyzed in 6 patients with active acromegaly (F/M: 2/4; age: 53 ± 9; BMI: 29 ± 4 kg/m2) and in 6 normolipidemic control subjects (matched for age, gender, BMI and apo E genotype). They underwent an oral vitamin A fat loading test. Baseline plasma triglycerides (TG) were not significant different in patients (1.75 ± 0.71 mM) and controls (1.15 ± 0.46 mM). Lipoprotein Lipase (LPL) activity was significantly lower in patients than in controls (108 ± 21 vs.141 ± 19 mU/mL, respectively; $p<0.05$). Baseline plasma apo E levels were higher in patients (60.8 ± 7.9 mg/L) than in controls (48.3 ± 5.9 mg/L; $p<0.05$). No differences were found in the area under the postprandial TG curve (AUC-TG), the incremental AUC-TG (DAUC-TG) and AUC-RE in the Sf<1000 remnant fraction. However, fasting plasma RLP-C concentrations, isolated by immunoseparation, were increased in patients with active acromegaly (0.41 ± 0.13 mM) as compared to control subjects (0.20 ± 0.07 mM; $p<0.05$). Incremental postprandial RLP-C response (corrected for fasting values) was also significantly elevated in patients (2.14 ± 1.19 mM*h) than in the controls (0.86 ± 0.34 mM*h; $P<0.05$). In both groups, the maximal RLP-C concentration was reached between 2 and 4 hours. In conclusion, the atherogenic postprandial remnants, represented by RLP-C, were significantly elevated at baseline and in the postprandial period, whereas the larger-sized remnants, represented by RE (Sf<1000), were not different from controls. The disturbances in the postprandial RLP-C response increased the susceptibility for premature atherosclerosis as observed in patients with acromegaly.

Acromegalic patients suffer from an increase in cardiovascular mortality (1) that is related both to an impairment of heart function (2) and premature atherosclerosis (3). The etiology of premature atherosclerosis in acromegaly is related to unfavorable changes in lipoprotein metabolism, insulin resistance and a disturbed fibrinolysis (4) (elevations of t-PA, PAI and fibrinogen (5)). Increased plasma levels of total cholesterol, LDL-cholesterol and triglycerides, as observed in patients with active acromegaly, characterize the dyslipidemia. In addition, there is increased evidence that lipoprotein remnants, dominating in the postprandial state, are highly atherogenic and related to increased susceptibility of coronary artery disease. In *in vitro* studies, lipoprotein remnants have an equivalent potency as oxidized-LDL to induce foam cells (6;7). In patients with manifest coronary artery disease (8) and patients with an increased cardiovascular risk (9-13) the postprandial TG-rich lipoprotein remnant levels were increased. No data are available yet on postprandial lipoprotein remnant metabolism in patients with acromegaly. We hypothesize that postprandial lipoprotein remnants in patients with active acromegaly are related to the etiology of premature atherosclerosis.

Most studies on postprandial dyslipidemia used retinyl-ester (RE) as a marker for chylomicrons and its remnants. However, the specificity of retinyl-esters as marker is under debate, as it appears that RE transfer to other lipoprotein fractions in the postprandial period. Recently, a separation procedure for lipoprotein remnants based on immunoseparation with specific apo B100 and apo AI antibodies (14;15) was introduced. This separation method enables us to isolate more specifically the remnant-like particles (RLP). We recently showed (Schreuder et al, Atherosclerosis in press) that remnant-like particle cholesterol (RLP-C) is an excellent marker for postprandial lipemia in vivo. Increased plasma postprandial RLP-C levels in untreated patients with heterozygous familial hypercholesterolemia (FH) were found, whereas statin treatment completely normalized fasting and postprandial plasma RLP-C levels (Twickler et al 2000). Furthermore, in patients with growth hormone deficiency we observed increased levels of fasting and postprandial RLP-C accompanied by an impaired endothelial function. Treatment with recombinant Growth Hormone resulted in a significant decrease of postprandial RLP-C and improvement of endothelial function (Twickler et al 2000).

In the present case-controlled study we investigated, whether in patients with active acromegaly, disturbances in postprandial lipoprotein remnant metabolism after an oral fat loading test, as reflected by abnormalities in RLP-C, occurred.

Patients and Methods

Patients

Acromegalic patients had been recruited from the outpatient clinic of the department of Internal Medicine and Endocrinology from the University Hospital Utrecht. Active acromegaly was defined as non suppressibility of (increased) plasma GH concentrations after an oral glucose load. The purpose of the present study is to study the effects of the presence of excess growth hormone on lipid metabolism, specifically on disturbances in postprandial lipoprotein remnant metabolism. Therefore we excluded patients with increased fasting plasma lipids (fasting plasma cholesterol > 6.5 mM and/or TG > 2.3 mM), Body Mass Index (BMI) > 30, renal and/or liver disease, diabetes mellitus (DM), apolipoprotein E2/E2 genotype, and a family history of premature atherosclerosis and/or non-insulin dependent diabetic mellitus. Before the start of the study, all patients had been on treatment with a short term acting Sandostatin®. The study was performed when the patient was at the end of a six-week off treatment period to restart a long acting Sandostatin Lar®. The hormone levels were measured at 13.00 pm. Because of the skewed distribution we present the average data and the range: cortisol: 0.24 μM (range: 0.1 - 0.4 μM, testosteron: 14.7 nM (range: 8.8 - 20 nM), TSH: 1.06 mU/L (range: 0.13 - 2.4 mU/L), and f-T4: 13.4 pM (range: 9 - 16 pM). Normolipidemic (fasting plasma cholesterol < 6.0 mM and TG < 2.3 mM) healthy control subjects, matched for age, gender, and BMI were selected by advertisement. They had no diabetes, no hepatic, renal, thyroid or cardiac dysfunction and a negative family history for cardiovascular diseases. The study protocol was approved by the human investigation review committee of the University Hospital Utrecht. Written informed consent was obtained from all participants.

Oral fat tolerance test

After an overnight fast of 12 h, participants were admitted to the metabolic ward at 7.30 h am. Cream (consisting of 40% fat (w/v) with a P/S ratio of 0.06, 0.001% cholesterol (w/v) and 2.8% carbohydrates (w/v)) was given as a single fat load in a dose of 50 g fat per m^2 body surface area. Vitamin A was added to the cream in a dose of 60.000 IU aqueous retinyl palmitate (RP) per 50 g fat (125 ml cream). After ingestion of the cream, venous blood samples were taken hourly from an indwelling catheter in the antecu-

bital vene during 10 hours. Blood was collected in EDTA containing tubes. All blood samples were immediately put on ice. During the test only water or tea without sugar were allowed to drink. None of the subjects vomited or suffered from diarrhea after drinking the cream.

Analytic methods

Plasma was obtained by centrifugation at 3000 rpm for 15 min at 4°C. Plasma TG and cholesterol were analysed with a colorimetric assay (Monotest cholesterol kit no. 237574 and GPO-PAP no. 701912, Boehringer Mannheim, Germany). Cholesterol was determined in the HDL fraction isolated by the permangate precipitation method (16). Low-density lipoprotein (LDL)-cholesterol was calculated with the Friedewald Formula (17). Apolipoprotein (apo) B concentrations were analysed on the Cobas Mira autoanalyzer (Unimate 3 Apo B, Roche Diagnostics). Plasma apo E concentrations and plasma apo CIII concentrations were determined with the use of electroimmunodiffusion (Hydragel LP E Ref. 4058 and LP CIII Ref., Sebia Inc. USA). The coefficient of variance for plasma total apo E and plasma apo CIII concentrations was less than 7.5%. Plasma Insulin and IGF-1 concentrations were determined with a radio-immuno assay. Apo E genotype was analysed as described (18). Postheparin lipolytic enzyme activities were measured as described before (Twickler et al 2000). HOMA-index (fasting glucose*fasting insulin/22.5) was calculated to estimate the insulin resistance. Body composition was performed with the bioimpedance measurements, as described before (19). The waist to hip ratio (WHR) was determined as the ratio of the circumference at the waist and hip level.

Assessment of postprandial lipoprotein remnants

Lipoproteins were separated by flotation in a Sf>1000 fraction which contains chylomicrons, large chylomicron remnants and large hepatic triglyceride rich lipoproteins, and a remaining infranatant fraction (Sf<1000) containing small chylomicron remnants and all the other lipoproteins (20;21). Retinyl Ester (RE) concentrations in plasma and in the Sf > 1000 and Sf<1000 fraction were measured with the high-performance liquid chromatography (HPLC) as described (22). Recoveries of RE in the Sf>1000 and Sf<1000 in comparison with plasma RE were between 80 and 105%.

The RLP fraction was prepared using an immunoseparation technique described by Nakajima et al (14;15). Briefly, 5 μl of serum was added to 300 μl of mixed immunoaffinity gel suspension containing monoclonal anti-human apo AI (H-12) and anti-human apo B100 (JI-H) antibodies (Japan Immunoresearch Laboratories, Takasaki, Japan). The reaction mixture was gently shaken for 120 minutes at room temperature followed by standing for 15 minutes. Then 200 μl of the supernatant was withdrawn for the assay of RLP-C. Cholesterol (CV% <3) in the RLP fraction was measured with the use of an enzymatic assay on a Cobas Mira S auto-analyzer (ABX Diagnostis, Montpellier, France).

Statistical Analysis

Data are presented as means ± SD, unless shown otherwise. Area under the integrated curve (and corrected for baseline values) was calculated for the postprandial TG, RE and RLP-C response using GraphPad Prism software (version 3.1, San Diego, California, USA). Baseline values between patients with acromegaly and control subjects were compared with the use of the unpaired t-test. Pearson's correlation or Spearman's rank correlation were applied to evaluate relationships between parameters. A two-sided P value of 0.05 was considered significant.

Results

Characteristics of the subjects

The patients with active acromegaly were strictly matched with the control subjects for age, gender, and BMI. Baseline characteristics are shown in table 1. No significant differences were found in fasting plasma cholesterol, plasma LDL-cholesterol, plasma HDL, plasma apo B and apo C-III between patients and controls. Although plasma triglycerides levels were higher in patients with active acromegaly than in controls, this difference did not reach the level of statistical significance. Total plasma apo E concentration was significantly higher in the acromegalic patients (60.8 ± 7.9 mg/L) than in the control subjects (48.3 ± 5.9 mg/L; $p<0.05$). The fasting plasma insulin levels and the HOMA-index were similar in both groups. As expected, the plasma IGF-1 levels were significantly higher in acromegalic patients than in the controls. Plasma IGF-1 concentration was positively correlated with HOMA-index ($r=0.64$;$p=0.02$), and plasma apo E concentration ($r=0.72$;$p=0.01$). Plasma postheparin HL activity was similar in both groups but the plasma postheparin LPL activity was significantly decreased in patients with active acromegaly ($P < 0.05$).

Postprandial TG responses

After the oral fat load, the maximal postprandial plasma TG levels (2.25 ± 0.80 mM) were reached at 3 hrs in the control subjects, whereas in the acromegalic patients maximal TG levels (2.97 ± 1.20 mM) were reached at 4 h as shown in Figure 1A. The areas under the curve (AUC-TG) and the incremental area under the curve (ΔAUC-TG) measured over an 8 h postprandial interval for plasma TG were not significant different between the acromegalic patients and the control subjects (Table 2). The postprandial plasma RE

Table 1: Characteristics in the acromegalic patients and the control subjects.

	Acomegaly	Controls
n	6	6
Male/Female	4/2	4/2
Age (yrs)	53 (9)	54 (10)
BMI (kg/m^2)	28.5 (3.7)	27.5 (2.9)
FM (kg)	21.38 (5.1)	20.3 (3.7)
FM%	24 (6)	25 (6)
W/H ratio	0.93 (0.18)	0.89 (0.07)
Cholesterol (mMl)	5.07 (0.86)	5.20 (0.90)
TG (mM)	1.75 (0.71)	1.15 (0.46)
HDL-cholesterol (mM)	1.27 (0.23)	1.40 (0.28)
LDL-cholesterol (mM)	3.03 (0.90)	3.33 (0.80)
Apo-B (g/l)	0.98 (0.22)	0.96 (0.27)
Apo-CIII (mg/l)	32.90 (6.62)	32.51 (5.28)
Apo-E (mg/l)	60.77(7.94)*	48.25 (5.93)
Insulin (mU/l)	12.0 (3.52)	10.7 (2.42)
HOMA-index	2.81 (1.07)	2.46 (0.52)
IGF-1 (ng/ml)	717(365)*	172 (29)
LPL(mU/ml)	108 (21)*	141 (19)
HL(mU/ml)	413 (204)	459 (187)

All values are expressed as mean (SD). Acromegaly vs controls: * $P<0.05$.

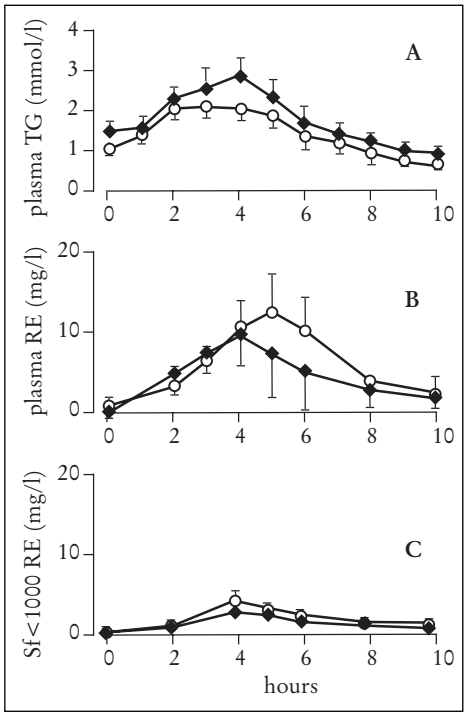

Figure 1. Postprandial responses for the acromegalic patients (u) and control subjects (m). Total plasma-TG (panel A), the incremental plasma RE (panel B) and RE concentrations in the non-chylomicron fraction (Sf<1000) (panel C) are presented. Values are presented as mean ± SEM

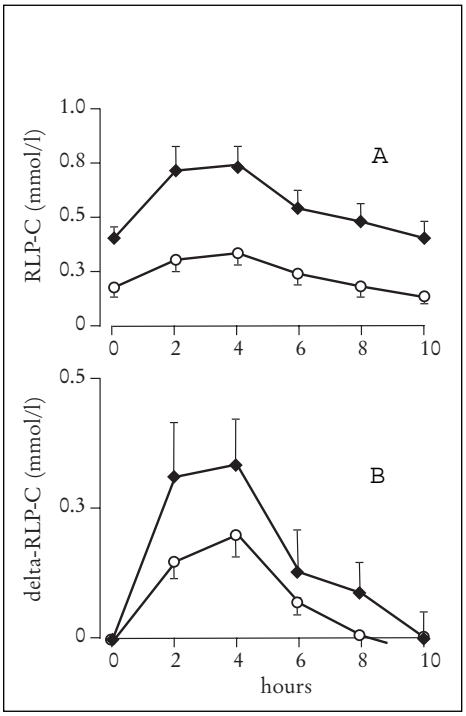

Figure 2. Postprandial remnant-like particle (RLP-C) responses for the acromegalic patients (u) and control subjects (m). Total plasma RLP-C (panel A) and the incremental plasma RLP-C (panel B) are presented. Values are presented as mean ± SEM.

response reached its maximum level at 5 h

Table 2: Integrated areas under the curve (AUC) of the postprandial TG response, postprandial plasma RE and non-chylomicron RE Sf<1000 response and postprandial RLP-C response.

	Acomegaly	**Controls**
Plasma TG	1.75 (0.71)	1.15 (0.46)
AUC-TG	17.09 (6.57)	14.14(6.01)
ΔAUC-TG	5.31 (3.6)	5.40 (2.55)
AUC-RE	42.00 (19.03)	39.05 (28.46)
Sf<1000 AUC-RE	16.65 (7.03)	14.18 (10.76)
Plasma RLP-C	0.40 (0.13)**	0.20 (0.07)
AUC RLP-C	4.78 (1.70)*	2.48 (0.74)
ΔAUC RLP-C***	2.14 (1.19)*	0.86 (0.34)

All values are expressed as mean (SD). Acromegaly vs controls: * P<0.05 and ** P<0.01. *** ΔAUC indicates area under the response curve after correction for baseline concentrations.

after the oral fat load in the control subjects and at 4 h in the acromegalic patients (Figure 1B). The area under the curve during an 8 hr postprandial interval (AUC-RE) for plasma RE was similar in the acromegalic patients (42 ± 19 mg*h/L) as compared to the control subjects (39.1 ± 28.5 mg*h /L). RE levels in the Sf<1000 remnant fraction (Table 2) were similar in controls and patients with active acromegaly. The AUC-RE (Sf<1000) was positively correlated with the BMI (r = 0.74; p<0.05).

Fasting plasma concentrations of RLP-cholesterol (RLP-C) were significantly elevated in acromegalic patients (0.41 ± 0.13 mM) compared to control subjects (0.20 ± 0.07 mM; P<0.05). Fasting plasma RLP-C concentrations were positively correlated with plasma apo E concentrations (r= 0.67; p = 0.02) and plasma triglycerides (r=0.88, P<0.02). In both groups, the maximum level of plasma RLP-C was reached between 2 and 4 hours after the oral fat load. The maximum concentration was 0.40 ± 0.14 mM in control subjects and 0.76 ± 0.27 mM in acromegaly patients (P<0.05). The AUC-RLP-C was significantly increased (4.78 ± 1.70 mM*h/L) compared to control subjects (2.48 ± 0.74 mM*h/L; P<0.05). If the AUC-RLP-C data were corrected for baseline values (ΔAUC-RLPC) the differences still remained statistically significant (Table 2). Positive correlations for AUC-RLPC were found for fasting plasma TG (r = 0.62;p = 0.03) and fasting RLP-C concentrations (r = 0.77; p = 0.003) and a negative correlation for postheparin LPL activity (r = -0.62; p = 0.04). Identical correlations were found for the ΔAUC RLP-C.

Discussion

In the present study, we showed that patients with active acromegaly, but without secondary causes of dyslipidemia, had elevated fasting and postprandial lipoprotein remnant (RLP-C) concentrations, whereas fasting concentrations of total, LDL-cholesterol, and HDL-cholesterol were similar to those measured in control subjects matched for age, gender, and BMI. The plasma TG levels were elevated in acromegaly although this did not reach the level of statistically significance. The importance of RLP-C as an atherogenic risk factor was established in a number of different studies. In patients with occlusive coronary artery disease the regression of the coronary diameter was strongly associated with the decline in plasma RLP-C (6). Moreover, increased fasting levels of RLP-C were found in patients with coronary artery disease and in non-insulin dependent type 2 diabetic patients with microalbuminuria (23). Increased fasting RLP-C concentrations were also found in non-diabetic, insulin-resistant, female subjects in contrast to insulin-sensitive subjects (24). In line with these observations, the increased fasting plasma RLP-C levels in our study could contribute to the increased susceptibility for atherogenesis leading to premature atherosclerosis in patients with acromegaly. We postulate that the increased fasting plasma RLP-C, in active acromegaly, could be partly explained by the relative increased state of insulin insensitivity in our patients. The plasma IGF-1 levels were positively associated with the homeostasis model insulin resistance index (HOMA-R). The HOMA-R index was highly correlated with the data obtained with euglycemic clamp studies in both non-diabetic and diabetic individuals (25) and is a simple measure for the state of insulin sensitivity. Additionally, insulin resistance decreased the activity of the hepatic LDL-receptor that plays an important role in the removal of lipoprotein remnants (26). Simultaneously, the secretion of VLDL apo B is increased (27). This phenomenon has

already been observed in acromegaly (28), although in our subset of patients no differences in plasma apo B levels were found. The elevation of fasting plasma RLP-C levels in patients with active acromegaly may also be the results of the slightly increased plasma triglyceride levels. It has been shown that plasma triglycerides are a major determinant for RLP-C levels because a correlation with increasing plasma triglyceride level was found ($r=0.88$, $P<0.02$). However, in a recent published study in healthy elderly men, it was found that RLP-C quantification is a better assessment for cardiovascular risk than measurements of LDL-cholesterol and plasma triglycerides (Karpe et al 2001). This observation is further established in the present study.

The postprandial RLP fraction consisted of apo B48 containing chylomicron remnants and of apo B100 containing triglyceride-rich remnants. Both apo B48 and apo B100 containing lipoprotein particles share the same lipolytic pathway mediated by LPL. However, apo B48 containing particles exert a higher affinity for LPL (29). We observed significant decreased postheparin LPL activities in patients with acromegaly. This is in agreement with an earlier observation of decreased LPL activity in acromegaly by Simsolo et al (30). The incremental area under the postprandial RLP-C curve was negative correlated with postheparin LPL activity, indicating that a low LPL activity is associated with an increased postprandial presence of RLP-C. No difference in postprandial profiles for retinyl esters as well as total triglycerides was found between the patients with active acromegaly and control subjects. This further showed that RLP-C measurement is a more specific marker for postprandial lipaemia than either total TG or retinyl esters. In patients with heterozygous Familial Hypercholesterolemia we observed an effect of statin treatment on the postprandial RLP-C levels but not on the postprandial Sf<1000 RE levels. Similarly, in patients with growth hormone deficiency, abnormalities in postprandial lipoprotein metabolism were found that were linked to specific abnormalities in RLP-C metabolism. Furthermore Karpe et al (31) found that only the early peak of ApoB48 remnants correlated to the severity of coronary atherosclerotic lesions and not the later postprandial peak of RE. Moreover Kugiyama et al showed a close association of the RLP-C with the impairment of endothelium-dependent vasomotor function in human coronary arteries (8). Taken together, recent studies established the importance of remnant-like particles in atherogenesis. In the present study, we clearly demonstrated that in active acromegaly more remnant-like particles were present in both the fasting and the postprandial state. We postulated that the increased fasting RLP-C levels could be a result of relative insulin insensitivity and increased plasma triglyceride levels, while the increased postprandial RLP-C levels could be additionally explained by the lower postheparin LPL activity. The increase in RLP-C levels may contribute to the increased susceptibility of premature atherosclerosis in acromegaly.

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GH/IGF and Pro-diabetic Phenotype

section B

2.1
The role of the IGF system in pancreatic b-cell function
(review)

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In this review, we highlight recent findings of the roles of various growth factors for pancreas beta cell function with emphasis on the Insulin-like Growth Factors (IGF). Signalling of insulin and the IGF system uses parallel (and interrelated) pathways. There is a complex interplay between insulin receptor-dependent signalling and the signalling of the IGF-system in the development of the islet cells, which has become apparent by various animal knock-out models. Recent knowledge about the influence of human gene polymorphisms relating to islet cell function also sheds more light on the importance of the IGF system for pancreas B-cell function.

Type 2 diabetes mellitus is generally due to a combination of insulin resistance and an impairment in insulin secretion by the pancreatic B-cell (1). Originally, the insulin secretion capacity of the pancreatic B-cell was considered as a communicative process of biological signals, such as derived from meal composition and neural input (2). Insulin secretion is known to adapt to systemic needs for insulin, and in humans systemic insulin profiles mostly reflect peripheral sensitivity to insulin action (3).

We will here describe the importance of growth factors, especially insulin-like growth factors I and II, and their signalling pathways for pancreas beta cell growth and function, which have become more clear by the use of animal models, and explore recent findings in humans.

Plasticity of high differentiated cells

Recent ontological studies show a high plasticity of highly differentiated cells with respect to maturation and differentiation. In contrast of what was thought before, highly differentiated cells have an ability to reenter the cell growth cycle on stimulation with specific growth factors; pancreatic beta cells have been shown to possess these properties (4). It has not been elucidated whether this interval of cell plasticity is limited in time (prenatal or also later in adult life). Barker hypothesized that especially the prenatal period is of importance for the development of functional tissues or organs and that diseases later in adult life (such as hypertension and type 2 diabetes) may have their origin in disturbances of growth and the development of functional tissue capacity (5). This would also imply that a disease later in life may result from a maladaptation due to reduced capacity of functional tissues (6). In support of Barker's hypothesis, lower birth weight has been shown to be associated with a prevalence of type 2 diabetes (7). Since overt diabetes mellitus results from a failure of pancreatic beta cells to adapt to environmental demands, i.e. insulin insensitivity (1), the effects of growth factors on pancreas beta cells are of growing interest. But also in case of a high plasticity of cells that continue to form functional tissue during adult life, defects in growth factors, such as the insulin-like growth factor system, may potentially give rise to a less adaptive capacity and therefore an increased susceptibility to disease (8). A prerequisite in this situation is an increase of a specific burden, i.e. insulin resistance related to obesity, that needs to be compensated for by functional tissues (i.e. B-cells) which are limited by a reduced capacity, and/or are limited in their adaptive growth.

Growth Factors in Humans

Expression of growth factors

GH, IGF-I and IGF-II are the principal growth factors in humans. Their biological action is dependent from the balance in the total IGF system, that consists of IGF-I, IGF-II and six IGF binding proteins (IGF BP 1-6). The growth hormone (GH) axis is functionally superimposed on the balances that exist in the IGF system. Primary disturbances in the secretion of GH are therefore associated with shifts in the balance of the IGF system. GH is secreted by the adenopi-

tuitary in a 24 hours pulsatile secretion profile, with a peak in the early morning. Other cerebral structures (such as hypothalamus) are involved in the regulation of GH secretion; stimulation by GH Releasing Hormone and ghrelin, and a reduction by somatostatin. GH increases the expression of IGF-I in the circulation (systemic IGF expression) and in many tissues (local IGF expression), while its effects on IGF-II are less clear. Moreover, plasma levels of IGFBP-3 are increased by GH stimulation. IGFBP-3 is the IGF binding protein that binds most of both IGF-I and IGF-II. Due to the fact that IGF-II is bound more avidly by IGFBP3, total plasma IGF-II levels are roughly three times higher than total plasma IGF-I in humans, and the IGF-II pool is therefore larger than the IGF-I pool. IGF-I has a negative feedback on GH secretion.

Shared receptors for insulin and IGF network

The distinctive molecules IGF-I, IGF-II and insulin bind to specific cell surface receptors. Due to structural homologies among these molecules, IGF-I also binds to insulin-receptors (but with a lower affinity than insulin), and recto-versa, IGF-I and IFG-II both bind to IGF-1 receptors. In addition to these cross-talks on the cell surface, insulin and IGF-1 receptors share common (post-receptor) intracellular signalling pathways, that were presented in recent reviews (9-12).

The insulin receptor is a transmembranous receptor, consisting of 2 extra-cellular alpha-subunits, that display the epitope for insulin, two membrane spanning beta-subunits and an intracellular tyrosine kinase related part. The IGF-receptor is related to the insulin receptor, with a subsequent 80% structural homology in the kinase domains (10).

Although the receptors are very common in structure, the distributions over the various tissues that are involved in the human insulin resistance syndrome differ. In adipose tissue, no IGF receptors are detected, in contrast to the liver and muscle. These non-equal distributions over several tissues have various implications, and especially in a system that is in imbalance, since signals may be become effective in tissues that are not "allowed" to receive.

I/IGF signaling

Insulin

Upon binding of insulin to extracellular domains of the insulin receptor, the signal transduction is mediated through intracellular beta-subunits that undergo autophosphorylation of tyrosine residues. After phosphorylation of these submembranous structures, a cascade of phosphorylations of so-called insulin receptor substrates (IRS) starts (13). An intracellular distribution of insulin-like signals is mediated along these IRS substrates. Hitherto, four intracellular IRS (IRS 1-4) proteins have been isolated and the distinctive IRS proteins direct to different intracellular pathways. In short, IRS-1 regulates somatic cell growth and is involved in insulin activity of muscle and adipose tissue (12). IRS-2 is involved in insulin activity mainly in liver, in brain growth, in reproduction, and in pancreas beta-cell growth (14). The pathways that are related to IRS-3 and IRS-4 are still not elucidated, but these IRS proteins are expressed in tissues of neuroendocrine and adipose origin. IRS-3 and IRS-4 disruption gives rise to normal (or even slightly lower) plasma glucose and normal insulin levels (13).

Upon phosphorylation of IRS proteins, the Phospho-Inositol 3' Kinase (PI3K) pathway is activated. This pathway consists of multiple subsequent phosphorylations, and gives rise to the expression of insulins effects on glucose homeostasis, such as enhancement of glucose uptake and glycogen synthesis in peripheral skeletal muscle, and inhibition of gluconeogenesis and glycogenolysis in hepatocytes, and inhibition of lipolysis in adipose tissue (9). Moreover, the signal transduction towards more downstream pathways of the PI3K pathway results in additional insulin-related effects, such as on protein synthesis, gene expression, mitogenesis, and cell growth (Table 1). Besides insulin-specific glucose metabolism related pathways (like

PI3K), several other intracellular cascades are activated. After the phosphorylation step of PI3K, several major pathways involves activation of grb2, SOS, RAS, and MAPK, whereas another pathway involves mTOR (mammalian Target Of Rapamycin) and PKB (PhosphoKinase B) activation (10).

IGF-I

IGF-I mainly acts via the IGF-1 receptor (IGF-1r). IGF-I has a critical role in fetal and post-natal growth. Knock-out mice homozygous null for either IGF-I or the IGF1R weigh only half the size of the wild-type animals (15). The IGF-1R has structural homology with the insulin receptor, and upon its activation, partly identical transduction pathways as for insulin are activated via IRS-2, followed by activation of PI3K and mTOR, but also of MAPK, both leading to mitogenesis (Table 1).

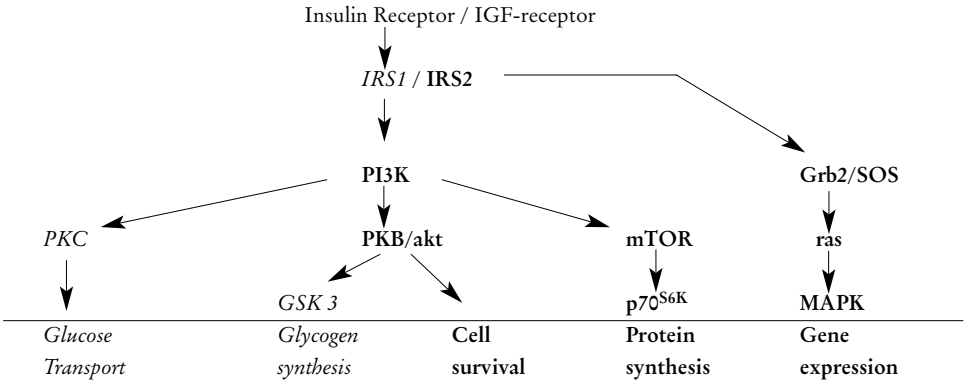
IGF-II

IGF-II can also bind to IGF-1R, but in addition also to the so-called Type-2 insulin-like growth factor receptor (IGF2R, also known as the IGF-6 mannose or IGF-M6 receptor), that has four distinct binding domains, which can bind IGF-II, mannose-6-phophate containing lysosomal enzymes, retinoic acid, and urokinase-type plasminogen activator receptor (16). This receptor has a role in the degradation of IGF-II, and may function as a tumor suppressor gene (16). In epigenetic phenomena occurring during gametogenesis, leading to silencing of either a maternal or a paternal allele, the expression of IGF-II and IGF-II related genes were recently found to be key factors in the development of tumors, such as Wilm's tumor and adenocarcinoma of the colon (17). However, also in adjacent colon tissue these epigenetic processes are found, which may suggest a more general field defect. So far, no extrapolation of these epigenetic results with IGF-II have been made to the field of insulin secretion or resistance.

Relationship with GH

As stated before, GH is a regulator of expression of IGF-I and IGF BP-3, in both circulation, liver and other tissues. Whether GH also regulates IGF-II production in humans has not been elucidated yet. Exogenous administration of growth hormone to healthy adults during 14 days, did not lead to appreciable changes in plasma IGF-II (18); on the other hand GH appears to stimulate the promoter region of IGF-II gene (19). Interestingly, GH has also cell growth promoting properties without the necessity to induce the local expression of IGFs. GH

Table 1: Simplified scheme of insulin/IGF signaling. Pathways used only by insulin signaling are given in *italics*; pathways presumably shared by both insulin and IGF are given in **bold**



binding to the GH receptor leads to phosphorylation of the Janus kinase 2 (JAK2), which will activate signal transducer and activator of transcription 5 (STAT 5). STAT 5 will then lead to mitogenesis. GH can therefore stimulate cell growth via various mechanisms, both more directly via its own receptor and/or indirectly via the IGF system. Interestingly, while cross talk between insulin-receptor and IGF receptor dependent mechanisms exist in various levels, there appears to be little or no cross talk between the JAK-STAT and the other pathways, at least in pancreas beta cells (20).

Growth factors and adaptive effects in adult pancreatic B-cells

Animal studies

Pancreatic beta cells are under influence of growth factors not only during the prenatal period. Also later in adult life, the influence of growth factors on the insulin secreting cells is considerable. Especially, knowledge about the involvement of the PI3K cascade in survival of pancreatic beta cells and the positive effect of MAPK activation on gene transcription show relationships between insulin receptor-mediated and IGF receptor-mediated effects with respect to beta-cell growth (9). Although insulin-secreting beta cells are well-differentiated in adult human life (less than 1% of pancreatic beta cells are in mitosis), they are still capable to undergo proliferative changes (9). Beta cell proliferation is stimulated by IGF-I via the activation of the post receptor IRS-2 proteins (21). In animal models, the IRS-2 knock-out mice have been shown to have not only impairment of insulin action, but also of beta cell development, with consequent development of diabetes (22). Those mice have a 60% reduction in pancreas islet cell mass with a relative beta cell deficiency; the number of islets is also half of normal. Disruption of the IRS-1 pathway, however, leads to a mild insulin resistance in peripheral muscle tissue with a two-fold increase in beta cells, that is associated with an hyperinsulinemia (10;12;13). IRS-1 binds with high affinity to calmodulin, a calcium-binding protein involved in insulin-secretion (23). IRS-1 deficient beta cells fail to increase the content of calcium in the cytosolic compartment, while the overexpression of IRS-1 protein increases the calcium levels in INS-1 beta cell lines (13). These observations suggest that a disruption in the IRS-1 pathway results in adaptation by doubling the pancreatic beta cells to overcome the decrease in calcium-driven secretion of insulin. Transgenic mouse models have indicated that subsequent phosphorylation of PI3K, Akt and mTOR are necessary for the transduction of this IGF-I signaling (21;24;25)(Table 1), while glucose has a strong modifying effect (26). Recent studies in mice with a beta-cell specific knockout of the IGF1Receptor indicate, that the mice showed normal growth and development of beta-cells but had a defective glucose-stimulated insulin secretion (with hyperinsulinemia) and had impaired glucose tolerance. These studies indicate that, even if IGFRI is not crucial for islet cell development, it is of importance for beta cell function (27).

Much less is known about the role(s) of IGF-II in pancreas beta cell mass and function. IGF-II is known to have anti-apoptotic properties. At least part of the effects of IGF-II may relate to its capability to bind to IGF-1 receptors. Recently, it was found that the Goto-Kakizaki (GK rat), which has been widely studied since it is a model for diabetes, displays defective IGF-II synthesis which may causative for its insufficient beta-cell development (28). However, transgenic mice overexpressing IGF-II develop frequently diabetes in spite of an increased beta cell mass, possibly due to an increase in glucagon producing alpha cells (13;29). Pancreas cells are capable of producing IGF-II themselves (30).

Other growth factors than IGF are presumably also at play. Fibroblast growth factors (FGF) have been proposed to be implicated already very early in the embryonic pancreas development (31). *In-vitro* studies suggest that they may influence the intestinal differ-

entiation program and the development of the dorsal and/or ventral part of the developing pancreas (32). FGF bind to extracellular FGF-receptors (FGFR) also belonging to the tyrosine kinase family. Recently, it was shown in in vitro studies, that FGF7 could control the development of exocrine pancreatic tissue, while removal of the FGF7 led to proliferation of endocrine tissue (33). Studies with transgenic mouse models, expressing a dominant negative version of FGFR1 show that these animals develop diabetes (34). These animal models have not only a reduced number of beta-cells, but they have also an impaired expression of the glucose transporter 2 (Glut-2), the most prominent glucose transporter of the pancreas and of the liver. Moreover, the expression of PC1/3, prohormone convertase 1/3, which is the enzyme catalyzing the final conversion of the prohormone proinsulin into the actual hormone insulin in the pancreas, is also decreased. Interestingly, the production of FGFs occurs in the beta-cells themselves under the influence of other transcription factors, notably Ipf1/Pdx1, insulin promoter factor1 or pancreatic duodenal homeobox gene 1. Ipf1 is known to stimulate insulin gene transcription and Glut 2 expression (30). In humans, a nonsense mutation of the Ipf1 gene is a known cause of Maturity Onset Diabetes of the Young type 4 (MODY4) (35).

Human studies

Recently, several reports mention the effect of disturbances in the expression of the IGF system on beta cell function in humans in relation to glucose metabolism (Table 2). Although still limited to some studies, most of them show an association between plasma levels of the IGF system or the presence of certain common gene polymorphisms and the release of insulin that are estimated by plasma insulin profiles during an oral glucose tolerance test (OGTT). However, plasma glucose levels change constantly during an OGTT. The use of hyperglycemic glucose clamping during which plasma glucose is kept constant, enables studying relationships between beta cell insulin release and influencing factors, such as the IGF system.

Table 2: Effect of IGF plasma levels or of common polymorphisms in the Insulin/IGF signaling molecules on beta cell function (or glucose homeostasis) in humans. Reference numbers are given in brackets

	Variant	Beta Cell Function	Glucose homeostasis
IRS1	Gly972Arg	contradictory findings (46;47)	possibly worsening (44)
IRS2	Gly1057Asp	no effect (47;47;49)	
IGF-I level		no effect (38)	present (36)
IGF-I gene		no data	no effect (39)
Promoter IGF-I gene		no data	possibly (40)(low birth weight)
IGF-1 receptor	GAG1013GAA		no effect (39)
IGF-II level		Stimulating (Twickler)	augmentation glucose (Twickler)
IGF-II gene		no data	
promoter IGF-II	IGF2-APA-I	possibly no effect	
IGF2 receptor		no data	

Relationship with IGF-I

The presence of low plasma IGF-I levels has been associated with an increased risk to develop IGT and type 2 diabetes (36). Both IGT and type 2 diabetes are related to a relative insufficiency of insulin secretion (37). In line with this observation, we also observed a negative correlation of the free IGF-I index with baseline and 2-h glucose response after an OGTT. However, we did not find a relationship of IGF-I with pancreas beta cell function as assessed with hyperglycaemic clamps in normal-glucose-tolerant subjects (38). In view of the above-mentioned findings in IGF1 receptor knock-out mice (27), it could well be that the role of this polymorphism in insulin secreti1on is more subtle, and would only become apparent during the development of IGT. It is of note, that the development of type 2 diabetes mellitus generally is the result of a combination of disturbances in insulin secretion and in sensitivity (35). Therefore, it could also be that the role of IGF-I in the development of IGT might possibly relate to an effect of IGF-I on peripheral tissue insulin sensitivity. Follow-up studies may be of relevance to clarify this point.

Relation with IGF-II

Very recently, we observed a significant association between both plasma IGF-II and IGFBP-3 levels and measures of beta cell function in subjects who have a normal glucose tolerance; these subjects were on average around 45 years of age, and were mildly obese (Twickler TB, de Sain- van der Velden MGM, van Doorn J, van Haeften TW. Relationship of insulin secretion with plasma Insulin-like Growth Factor-II. Submitted). Obesity (and age to a minor extent) is related to a certain amount of insulin resistance, but may also have an impact on beta-cell function. It is of note that the relationship of IGF-II with insulin secretion was still apparent after correction for obesity and age. It has previously already been shown that insulin secretion is elevated even in subjects with a BMI within normal ranges as compared to lean subjects (3). Our observation that IGF-II levels relate with beta-cell function in mildly obese subjects might therefore indicate a positive effect of IGF-II on beta-cell growth during embryogenesis which is in line with the Barker hypothesis. On the other hand, IGF-II may also have adaptive effects on adult pancreatic beta cells, which would compensate for changes related to increase of age and/or changes in glucose homeostasis due to obesity. However, to our knowledge, no additional data that deals with IGF-II plasma levels and beta cell function at a (very) young age in humans are available.

Gene polymorphisms and the relationship with glucose homeostasis

IGF-I

Several studies in type 2 diabetes patients have looked for the importance of common polymorphisms in genes involved in the IGF system. In a Danish study, DNA analysis of 82 probands of type 2 diabetes families showed no nonsense, frameshift or missense mutations in the IGF-I or IGF-1receptor genes; however, a number of silent or intron variants were noted (39). The most prevalent polymorphism (GAG1013GAA) of the IGF-receptor was not related to neither birth weight or insulin sensitivity index in a group of 349 healthy subjects. In addition, its prevalence was not higher in subjects with type 2 diabetes mellitus than in subjects with a normal glucose tolerance. Another common polymorphism in the IGF1 promoter region, however, has been reported to influence the weight at birth (40). A low birth weight is related to increased risks of insulin resistance, Impaired Glucose Tolerance and type 2 diabetes mellitus (6;41). Although insulin resistance is generally known to be the most important risk factor for the development of type 2 diabetes, disturbances in insulin secretion are almost always found in type 2 diabetes subjects (42). However, recent preliminary results from our group did not indicate any relationship between the presence of an IGF-1 gene polymorphism and beta cell function, neither in subjects

with normal glucose tolerance nor in subjects with IGT.

IGF-II
Little is known about the biological impact of polymorphisms in the IGF-II gene and the expression of a diabetic phenotype. In a recent study in a small group of subjects, a relatively frequent polymorphism of the untranslated part of the IGF2 gene was found to be associated with lower plasma insulin levels after an oral glucose tolerance test. (43). However, in preliminary studies in three groups of subjects we could not find a consistent effect of the polymorphism on either glucose levels after an OGTT or on insulin secretion during hyperglycemic glucose clamps.

Intracellular IRS proteins
Recent observations in humans indicate that carriers with a Gly972Arg substitution in IRS-1 proteins have a slightly decreased insulin sensitivity, and a slightly increased prevalence of type 2 diabetes mellitus (13;44). Conformational in-vitro studies show that RIN cells that have an increased expression of this variant have a reduced glucose-induced insulin release (45). Although one series of hyperglycemic clamps describes a marginally decreased beta cell function in humans (46), we were not able to confirm that observation in two larger cohorts (47). Very recently, in vitro studies point to a decreased conversion of proinsulin to insulin in human beta cells with the Gly972Arg variant. However, these data were obtained in cells from only two subjects with the variant, and compared to cells from only two controls (48), while insulin secretory function is known to differ markedly between subjects. Similarly, a common polymorphism (Gly1057Asp) in the IRS2 molecule does not appear to have an appreciable influence on pancreas beta cell function in man neither in subjects with normal glucose tolerance nor in subjects with IGT (46;49).

Conclusion

Taken together, animal studies showed a significant impact of growth factors, such as IGF-I and IGF-II, in physiological development of prenatal pancreatic beta cells, and in adaptive properties of beta cells on environmental changes in the postnatal period. In humans, the observation of a relationship between a low birth weight and an increased prevalence of impaired glucose tolerance and type 2 diabetes has prompted research into the effect of the IGF system on adult insulin secretion and the occurrence of derangements of glucose homeostasis. IGF-I levels have been shown to relate to the development of IGT. We have recently found evidence that plasma levels of IGF-II and IGFBP-3 are positively associated with insulin secretion. Others have observed a relationship with a promoter gene polymorphism of the IGF-I gene with (low) birth weight. However, no direct relationship has been found with type 2 diabetes, so far.
A major area of further research relates to the question whether the impact of growth factors on the insulin secretion capacity in humans is limited to the prenatal period or whether it is also related to coping processes of the beta cell in the postnatal period.

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2.2
Insulin-like growth factor-I and low birthweight

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Sir-Norbert Vaessen and colleagues (March 23, p 1036)(1) describe the interesting observation that a polymorphism of the insulin-like growth factor-I (IGF-I) gene is closely associated with low birth weight and an increased incidence of type 2 diabetes mellitus.

It is relevant in this context that a phenotypic association between low birthweight and increased incidence of type 2 diabetes is supported by a large Scandinavian study (2). From their results, Vaessen and colleagues propose that a specific IGF-I polymorphism is related to the extent of plasma IGF-I expression, a key factor in the development of pancreatic insulin-secreting cells. This proposal is supported by previous animal knock-out and transgenic models in which the concentration of plasma IGF-I is a determinant of the development and maturation of the insulin secreting B-cells in fetal life, and consequently affect insulin-secreting properties of the B-cells in adult-life (3). Most IGF-I in plasma is bound to IGF-binding protein-3 (IGFBP-3).

Due to the heritability of expression of IGF-I and IGFBP-3 (more with respect to IGFBP-3 than IGF-I (4)), we have assessed whether the relation between plasma IGF-I concentration, IGFBP-3 expression in insulin-secreting cells, or both is conserved in adult-life. We did a standard oral glucose tolerance test (75 g glucose), and a hyperglycaemic clamp (10 mmol/L during 180 min) with measurement of first and second (average plasma insulin in 140-80 min period) phase insulin secretion in 53 non-diabetic individuals (mean age 46 years, SD 6; 13 men, 40 women; mean body-mass index 25·9 kg/m^2, SD 3·8).

Plasma IGF-I concentrations were not related to parameters of insulin secretion, but plasma concentrations of IGFBP-3 were significantly correlated with second-phase insulin secretion ($p=0·025$) in the clamp, and with baseline ($p=0·056$) and 120 min plasma insulin after the oral glucose tolerance test ($p=0·037$). Multiple linear regression showed that the effect of IGFBP-3 on insulin secretion could be accounted for by body-mass index.

Thus, IGFBP-3 concentrations are closely related to insulin secretion in the adult pancreas. Several factors, including growth hormone status, age, nutrition, and hepatic function affect plasma concentrations of IGFBP-3. However, the variation between individuals of IGFBP-3 in the circulation of adults seems to be largely determined by a genetic component (4). Hence, at least to a certain extent, IGFBP-3 concentrations at the tissue level are also affected by genetic factors. IGFBP-3 plays an important part in the modulation (inhibitory or stimulatory) of IGF action at the cellular level.

During fetal development, local concentrations of IGF and IGFBP-3 may affect the balance between cell apoptosis and the maturing of functional B-cells (3), and hence the insulin secretory capacity in adult life. This may explain the intriguing finding that a relation exists between plasma IGFBP-3 and insulin secretion by the adult pancreas. The apparent effect of body-mass index on this relation remains puzzling. Considered together with the data of Vaessen and colleagues, we suggest that pancreatic B-cell function may be primarily determined early in life.

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2.3
Plasma IGF-II relates to insulin secretion in man

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To study the relationship between plasma IGF-II and insulin secretion in adult non-diabetic humans.
Methods. We evaluated relationships between plasma IGF-II and insulin secretion in 50 non-diabetic adults, estimated using an oral glucose tolerance test, and a hyperglycemic clamp (10 mmol/L, 180 minutes) with determination of first and second phase insulin secretion. Plasma IGF-II levels were positively associated with fasting plasma glucose (r=+0.37, p=0.004) and fasting insulin (r=+0.33, p=0.021) levels, and with second phase insulin secretion (r=+0.38; p=0.007). Multiple linear regression (with gender, age, BMI and waist-hip ratio as covariates) indicated that plasma IGF-II concentrations contributed significantly to the variance of baseline plasma glucose levels (partial coefficient r=+0.27, p=0.037), and of second phase insulin secretion (partial r= +0.28; p=0.044). Plasma IGF-II levels are significantly related to adult pancreas B-cell function.

Type 2 diabetes mellitus is generally due to a combination of insulin resistance and an impairment in insulin secretion by the pancreatic B-cell (1). A key step in the development of pancreatic B-cell function in animal studies, is the insulin / insulin-like growth factor (I-IGF) signalling pathway (2). Both IGF-I and IGF-II can activate this pathway through IGF-1 receptors (IGF-1r) resulting in cell processes, such as proliferation, differentiation, and prevention of apoptosis (2). In addition, IGF-II is also able to activate the type-2 insulin-like growth factor receptor (IGF2R), or IGF-6 mannose or IGF-M6 receptor. It has become apparent from these studies that pancreas B-cells are more plastic than was previously appreciated. A type II diabetes rat model without obesity (the Goto-Kakizaki (GK) rat) displays a defective IGF-II synthesis, which may account for its insufficient beta-cell development (3).

In humans, in accordance with Barker's hypothesis, it has been reported that aberrant foetal and infant growth is related with glucose homeostasis in adult life (4). Decreased plasma IGF-I levels are related to type II diabetes. Both IGF-I and -II are mostly bound to the IGF-binding proteins, mainly to IGFBP-3, and the biological activity of both growth factors is dependent on the not-bound fraction. In a preliminary report we noted that adult insulin secretion is related to IGFBP-3 (5); however, this significant association could be explained by the positive relation of plasma IGFBP-3 levels with BMI, presumably illustrating the well-known augmenting effect of overweight on insulin secretion (6). In the present studies, we aim to elucidate a possible relationship of plasma IGF-II levels with pancreatic insulin secretion in healthy non-diabetic adult subjects, as assessed by both an oral glucose tolerance test and a hyperglycemic glucose clamp.

Subjects, materials, methods

Subjects

Fifty healthy non-diabetic (OGTT) subjects took part in this study (Table 1). They are part of studies reported in earlier papers (7;8). The local Ethical Committee had approved the study, and informed written consent was obtained from each participant. All subjects had normal values for routine laboratory measurements for hematology, HbA1c (upper normal limit 6.1%), lipids, and kidney, liver, thyroid and adrenal function.

Oral glucose tolerance test (OGTT)

Blood samples for glucose and insulin determinations were taken at baseline and at 30

minute intervals after the oral administration of 75 grams glucose (in 300 ml water), and put on ice.

Hyperglycemic glucose clamp
A hyperglycemic glucose clamp was performed during 180 minutes aiming at a glucose level of 10 mmol/l (arterialized blood sampling, 55°C). Blood samples for insulin determination were taken at 2 minute intervals during the first 10 minutes, and at 20 minute intervals thereafter, for determination of first phase (summation from 0 to 10 minutes) and second phase (average plasma insulin levels from 140 to 180 minutes) insulin secretion.

Laboratory measurements
Blood glucose was determined immediately with a glucose analyzer (YSI, Yellow Springs, Ohio, USA). Plasma insulin was determined by radioimmunoassay with ^{125}I-labelled insulin (IM 166, RC Amersham, UK). Plasma IGF-II (nmol/L) was determined by a specific RIA, as described previously (9). Plasma IGF-II was also expressed as standard deviation score (SDS) corrected for age and gender, as reported previously (9).

Statistical analysis
Data are presented as mean with SD. Linear correlations of plasma IGF-II, and its SDS, with plasma glucose and insulin levels before and after the OGTT, and with first and second phase secretion during the clamp were investigated. Multiple linear regression (MLR) analysis was also performed with the use of age, gender, body mass index (BMI) and waist-hip (WH-) ratio, as covariates.

Results

Plasma IGF-II levels were positively correlated with baseline plasma glucose ($p=0.004$), and with baseline plasma insulin levels ($p=0.021$) (Table 2).
Multiple linear regression indicated, that plasma IGF-II contributed significantly to the variance of basal glucose (partial coefficient $r=+0.27$, $p=0.037$), but not to that of plasma insulin levels.
In the univariate analysis, no significant associations were found between the first phase insulin secretion and IGF-II, while second phase insulin secretion was positively related to plasma IGF-II levels ($p=0.007$) (Table 2).
Multiple linear regression analysis indicated

Table 1: Baseline characteristics of 50 subjects, fasting, 30 minute and 120 minute plasma glucose and insulin levels after an OGTT, and first and second phase insulin secretion as determined during a 3 hour hyperglycemic clamp (10 mmol/L, 180 minutes) in 50 healthy subjects. Data is Mean ± SD.

Gender (f/m)	38/12
Age (year)	46.2 ± 6.2
BMI (kg/m^2)	25.7 ± 3.5
Waist-Hip ratio	0.82 ± 0.07
Basal plasma Glucose (mmol/L)	5.2 ± 0.5
120 min plasma Glucose (mmol/L)	6.7 ± 1.6
Basal plasma Insulin (pmol/L)	41.3 ± 20.3
30 min plasma Insulin (pmol/L)	303 ± 182
120 min plasma Insulin (pmol/L)	337 ± 282
First phase (pmol/L*10min)	836 ±505
Second phase (pmol/L)	347 ±216
Plasma IGF-II (nmol/L)	54.4 ± 9.9

that BMI has the largest impact on second phase secretion (partial coefficient r= 0.38, p=0.015). Plasma IGF-II levels contributed significantly to the variance of second phase insulin secretion (r=+0.28, p=0.044).
In general, the use of Standard Deviation Scores (SDS) for univariate and multiple linear regression led to the same results (Table 2).

Discussion

To our knowledge, the present studies are the first to evaluate the relationships between IGF-II and pancreas B-cell function. They indicate that plasma IGF-II levels and second phase insulin secretion are related, also after correction for age, gender, BMI, and waist to hip ratio. Pancreas beta cell function is closely and negatively related with insulin sensitivity: in obesity, insulin sensitivity is decreased with a marked increase in the pancreatic beta-cell function (up till two- to three-fold) (6). Therefore, body weight is a major determinant of beta-cell function. In a preliminary report, we already showed that the relationship of IGFBP-3 with beta cell function is mainly due to an interaction with BMI (5).
The IGF system (and thus IGF-I and IGF-II) in both transgenic rat and mice models, is closely involved in the development of the fetal pancreas. The present observation is the first supporting this relationship in humans. Both IGF-I and IGF-II affect positively proliferation and maturation of developing B-cells, although partly through different receptors. In both fetal and neonatal life, the synthesis of IGF-II, is a common feature of isolated human islets cells (2). The role of IGF-II in the pancreas of the fetus and neonate is likely to be that of a paracrine or autocrine mitogen and anti-apoptosis agent, acting through the type 1 IGF-receptor.
Human plasma IGF-II levels increase moderately during early childhood and remain on a stable level during adult life, reaching plasma values exceeding those of plasma IGF-I more three-fold. Since the estimated heritability of plasma IGF-II concentrations is around 66%, there must be important genetic traits for the expression of IGF-II (10). Moreover, the expression of IGF-II specific receptors in fetal life is higher than IGF-I receptors (2), and it is thus plausible that the impact of (changes in) IGF-II on the definite functional properties of the pancreatic cells is important. (1).

We conclude that beta cell function is related with IGF-II, independent from other known key factors such as obesity. Although it is

Table 2: Linear correlation coefficients of relationships of IGF-II with fasting and post-glucose load glucose and insulin levels (OGTT), and first and second phase insulin secretion parameters as determined with a hyperglycemic clamp (10 mmol/L, 3 hours) in 50 non-diabetic subjects. SDS IGF-II denotes Standard Deviation Score for IGF-II according to previously determined SDS IGF-II values for age, gender and BMI.

	IGF-II	SDS IGF-II
Fasting Glucose	+0.37 [c]	+0.38 [c]
120 min Glucose	+0.05	+0.08
Fasting Insulin	+0.33 [a]	+0.34 [b]
30 min Increment Insulin	+0.19	+0.19
120 min Insulin	+0.18	+0.21
First Phase	-0.11	-0.15
Second Phase	+0.38 [c]	+0.35 [b]

[a] $p<0.05$, [b] $p<0.02$, [c] $p<0.01$

tempting to speculate that properties of the insulin secreting cells are determined in early life, obesity has also a marked influence on actual B-cell function. Therefore, IGF-II may have a role in B-cell adaptation to obesity (induced insulin resistance) during adult life. Consequently, a thorough analysis of the IGF system, from fetal to adult life, with respect to the properties of the insulin secreting cells may improve the understanding of (adult) insulin secretion, and possibly also shed new light on the disturbances of B-cell function related to the development of type 2 diabetes mellitus.

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2.4
Fasting Plasma IGF-1 Levels in AGHD Predict the Level of Insulin Resistance after initiation of rhGH Therapy

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Increased insulin resistance (IR) has been reported in patients with adult-onset of GH deficiency (AGHD) after starting rhGH therapy and this could oppose the beneficial cardiovascular effects of GH substitution. Therefore, a useful marker that predicts the increase of IR during rhGH therapy could be of clinical value. In 11 AGHD patients, baseline plasma IGF-1 levels (before rhGH substitution) were negatively correlated with the delta HOMA index. Moreover, the delta plasma IGF-1 levels were positively correlated with the delta HOMA index. In conclusion, a baseline plasma IGF-1 level already gives an impression of the level of IR in AGHD patients that is obtained during rhGH treatment and, additionally, the rhGH dosage need to be titrated to avoid an exaggerated increase in IR.

Recently, clinical concern arose about the increased IR in short-term substitution studies with recombinant growth hormone (rhGH) in adult-onset growth hormone deficiency (AGHD) patients. In several studies, the incidence of diabetes mellitus type II is higher in GH substituted patients with adult and/or childhood onset GHD (1) and it is known for a long time that an increased IR is associated with increased cardiovascular morbidity and mortality (2). On the opposite, increasing clinical evidence showed that premature atherosclerosis is a clinical feature in the AGHD syndrome and that rhGH substitution improved the initially increased femoral intima media thickness (IMT) and endothelial dysfunction (measured with Flow Mediated Dilation; FMD) (3-6). If IR increase after rhGH substitution, the beneficial effect of rhGH upon the cardiovascular parameters could consequently be counteracted. Nowadays, the substitution of rhGH is only according to sex and age (7;8) without a regular follow-up of a subsequent IR.

In this short communication, we report the observation that baseline plasma IGF-1 level in AGHD could prospect the insulin sensitivity that result after 6 months of rhGH substitution.

Subjects and Methods

Subjects

Eleven adult GHD patients (9 men and 2 women), aged 49 ± 5 years, BMI 28.3 ± 3.1 kg/m^2 (Mean ± SD) participated in this intervention study. After being optimally substituted for other deficient pituitary hormones, the patients were treated with daily subcutaneous rhGH during 6 months. Before the start and after six months of rhGH substitution venous blood was drawn to analyse. Dosages of rhGH were titrated upon the adjusted plasma IGF-1 levels, according to their age and sex. The daily adminestered rhGH was similar in all patients (between 0.8 and 1.1 IU/day). None of the patients had additional treatment in the study period, had a positive family history for type II Diabetes or suffered from renal and or liver disease. All patients obtained written information about the protocol (approved by the ethical committee) and from all patients an informed consent was obtained.

Methods

In fasting conditions, plasma glucose, insulin and IGF-1 concentrations were assessed before and after rhGH replacement. The IR was estimated by calculating HOMA index (fasting insulin times fasting plasma glucose divided by 22.5) (9). Measurement of insulin and IGF-1 were performed in plasma samples with a radio-immuno assay (10).

Statistical analysis

Data are presented as median (range). Effects of rhGH substitution in AGHD patients were analyzed by a paired (two tailed) t-test. Pearson's correlation or Spearman's rank correlations were applied to evaluate relationships between parameters. Correlations are mentioned in the text if they reached statistically significance. Differences between before and after rhGH treatment are expressed as delta. A P value of 0.05 was considered significant. Statistical analysis was performed with Sigma Stat (Jandel Corporation).

Results

The increase in insulin secretion (C-peptide was also increased) was not sufficient to prevent the small but statistically significant increase in fasting glucose (4.6 vs. 5.1 mmol/L) (Table 1). Although, the plasma insulin levels increased it did not reach the level of significance. Overall insulin resistance as determined by HOMA index, increased after rhGH. As expected, a significant increase in plasma IGF-1 levels was observed after treatment with rhGH.

Plasma IGF-1 levels, before rhGH substitution, were negatively correlated with the delta HOMA index (delta HOMA index= -0.01 * (basal IGF- I level) + 1.96; r = 0.56) (Figure 1).

The delta plasma IGF-1 levels were positively correlated with the delta HOMA index (delta HOMA index = 0.01(delta IGF-1) - 0.40; r = 0.73).

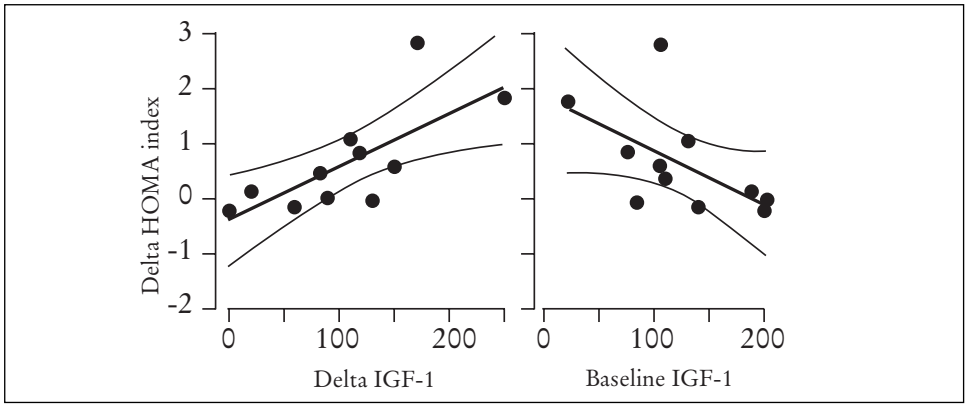

Figure 1. Relationships (with 95% confidence limits) between delta HOMA index and delta IGF-1 (ng/ml) (left) and between delta HOMA index and basal IGF-1 (ng/ml) (right).

Table 1: Effect of six months rhGH treatment on various plasma parameters

Parameter	Baseline	on rhGH treatment
Glucose (mmol/L)	4.6 (4.0-6.0)	5.1 (4.6-6.0) #
Insulin (mIE/L)	6 (3-17)	9 (2-21)
HOMA index	1.3 (0.7-4.5)	1.8 (0.6-5.6) #
IGF-1 (ng/ml)	102 (30-182)	212 (180-280) #

Results are Median (range). # P<0.05.

Discussion

In the present study, we showed that pre-treatment plasma levels of IGF-1 in AGHD patients could partially account for the definite change in HOMA index (basal insulin sensitivity) after rhGH therapy ($r^2 = 0.32$). Moreover, a decrease in insulin sensitivity in AGHD patients during treatment was associated with the incremental in plasma IGF-1 levels. Knowledge of plasma profiles of IGF-1 before and during rhGH substitution could therefore help in the individual titration of rhGH, and may optimize beneficial effects of rhGH treatment.

An increase in HOMA-index, due to elevation of both the plasma glucose and insulin levels, observed after rhGH substitution, reflect a decrease in insulin sensitivity. Short-term rhGH infusion results in hyperinsulinaemia, in impairment of insulin to suppress the hepatic de-novo glucose production and in stimulation of the peripheral glucose uptake and oxidation in skeletal muscles (11;12). Decreased insulin sensitivity is already described to be an independent cardiovascular risk factor, even in non-diabetic populations (13). The relation between hyperinsulinemia and cardiovascular mortality was confirmed in a large population during a 15-year follow-up study. In that study, the calculated HOMA index in subjects who died from cardiovascular disease was 2.11 (14). As previously noted the AGHD syndrome is associated with an increased mortality. While in the present study only one untreated patient exhibit a HOMA index above 2, the HOMA index was >2 in five patients on rhGH treatment. Therefore, the HOMA index in AGHD that results from GH substitution needs to be of concern during clinical follow-up.

In this study, lower plasma IGF-1 levels at the start of rhGH substitution result in more IR in rhGH treated AGHD patients. Plasma IGF-1 levels are for 35% genetically determined. A possible explanation could be a less intrinsic insulin secretion capacity by the pancreas due to initially low plasma IGF-1 levels. Prenatal, the development of pancreatic β cells is influenced by IGF-1; low plasma IGF-1 in the prenatal period result in a lower insulin secreting capacity. IGF-1 is an important regulator in the differentiation and proliferation of the pancreatic β cell (15). From index subjects and family members with IR, a genetically determined low IGF-1 expression was associated with a 7.5 fold increased risk for diabetes (95 percent confidence interval of odds ratio 2.8 to 16.2 (16). Therefore, the increase in IR after rhGH substitution may be interpreted as an in-born capacity reduction of the pancreatic gland that is not capable to oppose the increased glucose load (possibly due to increased gluconeogenesis that is related to rhGH substitution) in the general circulation. Therefore, the enhanced insulin response of the pancreatic β cell may be primarily considered to be genetically determined and only in a secondary manner, after the substitution of rhGH and the consequent elevation of the systemic glucose load will lead to IR.

From our results, one could consequently derive that less concern about IR is needed in these AGHD patients with higher plasma baseline IGF-1 levels. In line with this, AGHD patients with lower baseline IGF-1 levels will possibly need additional treatment to lower the insulin resistance by for example drugs like biguanides (Metformin) or insulin therapy. The increase in IR is also associated with the IGF-1 elevation after rhGH substitution and this observation advocate an optimal titration of rhGH substitution with special attention to the AGHD patients with initially lower plasma IGF-1 levels.

In conclusion, the increase of insulin resistance in AGHD after substitution of rhGH could be optimally managed with special attention to the baseline plasma IGF-1 levels.

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2.5
Endogenous glucose production rate during GH therapy in adult-onset growth hormone deficiency is maintained due to an elevated contribution of gluconeogenesis

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GH therapy in adult-onset growth hormone deficiency (AGHD) is associated with changes in glucose homeostasis. The circulating availability of glucose results from gluconeogenesis (GNG) and glycogenolysis (GL). In order to investigate GNG and GL in AGHD, GNG and GL in AGHD patients (n=5) were analyzed, before and after 6 and 12 months of GH therapy, in comparison to mild obese healthy volunteers (n=5), matched for age, sex and BMI. The total glucose turnover was estimated after intravenous infusion of 6,6 2H2 glucose, with a fractional contribution of gluconeogenesis after oral ingestion of 2H2O. The contribution of GNG via pyruvate pathway was determined through 2H enrichment in C6/body water, and via pyruvate + glycerol pathways through 2H enrichment in C5/body water. Glycogenolysis was calculated as the difference between glucose production and gluconeogenesis. On baseline, fasting plasma levels of insulin, glucose, C-peptide and HOMA did not differ between AGHD patients and control subjects. During GH therapy, insulin sensitivity decreased, but not significant. Total glucose turn-over in AGHD was not increased compared to control subjects and remained constant during GH therapy. Pretreatment, GL in AGHD was increased (and inverse for GNG), compared to control subjects. Due to GH therapy, the percentage contribution of GL decreases (from 34 % on baseline to 25% on rhGH (+12)), in favor for GNG (with pyruvate as a major substrate). Plasma glutamate levels increased during GH therapy. GNG was inversely associated with plasma IGF-2 levels ($r = -0.61$, $p < 0.01$).

Insulin sensitivity in AGHD patients tends to decrease during GH therapy. The total glucose turnover in AGHD patients remained stable, GL continue to be the major source of glucose in the postabsorptive phase, before and during GH therapy (in comparison to mild obese control subjects). Simultaneously, an increase in GNG (with pyruvate as a major precursor) is observed during GH therapy that may explained by an increase in fatty acid oxidation due to GH lipolytic effects.

Although results are conflicting, most reports note distinctive changes in glucose homeostasis in AGHD patients during GH therapy, such as a decrease in insulin sensitivity (1-3). Consequently, this may indicate a reduced availability of glucose for tissue metabolism (if not compensated by higher plasma insulin levels). An impaired glucose metabolism, and especially glycolysis, will limit cognitive functions, physical activity, and other glucose dependent processes. In humans, optimal plasma levels of endogenously derived glucose (in a range of 5 to 8 mmol/L) are maintained in a well-regulated balance in GNG, GL and glycolyse. In order to understand better the consequences of GH therapy on the molecular level of glucose metabolism, thorough analysis of both GNG and GL, with respect to EGP in AGHD patients, may therefore be of interest.

So far, no studies with a direct assessment of GNG and GL in AGHD patients are performed, or the effects of GH therapy on it. Moreover, the previous used methods to analyze GNG were insufficient and were an important limitation in the progress of knowledge about GNG and GL in humans. In healthy subjects who receive a 12 h GH infusion, Butler et al (4) measured carbon dioxide incorporation into glucose as quantitative estimation of GNG. However, this method is not sufficient, mainly due to dilution of marked labels at the oxalo-acetate level. This dilative effect can be avoided by recent developed method that uses deuterium incorporation into glucose (after oral

administration of ^2H_2O) that provides whole body estimate of total GNG (and by calculation GL).

In this study, we aim to analyze in AGHD patients the contribution of GNG and GL, in the total EGP, compared to that in matched healthy control subjects, and in AGHD patients during 6 and 12 months GH therapy.

Subjects and methods

Patients

The study population consisted of 5 male patients with AO-GH deficiency with a mean age of 50 years (range 44-58) and a mean body mass index of 29.2 kg/m^2 (range 25.0-33.8 kg/m^2). Patients were recruited from the department of endocrinology in UMC Utrecht, the Netherlands. The origin of the GH deficiency was panhypopituitarism after neurosurgery for a pituitary adenoma (time interval after surgery was at least 6 months). GH deficiency was confirmed by at least two GH stimulation tests. All patients were substituted for additional deficient pituitary axes, such as with thyroxin, corticosteroids, sex hormones and 3 patients receive desmopressin. Patients were only included in this study, if these hormones were optimally substituted. None of them were known with diabetes. The clinical characteristic are shown in table 1.

After inclusion and baseline measurements (including stable isotope studies), GH hormonal replacement (Norditropin, Novo, Nordisk) was started. After 6 months and 12 months GH therapy, the patients were subjected to a study day for the second time and third time, respectively. One patient could not complete the whole study because of surgery after 8 months of rhGH therapy. A weight maintaining diet has been described for three days prior to the study day to both the patients and control subjects. While on diet, excessive exercise and alcohol intake was not allowed.

The dietary compliance was evaluated from a diary record, which reports three days' diet prior to investigation in the metabolic ward. At baseline, the total energy intake in AGHD was 2088 ± 228 kcal/day, compared to 2150 ± 151 in control subjects (not significantly different). Moreover, GHD patients consumed 42 ± 3 % of carbohydrates, 36 ± 2 % fat and 21 ± 2 % protein (percentages were similar in control subjects). The patients following GH treatment (6 months) consumed a total of 2513 ± 188 kcal/day, 43 ± 3 % carbohydrates, 36 ± 2 % fat and 21 ±4 % protein. The patients following long term GH treatment (12 months) consumed a total of 2122 ± 188 kcal/day with a 44 ± 0 % carbohydrates, 36 ± 1 % fat and 21 ± 2 % protein.

Control subjects

The control subjects consisted of 5 men with an average age of 52 years (range 45-58) and BMI of 28.2 kg/m^2 (range 24.0-29.4 kg/m^2). The control subjects consumed 45 ± 1 % of carbohydrates, 35 ± 1 % fat and 19 ± 1 % protein.

The institutional Ethical committee approved the study protocol and each subject gave his or her informed written consent to participate.

Methods

Recently, a method has been developed in which Deuterium oxide (^2H_2O) can be used as tracer to measure gluconeogenesis (5;6). Briefly, the principle is as follows: ^2H is bound to the C3 of Phosphoenolpyruvate (PEP) that becomes eventually the C6 of glucose. GNG from glycerol also results in labeling of the C5 of glucose via incorporation of the label into C2 of glyceralaldehyde-3-P, which equilibrates with dihydroxyacetone-3-P. Because of rapid cycling between Glucose-6-P and fructose 1,6-P, there is an addition of hydrogen from body water to C2 by both GNG and glycogenolysis. Enrichment at C2 of glucose was approximately equal to the enrichment in body water in

both control subjects (5;5;7), in patients with malaria falciparum and cirrhosis (8;9) and in subjects in whom effects of fatty acids elevation on GNG was examined (10). Thus, enrichment of the deuterium bound to carbon 6 of glucose to that in body water following ^2H_2O administration equals the fraction of glucose formed by gluconeogenesis via pyruvate while enrichment of the deuterium bound to carbon 5 of glucose to that in body water equals the fraction of glucose formed by gluconeogenesis including glycerol. Use of non-recycling [6,6 $^2H_2$] glucose label provide a value for total hepatic glucose production. Because plasma deuterated glucose is analyzed, there is no discrimination between renal and hepatic GNG and thus whole body gluconeogenic fraction is measured.

Experimental design

One day before the study day, all subjects collected 24 hours urine specimen, which was analyzed for nitrogen (N) and for background of body water. Before the study day, participants have their last oral intake at 6.00 P.M. On the study day, subjects were admitted to the University Medical Center Utrecht, between 07.00 and 07.30 am. To avoid physical activity during the study, subjects came by bus or by car and were transported in a wheel chair in the hospital. Body weight was measured and a dorsal hand vein was cannulated for "arterialized" venous blood sampling (drawn from a hand vein in a heated box (60°C). Blood samples were drawn for measurement of basal enrichments and other parameters for insulin sensitivity (glucose, glucagon, HbA1c, insulin, C-peptide), additional hormones (free T4, free T3, cortisol), the IGF system (IGF-1, IGF-2, IGFBP-1, IGFBP-3), the precursors for GNG (pyruvate, lactate, alanine, glutamate, glutamine) and the lipolytic products (free fatty acids, glycerol).

A cubital vein in the contralateral arm was cannulated for the infusion of 6,6 $^2H_2$ glucose which started at 10.00 AM. At t=10.00 a priming 6,6 $^2H_2$ glucose 98% (26.4 μmol/kg prime) and a maintenance dose of 0.33 μmol/kg/hr min was given for two hours to determine the total rate of glucose appearance (Ra). Both catheters were flushed with heparin. During the study day, no food was given and subjects were allowed to drink water that was enriched to 0.5% with ^2H_2O to maintain steady state. To achieve an enrichment of ^2H in body water of approximately 0.5%, they were given orally ^2H_2O (1g/kg body water) (>99.8 % enriched; Cambridge Isotopes, Andover, MA) at t= 8.00, 8.30, 9.00, 9.30 en 10.00 AM. The next two hours were allowed for equilibration of the ^2H_2O. After emptying the bladder at t=12.00, urine have been collected between t=12.00 and t=13.00. At t= 10.00 AM, a blood sample for background enrichment was drawn. At t=12.00, 12.15 and 12.30 hr heparin blood samples were drawn from "arterialized" venous blood for the measurement of deuterium enrichments in blood glucose. After the last blood sample, the intravenous lines were removed and the subjects were given a regular meal.

Standard Analytical procedures

Plasma glucose was measured with standard laboratory methods on a Vitros 950 (Johnson & Johnson, Clinical Diagnostics, NY, USA). HbA1c was measured using a high-performed liquid chromatography method. Free T4 was measured with a full automatic competitive micro particle chemolumnisence immuno-assay. Free T3 was measured using an immuno enzymetric assay (a competitive ELISA using streptavidin technology performed on a ES300, Roche Diagnostics GmbH, D-68298 Mannheim). Glucagon was measured after alcohol extraction followed by a competitive radioimmmnoassay using a polyclonal antibody raised in rabbit against Pancreas Glucagon. Cortisol was measured with immuno chemiluminiscention. IGF1 was measured with an immuno chemolumnisence (Nichols Institute Diagnostics, San Juan Capistrano, USA). Concentrations of plasma IGF-2, IGFBP-3 (mg/L) and IGFBP-

1 (μg/L) were determined by specific RIAs, as described previously (11). Insulin was measured with a competitive radioimmunoassay using a polyclonal anti-insulin-antibody (CARIS46), 125I-Insulin (IM166, Amersham Nederland bv) as a tracer and Humuline (YV2632 AMV Lilly, Indpls,USA) as a standaard. C-peptide was measured with a competitive radioimmunoassay (MD315, Euro-Diagnostica, Malmö, Sweden). Pyruvate, FFA was measured on a Cobas Fara (Roche, Germany) using an enzymatical method. Lactate and glycerol were measured with standard laboratory method on a Hitachi-911 (Roche, Germany). Plasma alanine, glutamate and glutamine concentrations were measured on a Biochrom 20 automatic amino acid analyzer (Pharmacia Biotech, England).

Body composition measurements
Body height was measured to the nearest 1.0 cm by using a wall mounted stadiometer, and body weight to the nearest 0.05 kg. BMI was calculated as weight (kg) divided by height squared (m^2). Body fat was assessed by bioelectric impedance analysis (BIA) (tetrapolar BIA-101 analyzer: RJL-Systems, Detroit), based on resistance and reactance measurements. Resistance and reactance were measured (in) after appliction of an alternating current of 800 μA at 50 kHz with the electrodes placed as described by Lukaski et al (12). Body fat was calculated by using the manufacturer-supplied equation.

Indirect calorimetry
At t=0.900 A.M., ventilation, oxygen consumption and carbon dioxide production were calculated breath to breath (Oxycon Sigma (Mijnhardt, Jaeger)). Gas analyses were automatically performed by using room air as a reference. Subjects were asked to breath normally. Data were recorded at 30 seconds interval during 30 minutes. Before measuring, two minutes of adaptation period were introduced.Net glucose and lipid oxidation rates were estimated with the use of former described method (13) and protein oxidation rates were estimated by the assessment of serum nitrogen (N) concentrations and urinary excretion rates of N.

Nitrogen measurement
The nitrogen was converted to ammonia by wet oxidation according to the Kjeldahl technique in a digestion mixture of sulfuric acid, sodiumsulphate and mercuric sulphate.

Procedures for measuring glucose metabolism
In order to measure glucose kinetics 5 ml plasma for enrichment on C5 and 2 ml to determine enrichment on C6 was immediately deproteinized using equal volumes of prechilled 10% perchloric acid and stored at -20°C. The other samples were immediately measured or stored below -20°C. The deuterium enrichment in bound to carbon 5 and 6 of blood glucose was determined as described elsewhere (5;6) with some slight modifications. In short, supernatant obtained after deproteinizing blood samples was passed through a mixed cation of AG1-X8 (formate form) and AG 50 w-X8 (H+). Glucose in the effluent was isolated with use of high pressure liquid chromatography using a Aminex HPX-87c column (Bio-Rad, the Netherlands) with water at 80°C as solvent. In order to isolate C6, 0.5-1mg was oxidized with periodate to form formaldehyde. This was converted to hexamethylenetetramine (HMT). Enrichment at C5 of a portion of glucose is determined chemically by conversion of glucose to xylose with removal of C6. The xylose is oxidized with periodate to form formaldehyde. Plasma glucose enrichment was determined by gas chromatography mass spectrometry (GC-MS) on a Hewlett-Packard HP 5890 type II gas chromatograph interfaced to a HP 5989B mass spectrometer. The gas chromatograph was equipped with a coating CP Sil 19CB capillary column (Chrompack, Bergen op Zoom, the Netherlands). Injection (2 μl) was performed in a split mode (1:20). The flow rate of carrier gas (helium) was 1 ml/min. Injector temperature was 240°C and oven temperature was programmed starting at 210°C for 1 minute,

then increased from 210°C to 280°C at 25°C/min and maintained at 280°C for three minutes. The HMT eluted at approximately 4.5 minutes. The source and quadruple temperature were 250°C and 150°C, respectively. Fragments 140, 141 and 142 were monitored and enrichment was determined using a calibration curve whereby measured ion abundance ratios are correlated with enrichment of standards of known isotopic composition. The distribution of different masses in HMT was used to calculate deuterium enrichments by mass isotope distribution analysis(14). Comparing both methods revealed a correlation of r^2=0.995 (y=0.908x-0.0061, n=54). Since we observed that the tracer/tracee measurements are affected by the quantity of HMT analyzed, an initial "pre-run" was performed to determine relative sample concentrations. The second run was performed after adding appropiate volumes of solvent to each vial in order to narrow sample concentration within the concentration measured within the calibration curve. All samples were measured in duplicate, with a CV<3%.

The enrichment in urinary water was determined after reaction with calcium carbide to form acetylene (15). The *m/z* signal ratio for acetylene measurements were performed in triplicate (different vials) with a CV% <4%.

Calculations and statistics

To measure glucose production, an infusion of [6,6 $^2H_2$] glucose is given simultaneously with ^2H_2O. The percentages of the HMT molecules, with two 2H bound to C6, are determined and are measured as percentage of the molecules of molecular mass 142 (m+2).Glucose production is calculated as:

Table 1: Clinical and fasting biochemical characteristics of GHD patients before and after respectively 6 and 12 months of rhGH replacement therapy compared with age, sex and BMI matched control subjects

	Baseline	6 months rGH	12 months rGH	Control subjects
N	5	5	4	5
Age	50 ± 2	50 ± 2	50 ± 1	52 ± 2
Body weight (Kg)	96.2 ± 5.1	97.4 ± 6.9	95.7 ± 6.5	85.3 ± 3.6
BMI (Kg/m^2)	29.2 ± 1.7	29.5 ± 1.9	29.8 ± 2.4	28.2 ± 1.1
FM (Kg)	23 ± 3	22 ± 3	23 ± 4	22 ± 2
FFM (Kg)	73 ± 5	75 ± 5	73 ± 5	64 ± 3
FM (%)	24 ± 3	23 ± 3	23 ± 4	25 ± 2
FFM (%)	76 ± 3	77 ± 3	77 ± 4	75 ± 2
WHR	0.96 ± 0.03	0.94 ± 0.04	0.93 ± 0.05	0.94 ± 0.02
RQ	0.80 ± 0.02	0.79 ± 0.02	0.78 ± 0.02	0.79 ± 0.03
M (kcal/24hr)	1038 ± 78	1269 ± 139	1242 ± 191	1138 ± 95
Glucose oxidation (mg/kg/min)	0.81 ± 0.28	0.81 ± 0.25	0.73 ± 0.24	1.13 ± 0.07
Fat oxidation (mg/kg/min)	0.64 ± 0.11	0.81 ± 0.34	0.83 ± 0.18	0.77 ± 0.08
Protein oxidation (mg/kg/min)	0.60 ± 0.02	0.51 ± 0.05	0.51 ± 0.11	0.58 ± 0.07

Data respresent Mean ± SEM. BMI= Body mass index, FM= Fat mass, FFM= Fat free mass, WHR=waist-hip ratio, RQ= respiratoir quofficient, M= Metabolic rate.

Data represent mean ± SEM

Infusion rate (0.33 μmol/kg/min)/ enrichment (M+2) at blood glucose C-6. The fraction of blood glucose formed by GNG from pyruvate was calculated by one half of the enrichment at carbon 6 (M+1) divided by the enrichment in body water. A factor of 0.5 is used since two hydrogen atoms are bound to carbon 6. The percent contribution of GNG from pyruvate + glycerol to plasma glucose was calculated as the ratio of the enrichment at C5/enrichment at body water in each subject. The rate of GNG from both pyruvate and glycerol and pyruvate was calculated by multiplication of the total glucose production by fractional GNG. Glycogenolysis was calculated from the difference between the rates of total glucose production and GNG (from glycerol and pyruvate). Data are represented as mean ± SEM, expressed as %, μmol/kg/min and μmol/kg FFM/min. Statistically significance (which was set at $p<0.05$) of difference was tested using a paired t-test for patients and a two sample t-test was used to compare patients with control subjects. Log transformation was used in non-normally distributed parameters.

Results

Body composition

Body weight (kg), BMI (kg/m^2), WHR and Fat Free Mass (FFM) were not significantly different between AGHD patients (on baseline, rhGH (+6) and rhGH(+12)), and control subjects (table 1).

Indirect calorimetry

The resting metabolic rate in AGHD tended to increase from 1038 ± 78 kcal/24 h to 1269 ± 139 kcal/24 h after 6 months to 1242 ± 191 kcal/24 h after 12 months rhGH therapy (table 1). No difference in resting metabolic rate was found at baseline in AGHD, compared to control subjects. Glucose oxidation was higher than fat oxidation in both AGHD at baseline and control subjects. The oxidation of fat in AGHD patients increased during GH therapy, with a decrease in glucose oxidation, however not significant (table1).

Circulating metabolites and hormones

Glucose and Insulin

Fasting plasma glucose levels in AGHD were not increased, as compared to BMI matched control subjects. GH therapy tended to increase plasma glucose levels (table 2). Insuline, C-peptide, pyruvate and HOMA-ratio were not significantly different between patients (before and during GH therapy) and control subjects (table 2). Glycosylated hemogloblin (Glyc-HB) was unchanged during GH therapy (table 2). Fasting insulin levels in AGHD tended to increase during GH therapy, but at baseline no difference with control subjects was found. Fasting plasma insulin concentrations correlated positively with the degree of fat mass (Kg) ($r= 0.75$, $p<0.0001$; grouped results of all participants).

Hormones

No change in plasma free T4 and free T3 levels in AGHD was found during GH therapy. Plasma glucagon and cortisol levels tend to be higher in GH treated AGHD patients compared to control subjects, but no significant difference was found.

IGF system

The plasma levels of IGF-1, IGF-2 and IGFBP-3 were lower in AGHD patients than in the control subjects. The IGF-1 concentration was significantly higher at 6 months and at 12 months rhGH therapy, compared to pretreatment levels ($p<0.05$), and significantly higher at 12 months rhGH therapy, compared to 6 months rhGH treatment ($p<0.01$) (table 2). Both IGF-2 and IGF BP-3 increased during GH therapy.

Glucose precursors

FFA (table 2) as well as the other gluconeogenic substrate precursors, such as lactate, alanine, glutamine and glycerol (table 3), were not significantly different between AGHD patients and control subjects and

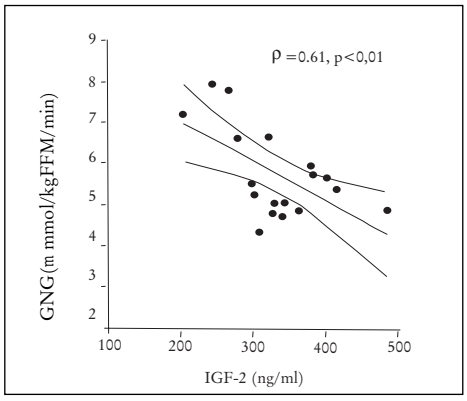

Figure 1. The association-curve between the GNG (y-axis) and plasma IGF-2 levels (x-axis).

these parameters did not change during GH therapy. In contrast, glutamate was significantly higher (P<0.05) at study entry, compared with control subjects, and sustained higher during GH therapy for 6 and 12 months (table 3). There was a significant decrease in the urinary N excretion after 6 months therapy (but not after 12 months GH therapy due to higher variance), compared to pretreatment levels.

Glucose metabolism

The postabsorptive rates of endogenous glucose production, GNG and glycogenolysis are presented in table 4. There was no difference between AGHD patients and control subjects in endogenous glucose production, expressed as μmol/kg/min or as as Mmol/kg FFM/min. During GH therapy, total glucose rate remained constant. GL, expressed as μmol/kg FFM/min, was significantly increased in pretreatment GH period (2.7 ± 0.3 μmol/kg

Table 2: Fasting biochemical charactersitics of GHD patients before and after respectively 6 and 12 months of rhGH replacement therapy compared with age, sex and BMI matched control subjects.

	Baseline	6 months	12 months	Control subjects
Glucose (mmol/L)	5.0 ± 0.3	5.2 ± 0.2	5.6 ± 0.5	5.4 ± 0.2
Gly-Hb (%)	5.8 ± 0.1	5.6 ± 0.2	5.6 ± 0.1	5.5 ± 0.2
Free T4 (pmol/L)	14 ± 2	15 ± 1	15 ± 3	15 ± 1
Free T3 (pmol/L)	6.2 ± 0.4	7.8 ± 1.0	6.9 ± 0.7	6.7 ± 0.3
Glucagon (pmol/L)	29 ± 4	32 ± 6	34 ± 18 [&]	29 ± 3
Cortisol (μmol/L)	0.60 ± 0.09	0.61 ± 0.12	0.61 ± 0.06	0.45 ± 0.10
IGF-1 (ng/ml)	117 ± 25	198 ± 27 *	154 ± 23 * #	133 ± 12
IGFBP-1 (μg/L)	33 ± 7	28 ± 7	42 ± 25	23 ± 7
IGFBP-3 (mg/L)	1.35 ± 0.15	1.62 ± 0.06	1.76 ± 0.29	1.86 ± 0.18
IGF-2 (ng/ml)	301 ± 27	369 ± 36	330 ± 25	382 ± 23
Insuline (mE/L)	8 ± 3	12 ± 5	13 ± 7	12 ± 3
C-peptide (nmol/L)	0.92 ± 0.19	1.1 ± 0.3	1.1 ± 0.4	0.97 ± 0.12
Pyruvate (μmol/L)	83 ± 14	70 ± 7	74 ± 7	62 ± 5
FFA (μmol/L)	1095 ± 172	1255 ± 73	1244 ± 253	1513 ± 341
HOMA-ratio	1.9 ± 0.7	3.0 ± 1.2	3.6 ± 2.3	3.0 ± 0.5
N (g/day)	12.9 ± 0.8 [@]	11.7 ± 0.8 *	10.5 ± 2.9	11.6 ± 0.8

Data represent mean ± SEM

[&]: 1 data ontbreekt nog (infusie 21)

* p<0.05 compared to basal situation

[#] p<0.01 compared to 6 months treatment

[@] p <0.05 compared to control subjects

FFM/min; p<0.01), and after 6 months therapy (2.5 ± 0.3 μmol/kg FFM/min; p<0.05), compared to control subjects (1.5 ± 0.1 μmol/kg FFM/min). The percent contribution of GNG from pyruvate to glucose production was significantly (P<0.05) lower at basal situation (44.6 ± 2.4 %) compared to control subjects (55.7 ± 3.2%). This is also true when expressed in μmol/kg FFM/min although this was not statistically different (table 4). The percent contribution of GNG from pyruvate + glycerol to glucose production was statistically lower at baseline and at 6 months treatment compared to control subjects (P<0.05) and gradually increased during rhGH treatment; 66.7 ± 3.1 at baseline to 68.5 ± 3.8 and 75.6 ± 1.9 at 6 and 12 months of rhGH treatment respectively. However, when expressed as μmol/kg FFM/min it was not significantly different. In the whole data set, GNG (μmol/kg FFM/min) was negatively associated with plasma IGF-2 levels (R=-0.605, p=0.006) (fig.1).

Table 3: Plasma GNG precursors levels in GHD subjects and on respectively 6 and 12 months treatment on rhGH

	Baseline	6 months	12 months	Control subjects
Lactate (mmol/L)	1.6 ± 0.3	1.5 ± 0.3	1.4 ± 0.1	1.4 ± 0.1
Alanine (μmol/L)	304 ± 16	324 ± 27	335 ± 60	318 ± 14
Glutamate (μmol/L)	125 ± 12 @	114 ± 11 @	124 ± 12 @	78 ± 10
Glutamine (μmol/L)	451 ± 33	400 ± 78	482 ± 17	517 ± 23
Glycerol (mmol/L)	0.16 ± 0.05	0.18 ± 0.03	0.24 ± 0.05	0.13 ± 0.04

Data represent mean ± SEM

@ p<0.05 compared to control subjects

Table 4: Glucose turnover of GHD patients before and after respectively 6 and 12 months of rhGH replacement therapy compared with age, sex and BMI matched control subjects

	Baseline	6 months	12 months	Control subjects
Glucose Ra (μmol/kg/min)	10.5 ± 0.3	10.8 ± 0.5	9.5 ± 0.7	10.2 ± 0.3
Glucose Ra (μmol/kg FFM/min)	8.0 ± 0.5	8.4 ± 0.7	7.3 ± 0.9	7.6 ± 0.4
GNG Pyruvate (%)	44.6 ± 2.4 @	47.4 ± 1.2	53.2 ± 4.2	55.7 ± 3.2
GNG Pyruvate (μmol/kg FFM/min)	3.6 ± 0.2	4.0 ± 0.3	3.6 ± 0.4	4.3 ± 0.4
GNG pyruvate+glycerol (%)	66.7 ± 3.1 #	68.5 ± 3.8 @	75.6 ± 1.9	79.8 ± 2.2
GNG Pyruvate +glycerol (μmol/kg FFM/min)	5.4 ± 0.5	5.7 ± 0.4	5.5 ± 0.8	6.1 ± 0.5
Glycogenolysis (μmol/kg FFM/min)	2.7 ± 0.3 #	2.5 ± 0.3 @	1.8 ± 0.2	1.5 ± 0.1

@ p<0.05 compared to control subjects.

# P<0.01 compared to control subjects.

Discussion

Endogenous glucose production in AGHD patients is maintained on a stable level due to an increase in gluconeogenesis during GH therapy. Probably, this increase in GNG during GH therapy is the consequence of a decrease in insulin sensitivity. On the other hand, less potency to compensate the decrease in insulin sensitivity (by components of the IGF system, such as IGF-2) during GH therapy may be another explanation. Animal models with defects in the expression of IGF-2 receptors or with defects in the related IRS-2 pathway showed no capacity in the compensation for a prodiabetic phenotype (16;17). Indeed, in our study pooled plasma IGF-2 levels were found to be negatively associated with pooled GNG.

Although several earlier reports noted decreased insulin sensitivity during GH therapy (18;19), a recent report found that the higher start dosages of GH in those early days of GH treatment were related to these negative effects on glucose homeostasis (20). Even opposite effects of GH therapy on insulin sensitivity were found; a study with a hyperinsulinemic-euglycemic clamp in AGHD patient showed that 7 years of GH treatment may prevent from an age-related decline in insulin sensitivity (19). Indeed, in this study no increase in HOMA index was observed, although a tendency existed for higher levels of insulin during GH therapy. In one study, we compared AGHD patients with BMI- and age matched control subjects on baseline, and insulin sensitivity was in the mild obese control subjects higher than in AGHD patients (although not significant).

The metabolic rate in AGHD patients increased slightly, but not significantly, during GH therapy. In previous studies, rest metabolic rate (RMR) in AGHD patients increased after 3 to 6 months of GH therapy, and the change in RMR could be explained for about 60% by the change in FFM (21;22). Moreover, a decrease in FFM in AGHD patients was observed in the first 6 weeks of the 24 weeks GH therapy, with a simultaneous increase in RMR (23). In line with this, we also found no change in FFM during GH therapy. The glucose oxidation, as a principal energy source in humans, (as estimated by RQ measurements) is about 60% in both AGHD patients and control subjects with only a small decrease during GH therapy. Fat oxidation in AGHD patients increased during GH therapy, but not significant. Recombinant GH substitution in fasting healthy volunteers increased oxidation of fatty acids with a related decrease in serum alanine levels (24). Our observations are in concordance with Hoffman et al, who showed no difference in energy expenditure (by indirect calorimetry) and fuel utilization (fat and glucose oxidation) between AGHD patients and control subjects (25).

In this study, total glucose turnover was not different between AGHD patients and control subjects. This observation is in line with previous reports that explore post absorptive state glucose metabolic clearance rate and glucose turnover in AGHD (26), also after start of GH therapy no significant change in glucose turnover was found (27). Additionally, so far, no other study showed that the percentage of GNG is markedly decreased in pretreatment AGHD patients in comparison to mild obese control subjects. On the other hand, GL in AGHD was increased. In physiological conditions, a decrease in GNG is directly related to an increase in glycogenolysis to prevent harmful hypoglycemic events. This inverse relationship between GNG and GL reflect an autoregulatory pathway, that activates GL in response to a decreased level in GNG. Since control subjects were matched for BMI, the difference in GNG can consequently not explained by the mild obesity. Other factors, probably induced by GH and translated towards the IGF system, may be more adjusted. While previous reports mention normal insulin sensitivity in GH

deficient subjects, some other studies report an increased insulin resistance in obese GH deficient subjects (28), and an impaired glucose tolerance, in especially female GH deficient subjects. GNG is under influence of the hormone glucagon, that slightly increases in parallel with GNG in this study. The increased amounts of lipolytic products, such as glycerol and fatty acids, during GH therapy will decrease the entry of citrate in the Kreb cyclus, and favours acetyl-Co A to the oxaloacetate-malate reaction with formation of GNG substrate. In line with conditions that are associated with an accelerated lipolysis, such as a prolonged fasting period (29) and in diabetes mellitus (30) glycerol acts as a precursor in gluconeogenic processes. Although, FFAs serve as a substrate for de-novo glucose synthesis (31), high FFA levels also inhibit insulin action on the receptor level. Consequently, activation of glycogen synthase by insulin is inhibited (32).

The significant decreased GNG in AGHD patients may also be due to failure of the expression of rate determining enzymes in GNG, such as phosphoenol pyruvate carboxy kinase, Gluc-6-phosphatase and fructose 1,6 biphosphatase, or by reduced supply of amino acids that serve as glucose precursors in GNG. In AGHD patients, severe defects of in vivo insulin sensitivity and skeletal muscle intracellular glucose phosphorylation and glycogen synthase activity persist with 24 months rhGH therapy (approximately 0.22 IU/kg week) (33;34). Although limited amounts of glycogene in AGHD patients are present, GL contributed most to EGP. After start of GH therapy, the increase in GNG in AGHD is mostly related to pyruvate as a C3 GNG precursor. The amino acid alanin in humans provides most of the substrate, that is needed to form pyruvate with subsequent GNG (Felig cycle), and plasma levels of alanine in AGHD patients tend to increase during GH therapy. GH has a nitrogen sparing effect; and this related to a decrease in N urine and an increase in glutamate efflux from the liver (35;36). Indeed, plasma glutamate levels in AGHD patients were higher and a decrease of N in 24 hour collected urine was found during GH therapy. Interesting, the plasma glutamate levels in AGHD patients were already increased before treatment, and this suggests that not only GH induces the glutamate efflux. Although baseline plasma levels of alanine (most important GNG substrate) and lactate were comparable in AGHD patients and control subjects, the plasma glutamine levels were decreased in GHD patients and glutamate significantly increased in those on therapy (table 3). In terms of adding new (non-glucose derived) carbons to the plasma glucose pool, glutamine appears to be as important as lactate (37). In humans in a postabsorptive condition, glutamine is an important precursor for renal GNG which contributes 20% to 25% of the whole body glucose production (38).

Taken together, in comparison with control subjects that are matched for BMI, the level of peripheral insulin resistance, the glucose production rate and level of glucose oxidation are comparable to AGHD patients, before and during GH therapy. However, the contribution of GNG as part of total glucose production rate in AGHD patients increase with alanine, pyruvate and glutamine (with a possible increase of renal GNG) as probable precursors, in parallel with a decrease in urinary nitrogen excretion (that is a nitrogen sparing effect of GH). The negative relationship between GNG and plasma IGF-2 levels is not elucidated yet, but suggests an interesting relationship, also in humans, between components of the IGF system and glucose homeostasis.

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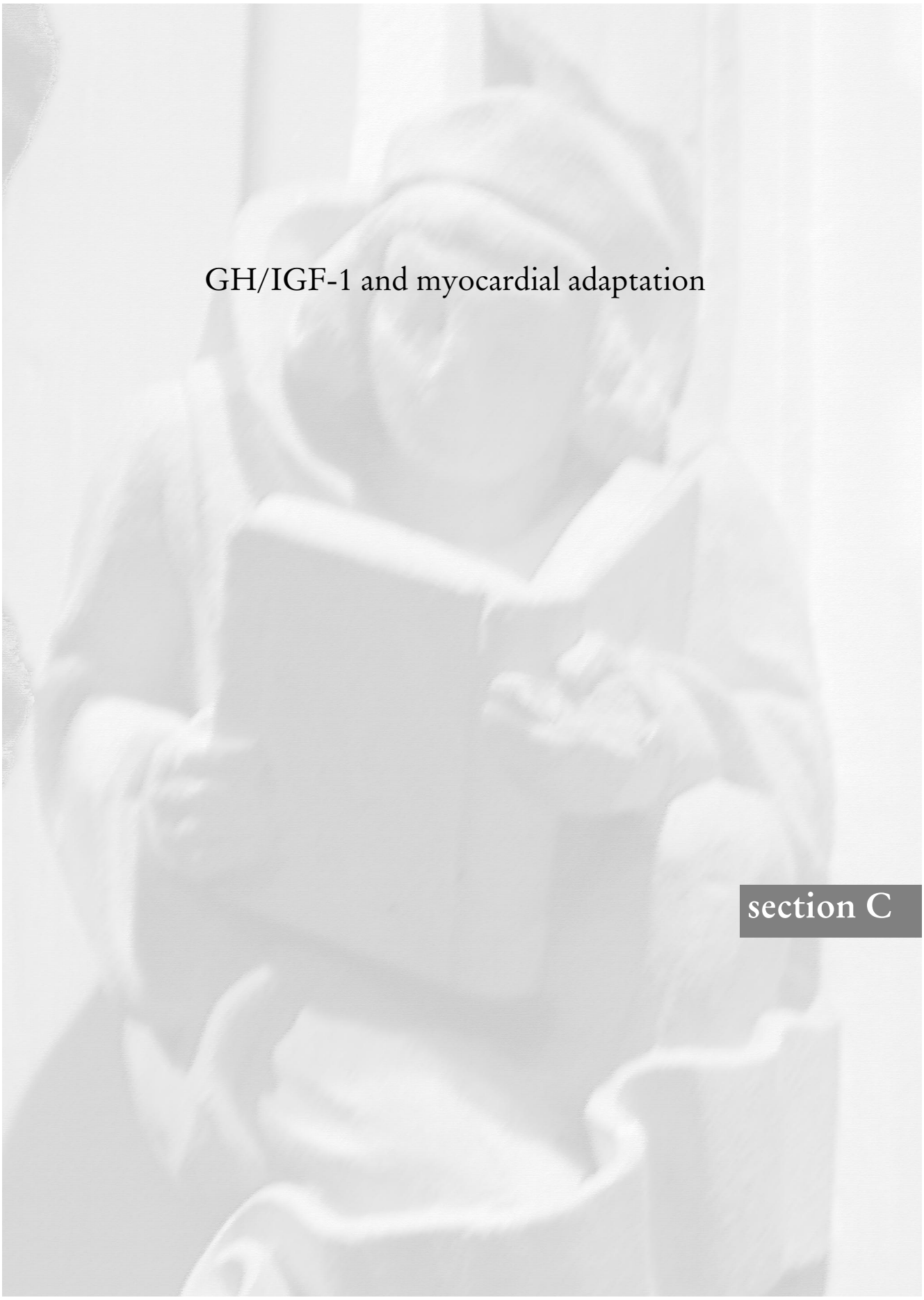

GH/IGF-1 and myocardial adaptation

section C

3.1
Acromegaly and Heart Failure
"Revisions on the growth hormone (GH) / Insulin-like growth factor (IGF) axis in its relation with the cardiovascular system"

(review)

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Many diseases in endocrinology are characterised by an increase in cardiovascular mortality. In this Current Perspective, we highlight the cardiovascular pathology in active acromegaly (circulating growth hormone excess) that is responsible for an increase in cardiovascular mortality, with recent developments in molecular endocrinology and cardiology. Afterwards, possible relationships between growth hormone (GH) / insulin-like growth factor (IGF) system and heart function in ischemic heart failure, in order to underline a concept of cardiovascular endocrinology, will be discussed.

Cardiovascular Mortality in Acromegaly

In patients with active acromegaly, clinical studies noted an increase in cardiovascular mortality, that is determined mostly by heart failure (1;2). Premature atherosclerosis, as a second contributor of the increased cardiovascular mortality in acromegaly, may be due to disturbances in lipoprotein remnant metabolism or other present cardiovascular risk factors (1;3).

Aggressive treatment of active acromegaly may be able to reverse cardiovascular pathology. A retrospective study by Orme et al reported a decrease in cardiovascular morbidity and mortality in acromegaly after lowering the plasma concentration of GH under 7.5 mU/l and of IGF compatible with reference levels that are age and gender related (4). In addition to observations, suggesting that ultimate levels of plasma GH and IGF determine the cardiovascular and consequently the clinical outcome, also the mode of treatment and the delay in obtaining "normal" GH and IGF levels are relevant. Neurosurgical treatment with total removal of the GH-producing adenoma, with supplementary drug treatment, will result in a reduction of cardiovascular mortality (5). The highest death rates are observed in acromegalic patients with aged over 60 years (75% of the total deceased patients) and with persistent acromegaly despite medical treatment (3.5 fold increase in relative mortality rate). During treatment with Octreotide retard " (Lanreotide) in postoperative persistent disease, an improvement of the diastolic left ventricular function (decrease of the isovolumetric relaxation time) and geometry (decrease of left ventricular mass) was found after 3 months' treatment period (6). This improvement in left ventricle function was stable during a one year follow-up treatment, but the significance for life expectancy needs to be evaluated. Unfortunately, treatment with Octreotide alone lowers the plasma GH and IGF levels in only 70% of all treated acromegalic patients (7). In contradiction to supplementary medical therapy that lowers systemic plasma GH and IGF-1, pituitary radiotherapy alone detoriated heart function (as measured by pulse-wave Doppler echocardiography and electrocardiography) over a 10 year' follow-up treatment period (8). Probably, the consequence of radiotherapy on mortality may be explained by the observation that the threshold for plasma GH concentration was thought to be under 2.5 microgram/L to obtain similar cardiovascular mortality rates compared to a healthy unaffected population. However radiotherapy alone was able to decrease plasma GH concentrations to those threshold levels in about 20% of acromegalic patients after a 10-year treatment period (9).

To consider together, an active and postoperative persistent disease in acromegaly give rise to increased cardiovascular morbidity and mortality. Cardiovascular mortality in acromegaly could be reduced if the threshold for plasma GH under 2.5 microgram/L is reached and this limits treatment with a combination therapy or long acting Octreotide.

Development of Cardiomyopathy in Acromegaly

An untreated acromegaly or persistent postoperative disease results primarily in a concentric hypertrophic cardiomyopathy that develops over several years (for a short review (10)). The development of cardiomyopathy in active acromegaly is determined by several periods (figure 1).

The Hyperkinetic Hemodynamic Period

At an early phase in acromegaly, cardiovascular symptoms are explained by a high cardiac output that is caused by a low systemic resistance. Such dilation of the peripheral arterial system is probably due to elevated plasma levels of GH and IGF that are able to induce nitric oxide (NO) in endothelial cells; increased endothelial NO gives rise to significant vasodilatation (11). This initial period is called the hyperkinetic hemodynamic period. The fall in afterload is associated with an increase in the activation of both the renin-angiotensin-aldosteron system (RAAS) and the beta-adrenergic system. High plasma levels of angiotensin II, and also high plasma levels of aldosteron, have maladaptive effects on the heart muscle. Therefore, high circulating levels of angiotensin II and aldosteron may worsen the cardiac performance by an increase of myocardial hypertrophy and fibrosis (12). In addition to after load reduction, the condition of the cardiac muscle to respond properly may also be limited. In *in vvo* models, GH is required for normal intrinsic function of cardiac muscle (reflected by its capacity to contract) by maintaining Ca(2+)- and beta-adrenergic responsiveness (13), although no relation was found in a different rat model (14). Moreover, in long standing acromegaly, no increased adrenergic activity was found (15).

In conclusion, the increased circulating GH and IGF-I, that induce high endothelial NO levels with vasodilation, result through systemic maladaptation in an increased workload of the heart muscle. Together with the trophic effect of the elevated plasma GH and IGF-1 levels on the myocardium, the heart chamber in acromegaly is characterised by a normal size with increased relative wall thickness (concentric remodelling).

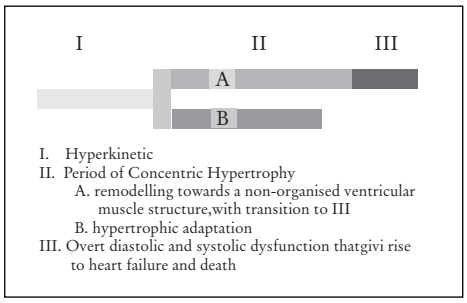

Figure 1. Different periods that determine the development of cardiovascular disease in persistent acromegaly that finally give rise to heart failure.

The period of development of concentric hypertrophic cardiomyopathy

As a result of the increased left ventricular workload and the direct trophic effects of GH and IGF-1, concentric hypertrophic changes of the cardiac muscle occur (16). In parallel with concentric remodelling, local processes in the ventricular wall result in an increase of interstitial fibrosis, an influx of polymorphonuclear cells and a replacement of (functional) cardiomyocytes (17). The

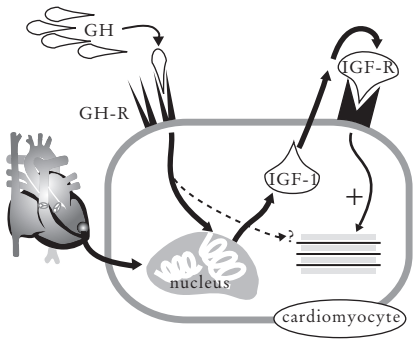

Figure 2. Distinctive regions in the heart interact with the GH axis/IGF system, resulting in an increased expression of myofibrils..

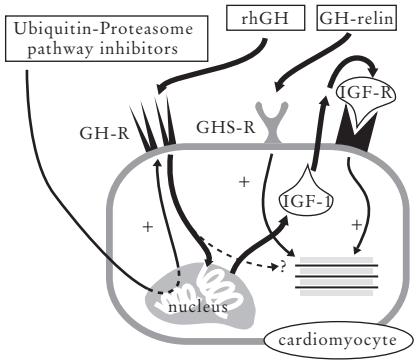

Figure 3. Possible interventions for future therapy in heart failure

continuous deposition of interstitial fibrosis and ventricular hypertrophy in combination with the hyperkinetic haemodynamic period result in a left ventricular diastolic dysfunction, as determined by measurement of the isovolumetric relaxation time (prolonged) and an inversed ratio of the early (Ev) and late (Av) diastolic peak velocity (18). Long term persistence and inability to compensate the hemodynamic profile result in a transition from a compensated hypertrophy to heart failure (with ultimately an overt diastolic left ventricular incompetence with accompanying systolic failure)(10) . Not only the left ventricular function may be affected, but also the right ventricular function could be involved in acromegalic cardiomyopathy (biventricular heart failure).

Pathophysiological Concept of Cardiomyopathy in Acromegaly

In-Vitro and Animal Studies

The heart, and its functional units the cardiomyocytes, is integrated in the body by several extrinsic pathways (such as by the endocrinological and neurological pathways) and by the circulation itself. Deprived of blood plasma in in-vitro experiments, cardiomyocytes demonstrated increased apoptosis that is prevented by addition of IGF-1, which inhibits apoptosis by activation of Bax induction and Caspase 3 activation (19). This action of IGF-1 further progresses the cardiomyocyte in the cell growth period. This in-vitro observation supports a relation of IGF with cell growth and cardiomyocyte hypertrophy. Indeed, cardiomyocytes possess large amounts of GH (GH-R) (20)and GH secretagogue receptors (GHS-R)(21). In a physiological state, the binding of systemically circulating plasma GH to the GH-R is a signal to the cardiomyocyte to produce and secrete local IGF in an autocrine and/or paracrine way. This autocrine / paracrine secreted IGF binds to IGF-receptors of cardiomyocytes and induces an increase of protein transcription (myofibrils) and growth of cardiomyocytes (17). Due to the systemic high levels of GH in acromegaly, local IGF is overexpressed with prevention of apoptosis, but with ventricular hypertrophy (22). Probably, this inverse relation between apoptosis and ventricular hypertrophy serves to protect the early cardiac adaptive system. Therefore, these previous results confirm that imbalances in systemic and local GH/IGF systems cause final damage to cardiomyocytes (23). This may explain the effects of systemic excess of GH (and IGF-1), occurring in active acromegaly (24). The contribution of IGF-I to unfavourable changes in cardiac performance in acromegaly can be indirectly confirmed in knock-out animal models for hepatic IGF-I production. With this model, no cardiac malfunction is observed, although plasma GH levels reach excessive levels (25). The marked contribution of IGF-1 in acromegaly, and not specifically GH, is once again proven with these experiments. In the development of hypertrophic cardiomyopathy, not only systemic IGF-1 but also locally produced IGF-I may play a role; *in vitro* experiments showed an increase in tissue expression of IGF-I receptor and IGF-I mRNA of cardiac muscle tissue in the case of an elevated pressure or volume load (25). Increase of left ventricular hypertrophy results in a decrease of wall compliance and subsequently to an increased end-diastolic left ventricular pressure. Consequently, local increase of IGF-I in cardiac muscle gives rise to further hypertrophic changes, but in addition to adaptive hypertrophic changes another factor need to be present to give rise to cardiomyopathy, such as a decreased property of muscle contraction. GH regulates the contractility of the heart muscle by its influence on Ca^2+ dependent processes. But in-vitro, Hexarelin (a synthetic GH secretagogue) had no significant effect on calcium transients and on the Ca flux measured in isolated ventricular cells (26). In line with this observation, acromegaly deteriorates the cardiac ventricular relaxation (diastolic phase) while it has no influence on contractility (systolic phase)(27), and the progression of cardiomyopathy. Also in concordance with strict lowering of both GH (because

this will decrease local IGF-I) and IGF-I with aggressive octreotide therapy (such as Slow Release Lanreotide and Long-Acting Release Octreotide) that results in improvement of the end-diastolic pressure (after three months of therapy)(6).

In Humans

Most previous proposed pathophysiological mechanisms of the hypertrophic cardiomyopathy in acromegaly are constructed from several non-human models (such as animal models and in-vitro experiments). On the other hand, cardiomyocytes obtained from cardiac muscle biopsies from acromegalic patients, reveal an increase of apoptosis. Moreover, the increase of apoptosis is functionally related to a decrease in ejection fraction, but not related to plasma GH or plasma IGF-1 levels (Fristaci et al 1999 Circulation). This observation is in contrast with results as described in animal models: IGF protects for cardiomyocyte apoptosis, while in GH deficiency an increased apoptosis and increased occurrence of enhanced heart performance is found (28). Possibly, this contradiction may partly be explained by a down-regulated GH-R, in case of GH excess because that may give rise to an increased apoptosis although high circulating GH and IGF levels exist. Therefore, we hypothesize that increased plasma levels of GH and IGF may exist, with a low local expression of GH-R on the cardiomyocyte, which give rise to a relatively low local biological activity of IGF-1. The decrease in GH-R results in a reduction of intracellular signal transmission towards intracellular production of IGF-1 and, therefore, with few or no release of local and paracrine and autocrine active IGF-I. In addition, GH receptors on the surface of cardiomyocytes are regionally expressed in different amounts, mediated through the intracellular ubiquitin-proteasome (U-P) system (29). Increase of the U-P system activity results in a decrease of GH-R expression (such as occurs in cachectic patients with a malignant disease (30)) and by intervention with antagonists of the U-P system, GH resistance may be corrected. The coexisting excessive amounts of systemic IGF-1 are functional in the negative hypothalamic feedback route and not in local tissue effects. Hitherto, this analysis of IGF-1 in acromegaly and its effect on tissue level is still under study. More efforts need to be made in human models in order to evaluate the balance between local and systemic IGF-1 in cardiomyopathy during acromegaly.

To conclude, the pathophysiological concept of hypertrophic cardiomyopathy in acromegaly based on animal models and in vitro experiments needs to be reassessed with results that are derived from human studies. Completion of the pathophysiological basis of the cardiomyopathy by the rapid development of non-invasive techniques (such as metabolic studies with MRI of the heart) and the increase of knowledge of GH-R expression and apoptosis will be an impulse in the creation of future interventions.

New Insights in Heart Failure Pathophysiology: Role for GH/IGF-1 axis?

GH substitution and heart failure

Cardiomyopathy and subsequent heart failure, is mostly the consequence of ischemic heart disease (31). In a recent editorial written by Cuneo (32), the substitution of rhGH in patients with ischemic heart disease was favourable for cardiac performance, such as ejection fraction and wall kinetics. Especially its effect on trophic stimulation of the heart muscle and the subsequent increase of contractility is put forward to be principally important. One study showed an additional effect on ACE inhibition of GH in improving cardiac performance after mild cardiac ischemia (33). Although these positive effects on left ventricular function during rhGH substitution in short term intervention studies, there are no randomised placebo controlled studies that show a decrease in cardiovascular morbidity and mortality after rhGH substitution in patients with coronary artery disease (CAD).

Acquired GH insensitivity in heart failure
Besides substitution of rhGH in CAD patients to prevent the definite decrease in heart performance, subcutaneous substitution of GHrelin may be cautiously considered as another progress in the treatment of heart failure. GHrelin is an endogenous ligand of the growth hormone secretagogue receptor (GHS) (34)and in this way acts additionally in the GH/IGF-1 axis (35). In a rat model with ischemic heart failure, left ventricular dysfunction and cardiac cachexia ameliorated during long-term application of subcutanous Ghrelin (36). In patients with chronic ischemic heart failure, Ghrelin administration improved cardiac function without a negative influence on renal function (21). In other studies, plasma Ghrelin levels were elevated in patients with congestive heart failure and cachexia, compared to patients with only congestive heart failure. These plasma levels of Ghrelin are significantly associated with plasma GH and TNF-alpha levels and body mass index (37). Elevated plasma GH, that suggests a decreased local GH sensitivity (38), and Ghrelin levels compensate for low expression of cardiomyocyte GH-R in order to increase intracellular IGF production. Increased secretion of IGF-1 will induce trophic changes that may sustain cardiac performance. Therefore, application of synthetic Ghrelin in a condition of a decreased cardiac performance due to ischemic heart disease, may correct simultaneously an acquired GH resistance state on the level of cardiac tissue. Anand et al (39) and Anker et al (40)who have shown an increase in plasma levels of GH and a decrease in GH-BP, IGF-1 and IGF-BP3 supported a GH resistant state in case of ischemic heart failure.

Oxidative stress
In vitro and animal studies, congestive heart failure is considered as a state of oxidative stress. GH has a direct protective effect on oxidative stress-induced apoptosis in cardiac myocytes and that the effect of GH is attributed at least in part to the activation of ERKs through Ras and PTKs including JAK2, Src, and EGF receptor tyrosine kinase(41). However, human studies do not support this oxidative state as the origin of congestive heart failure (42).

In conclusion
The GH axis/IGF system is related to hypertrophic cardiomyopathy (in acromegaly) and heart failure (in ischemic heart disease) in distinctive ways. Although several local processes in the cardiomyocyte play a role in the adaptation of heart function, similarities in coping are observed. Control of apoptosis appears to be a key factor, and that is partly related to GH/IGF balances in local heart tissue. Specific interventions (such as Ghrelin administration) may modulate these balances and give rise to a reversal of deranged coping mechanism of the heart. Our contribution aims to express the need for a multidisciplinary approach in cardiovascular disease, and its relation with hormonal balances: the concept of cardiovascular endocrinology (43).

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3.2
Significant improvement of acromegaly-induced cardiomyopathy after normalisation of GH levels
- A case report and review -

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Cardiomyopathy is known to originate from several factors, including hormonal disturbances. In this report we describe a 49-year old woman that suffered from a disabling cardiomyopathy, for which she was referred to our heart transplantation unit. Clinical and laboratory evaluation revealed that the patient suffered from acromegaly. The subsequent treatment that consisted of neurosurgery, pituitary irradiation and somatostatin analogue therapy resulted in a significant decrease of both the plasma IGF-1 and GH levels. In parallel with this decrease, the decreased left ventricle function improved dramatically to normal. This case shows that aggressive treatment of acromegaly can result in major improvement of cardiac dysfunction. The most remarkable results with respect to the cardiac performance in acromegaly are obtained if plasma GH levels are strictly reduced to less than 2,5 mU/L. Indeed, in this case report we also show an impressive improvement of the diminished left ventricle function following extensive lowering of plasma GH, despite the absence of full normalisation of plasma IGF-1 levels. Myocardial dysfunction therefore warrants an extensive analysis of possible hormonal disturbances, especially of the GH/IGF system.

Each year, approximately one hundred patients are candidates to be evaluated for a cardiac transplantation procedure in the Netherlands, and several thousand patients worldwide. In most patients, an underlying cardiomyopathy (in 40-74% the result of ischemic heart disease), is the major reason for transplantation (1). Although it is the leading cause, not only ischemic heart disease may be the reason of (severe) cardiomyopathy and an elaborate analysis of the cardiomyopathy may identify reversible causes. Cardiovascular morbidity and mortality has been associated with hormonal disturbances, such as occurs in subclinical hypothyroidism (2;3), subclinical hyperthyroidism (4) and adult-onset growth hormone (GH) deficiency (5-7). Although these hormonal disturbances are frequently observed in premature cardiovascular disease, a more rare but impressive effect of GH excess (acromegaly) on cardiac function is known. However, the clinical presentation and recognition of acromegaly and especially its relation with overt cardiovascular disease is not easy. In general, careful clinical observation is necessary to diagnose a possible hormonal disturbance that underlies cardiovascular disease, preferably at an early stage.

With this case report, we focus on GH excess as a primary cause of overt and disabling cardiovascular disease. In addition, we will discuss the effects of the GH/IGF system on the cardiovascular system and its implication in our understanding of the development into end stage cardiomyopathy.

Case

A 49-years old woman was referred to our heart transplantation unit because of an idiopathic cardiomyopathy, first diagnosed one year before the referral. Two months before, she was hospitalized in a local hospital due to manifest cardiac failure that was treated with intravenous dehydration therapy in the acute period. She continued the medication she used before the heart failure period such as diuretics, Angiotensin-Converting-Enzyme (ACE) inhibitors, oral nitrates, selective antagonist of alpha 1 and alpha 2 receptors and lipid lowering medication. In parallel, she also developed diabetes, which had become insulin-dependent the moment of presentation. Before the observation of the cardiomyopathy, she had always been “in excellent health”. She had no other risk factors for cardiovascular disease besides diabetes. Routine laboratory tests were within normal limits. No definite cause for the cardiomyopathy was found at that time. Echocardiography

showed poor left ventricular function and mild secondary regurgitation of the mitral and tricuspid valves. The angiographic procedure (performed in the local hospital) showed a severely dilated left ventricle with only poor contractions of the left ventricle. The coronary arteries were normal. The pressure in the right cardiac system was not increased.

At our heart transplantation unit, extensive investigations confirmed the dilated left ventricle with a low ejection fraction (figure 1, period A). Her body mass index was 34 kg/m^2 (height: 1,62 m; weight: 90 kg). Blood pressure was 110/65 mmHg. Glycosylated Hb was 9.7% with a fasting plasma glucose level of 16.6 mmol/l. Baseline fasting plasma cholesterol, HDL-cholesterol and triglycerides were 6.7 mmol/l, 1.02 mmol/l and 8.5 mmol/l, respectively. As a consequence of her stable clinical condition, no immediate measures were taken to prepare her for heart transplantation and consequently she was followed in the outpatient clinic. In this period life style measures, fluid restriction and optimalisation of her medication improved further her clinical condition and exercise tolerance. From the cardiology department, she was referred to the department of endocrinology because of increasing dosages of insulin which were needed to correct the hyperglycemia.

At the endocrinology department, she complained of increased sweating and diminished sensitivity in both her feet and hands during the last year. Also, shoe size had increased during that time. She did not smoke or drink alcohol in large amounts (less than 2 units a day). Menses were regular and she had no (severe) headaches. At physical examination, she had an evident acromegalic appearance that consisted of manifestations of soft tissue swelling (thickened fingers and no fit of her rings) and acral overgrowth. In addition, she had a carpal tunnel syndrome. Previous ophtalmological evaluations had revealed visual disturbances that were thought to be related to the DM type 2; no retinal disease or visual field defects had been detected. In line with the clinical diagnosis of acromegaly, both the plasma levels of plasma insulin like growth factor-1 (IGF-1) and GH were increased (fig 1A). The MRI scan of the pituitary region showed an adenoma with a size of 1.5 cm, which was resected by transsphenoidal surgery after pretreatment with Octreotide LAR (20 mg during 4 weeks). Histologic examination revealed a GH and prolactin secreting adenoma. Both the type 2 diabetes and the hyperlipidemia with which she initially presented, were considered to be part of the acromegaly syndrome.

Postoperative evaluation showed significant reduction but no normalisation of plasma GH and IGF-1 levels (fig 1B). MRI scanning of the pituitary region showed a contrast filling remnant adenoma tissue next to the left internal carotid artery. Octreotide LAR treatment was restarted and she underwent local radiotherapy (RT: rotation technique, 50 Gy in 25 fractions). In the year following RT, Octreotide LAR dosages had to be increased from 20 to 30 mg/weeks and additional cabergolin (1mg/wk) treatment was initiated (fig 1C) because of persisting remnant GH overproduction

Echocardiography during the treatment of

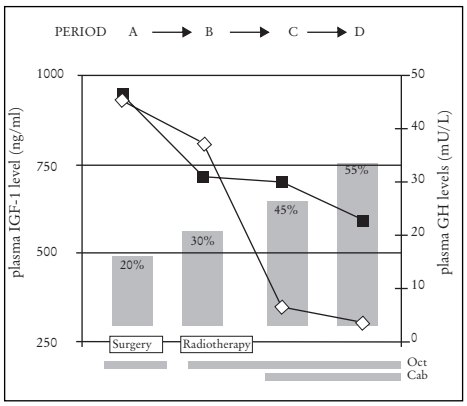
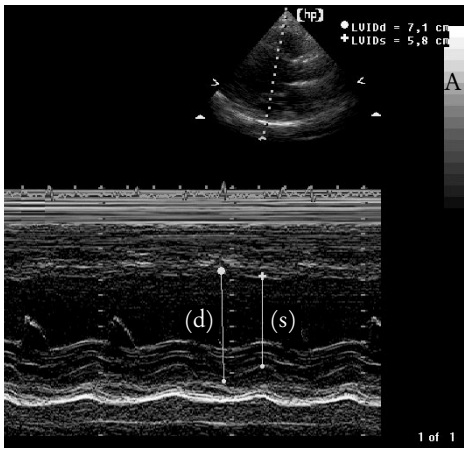


Figure 1. Figure 1. An overview of the several parameters measured in the patient with acromegaly during clinical follow-up (from A to B to C to D; in the upper part) in the cardiology and endocrinology department. Plasma levels of IGF-1 (left y-axis; closed boxes) and of GH (right axis; open triangles) are shown. The ejection fraction of the left ventricle is presented by a bar with an indication of its actual value (in percentage). Closed horizontal bars beneath the graph express the: Oct: Octreotide treatment, Cab: Cabergolin treatment

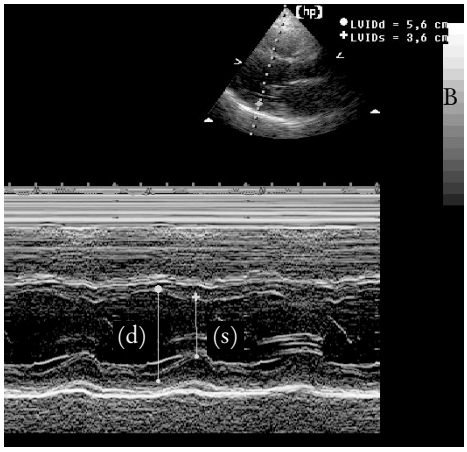
the acromegaly showed a dramatic improvement in left ventricular function. Initially, the left ventricle was severely dilated with an impaired function (ejection fraction at rest was 20%, fig 1 period [A]). No significant abnormalities of the right ventricle were observed. After one year, the left ventricle was less dilated in combination with a mild concentric hypertrophy and a moderate diffuse hypokinesia of the left ventricle. In the third year of evaluation the left ventricle size was within normal limits. Only slight concentric left ventricle hypertrophy and further reduction of global hypokinesia were observed. The ejection fraction of the left ventricle at rest increased to 45 % (Fig 1 period [C]). The most recent evaluation (Fig 1 period [D]) showed a normalization of the left ventricle size and left ventricle wall thickness with only a slightly impaired left ventricular systolic function with an ejection fraction of 55%. Fractional shortening [defined as [(Left Ventricular Intrinsic Diameter in diastole-Left Ventricular Intrinsic Diameter in systole / Left Ventricular Intrinsic Diameter in diastole)*100%]], another parameter of contractility, increased from 18% (period A) to 36% in period D (figure 2). The left atrium was mildly dilated during the first two years of evaluation, which was normalized at the third evaluation.

Figure 2a



"*Parasternal long axis M-mode echocardiographic recordings. Figure 2a depicts a severely dilated left ventricle with a global hypokinesia. Fig 2 b demonstrates the normalization of the left ventricle size, with a slightly impaired left ventricular systolic function. In both ultrasound pictures (figure 2a and 2b), the left vertical white line indicates the left ventricular intrinsic diameter (LVID) in the diastolic period(d), and the right vertical white line the LVID in the systolic period(s).*"

Figure 2b



Discussion

Disturbances in the GH/IGF-1 axis, as in a deficient (GH deficiency) and excessive (acromegaly) state, are associated with disturbances in cardiovascular performance, in body composition and in lipoprotein remnant metabolism (8;9), with a consequent increased cardiovascular morbidity and mortality (10-12). Suppression of plasma GH levels below 5.0-7.5 mU/l and lowering of plasma IGF-1 levels to age and sex adjusted normal ranges reduce the cardiovascular morbidity and mortality to that of the general population (13).

In our patient, all three therapeutic options (neuro-surgery, radiotherapy and medication) were needed to control the increased activity of the plasma GH/IGF-1 axis. Despite aggressive therapeutic strategy, plasma IGF-1 levels could not be normalised, in contrast to the plasma GH levels. Limitations of the results of medical treatment after neuro-surgery and radiotherapy in acromegaly are well known. Octreotide therapy only reduces plasma IGF-1 levels in at least 60- 70% of the total acromegalic population (14). Suppression of systemic levels of plasma IGF-1 and GH, such as with octreotide therapy (slow release lanreotide and long-acting release octreotide) result in improvement of diastolic function after 3 months of treatment (15).

In most cases, GH excess (acromegaly) results in a concentric hypertrophic cardiomyopathy (16) that slowly develops during several years. The origin of this cardiomyopathy results from a high cardiac output, due to an initially low systemic vascular resistance, together with a high diastolic capacity of the left ventricle, that is also described as a hyperkinetic hemodynamic syndrome (17). The decrease in afterload can be explained by the inductive effect of IGF-1 on the nitric oxide (NO) system in the arterial endothelium that results in a vasodilation (18). As a consequence of the high output state, the workload of the left ventricle is continuously elevated, resulting in hypertrophic changes. In addition, GH excess induces interstitial fibrosis of the ventricular myocardium (19). Finally, in chronic GH excess state, the impairment of diastolic competence accounts for the definite systolic dysfunction [16]. In our patient, both the severe impairment of the contractility (with an ejection fraction of 20 % at rest) and the increase in the left ventricle diameter in both the systolic and diastolic period indicate that similar processes in the development of left ventricle dysfunction took place. Although Fazio et al (20) reported a biventricular involvement in the cardiac dysfunction in acromegalic patients, no impairment of the right ventricle function could be observed in our patient. Both hypertension (especially elevated diastolic blood pressure)(21) and glucose intolerance [16] are aggravating factors in cardiac dysfunction in acromegaly. In our patient the insulin resistance progressively increased; this may have contributed to the severity of the impaired left ventricle function at admission. No hypertension was observed at that stage, probably due to severely impaired ejection fraction (20%) and GH-related peripheral vasodilation.

In order to explain in acromegaly the transition from the initial adaptive hypertrophy towards heart failure, recent observations show that local expression of distinctive growth factors increase the susceptibility of a failing myocardium. The cross-talk between these growth factors and the myocardium determine the definite response of the heart muscle on haemodynamic changes, with a different response in case of a volume or a pressure overload (22;23). Several local growth factors (such as IGF-I, endothelin-I and Angiotensin-II) are expressed in a closely coordinated and sequential order (24). In acromegaly, the early diastolic dysfunction (with a progressive increase in volume load and a subsequent overload) is compensated by hypertrophy of the left ventricle. In the process of adaptation in case of volume overload, the local expression of both the IGF-I mRNA and protein are necessary (25). After a certain level of wall stress (threshold) is

reached, the local expression of IGF-I will diminish. Reciprocally, with an increase in transmural tension the local tissue expression of Angiotensin-II will be upregulated with a subsequent harmful effect on the function of the cardiomyocyte (26). Apoptosis of cardiomyocytes and the development of myocardial fibrosis are positively associated with local tissue levels of this inducible Angiotensin-II (27;28). In line with the knowledge about local expression of growth factors in the heart, the transition towards heart failure in overt acromegaly could be related to the actual expression of the local IGF system. An adequate expression of local IGF-I in the cardiomyocyte prevent the cell from apoptosis (29;30). The capability to express the local IGF system may be intrinsically (such as polymorphisms for IGF-1 receptor)(31) or extrinsically (such as acquired GH insensitivity) (32) related. The non-availability of the GH receptor on the cardiomyocyte is indirectly suggested by positive effects on the heart function in heart failure after stimulation of the GH secretagogue receptors (GHS-R). These GHS-Rs are abundantly, and in parallel with the GH receptor, expressed on the myocardial surface (33). Stimulation of the GHS-R by the natural GHS ligand, ghrelin, improves left ventricle dysfunction in rats (34) and in humans in end stage heart failure (33;35). Therefore, in the final stage of heart failure not GH itself but the GHS ligand is able to transmit a positive signal directing maladaptation towards adaptation of the left ventricle, probably through local induction of IGF-I.

In conclusion, in case of severe non-ischemic cardiomyopathy reversible defects in the activity of hormonal systems (such as the GH/IGF system) need to be thoroughly investigated. The cardiac dysfunction as observed in acromegaly depends on the effect of the influence of systemic GH on the local GH/IGF balance in the cardiac tissue. Therefore, plasma GH levels may better reflect the consequences of acromegaly on the local equilibrium of the cardiac muscle than plasma IGF-1 does. Cardiovascular dysfunction is reversible after optimal lowering of the plasma GH levels, probably as a consequence of normalization of the local GH/IGF balance in the cardiac tissue.

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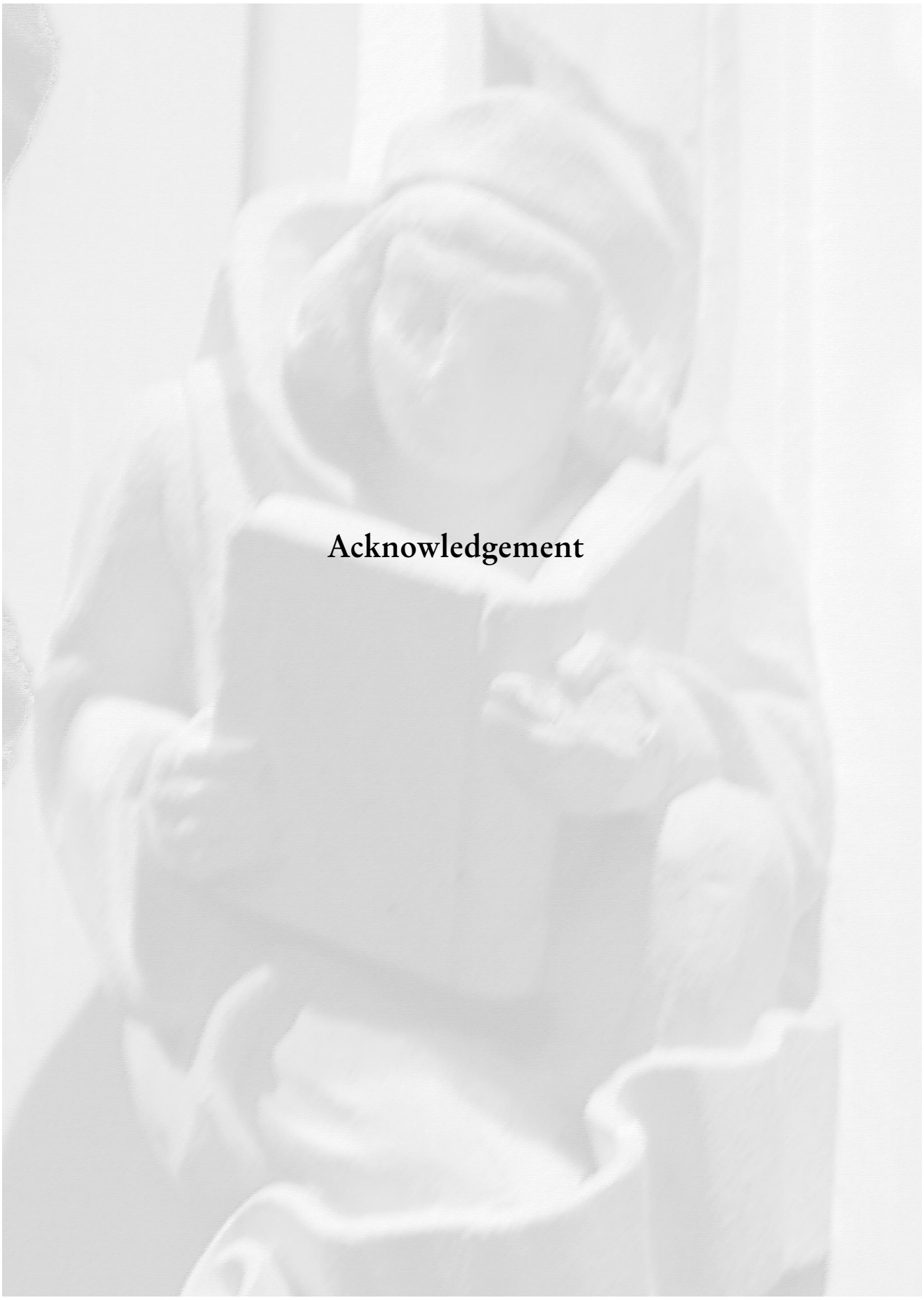

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Dr TW van Haeften. Beste Timon, niemand weet zoveel van de insuline secretie en het glucose metabolisme als jij. Jouw energie is enorm en tevens aanstekelijk. Daarnaast is jouw cabareteske woordenspel immer een verademing bij het soms langdurig wetenschappelijke steekspel. Ondanks jouw voorkeur voor 1e klas vliegtickets KLM, hoop ik dat we nog menig wetenschappelijke reis samen zullen ondernemen.

Dr. J. van Doorn. Beste Jaap, jouw kennis rondom het IGFsysteem heeft me veel geholpen. Zoals je zelf al aangaf is het nu de tijd om de data naar de mens te extrapoleren. We gaan hard aan de slag.

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Dr MC Castro-Cabezas. Beste Manuel, ondanks onze verschillen van mening, was het jouw initiërende en doortastende enthousiasme dat me op dit spoor van het onderzoek naar afwijkingen in het triglyceride lipoproteinen metabolisme heeft gezet. Ik ben je hier oprecht dankbaar voor.

Dr P de Sauvage Nolting, beste Pernette. De prettige samenwerking met de groep van professor dr John Kastelein in het AMC liep altijd voorspoedig via jou en vooral in een prettige ambiance. De vele plasma's die jij tijdens de Express studie verzamelde, werden met enthousiasme gedeeld om er de lipoproteinen remnants in te bepalen. Zelfs toen je al in de kliniek werkte, kostte het samenwerken je geen moeite. Via jou wil ik de gehele Express studie groep, die bestaat uit vele collegae uit talrijke Nederlandse klinieken, bedanken, met natuurlijk dr Koos Zwinderman.

Een dokter in het laboratorium zorgt altijd voor onrust en in mijn geval was dit niet anders. Menig analist had zijn/haar eigen invulling van dit bijzondere gedoogbeleid. De koffiekamer echter was altijd een bron van plezier en extra calorieën (Huffels deed goede zaken en taartjes moesten in die periode dan ook vroeg gehaald worden). Het was een bijzonder leerzame en ook leuke tijd; waarvoor iedereen bedankt!
Een aantal wil ik in het bijzonder noemen. Florianne de Ruijter-Heijstek heeft me uitstekend ingewerkt en me later begeleid in alle methoden rondom de bepaling van lipiden. Jouw geduld was enorm. Nadat we aan elkaar gewend waren, vond ik onze gesprekken over het leven en de drijfveer voor het doen van klinisch wetenschappelijk onderzoek mooie momenten van een geslaagde onderzoeksdag. Met Berthil Prinsen startte ik in het laboratorium, en daarom zijn we bijna altijd parallel aan elkaar opgegaan. Jouw kennis over de chemie en de achterliggende mechanismen van de methoden waren voor mij een licht. Natuurlijk mag niet onvermeld dat we allebei op dezelfde middelbare school in Brabant zaten. Het moment dat ik dit schrijf zit je zonder twijfel diep in het carnaval. Over een tijdje zal jouw promotie zijn; ik kijk er naar uit. Hopelijk blijf je in de buurt erna!
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Tous les membres de l'unité 551 d'INSERM, mon séjour à votre endroit m'a fait beaucoup

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Kilian, samen zijn we christen-democraat èn katholiek in hart en nieren. Menig politiek dispuut is door ons met een biertje gevoerd, natuurlijk deels binnen de Neude-groep (opperbeste avonden; heren onder elkaar). De uitnodiging om paranimf te zijn werd door jou beantwoord met de gedenkwaardige opmerking: "Ik voel me ten huwelijk gevraagd". Wie verzorgt er na de Laudatio de rijst?

Beste en lieve ouders. Gerard en Irene, een kind is altijd afhankelijk van zijn start en die was bij jullie in goede handen. Behoedzaam stuurden jullie me de grote wereld in. Nooit voelde ik oneigenlijk druk, wel was er altijd jullie steun. Hoe vanzelfsprekend misschien, toch wil ik jullie voor al het geluk van de afgelopen 33 jaar bedanken.

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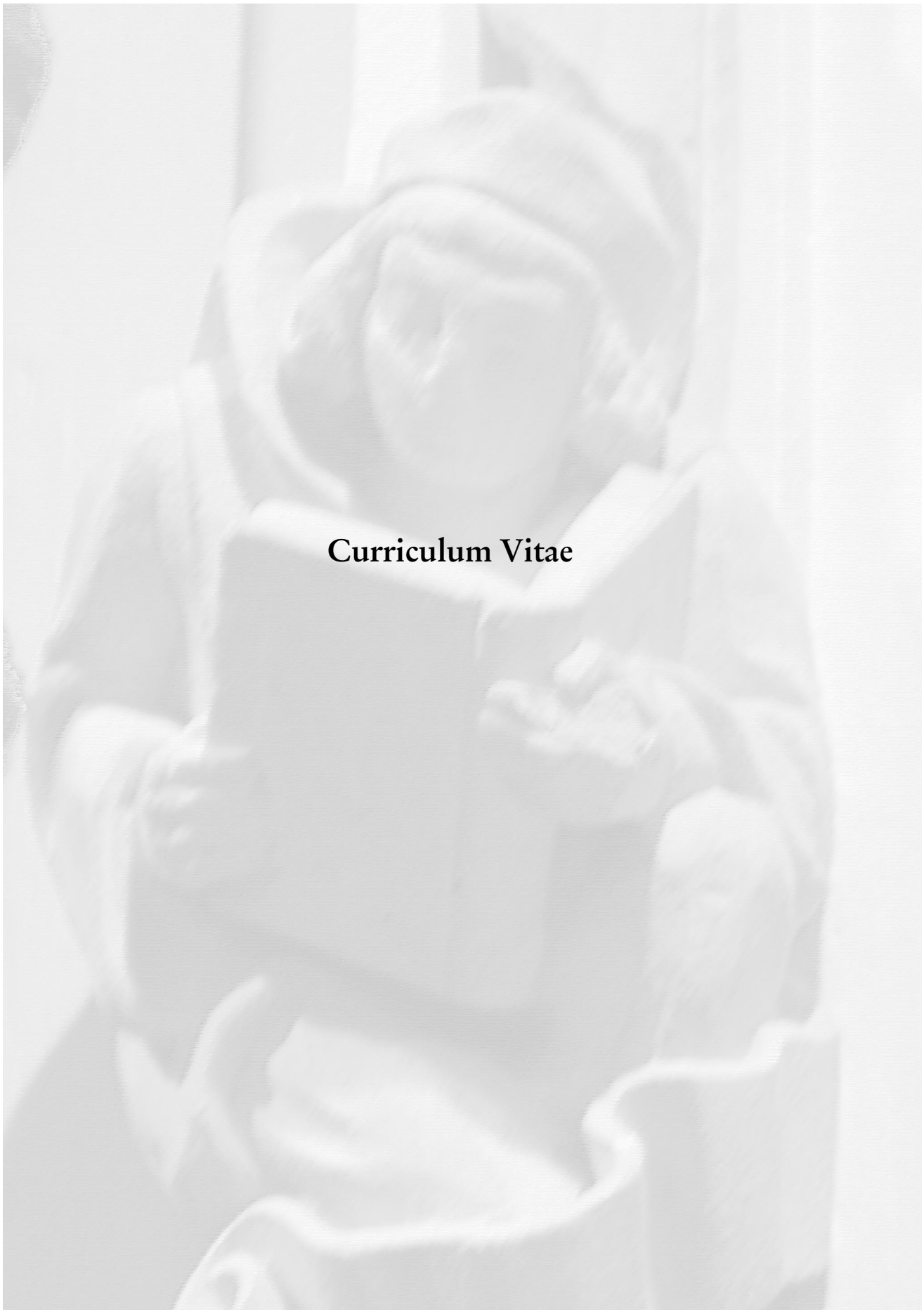
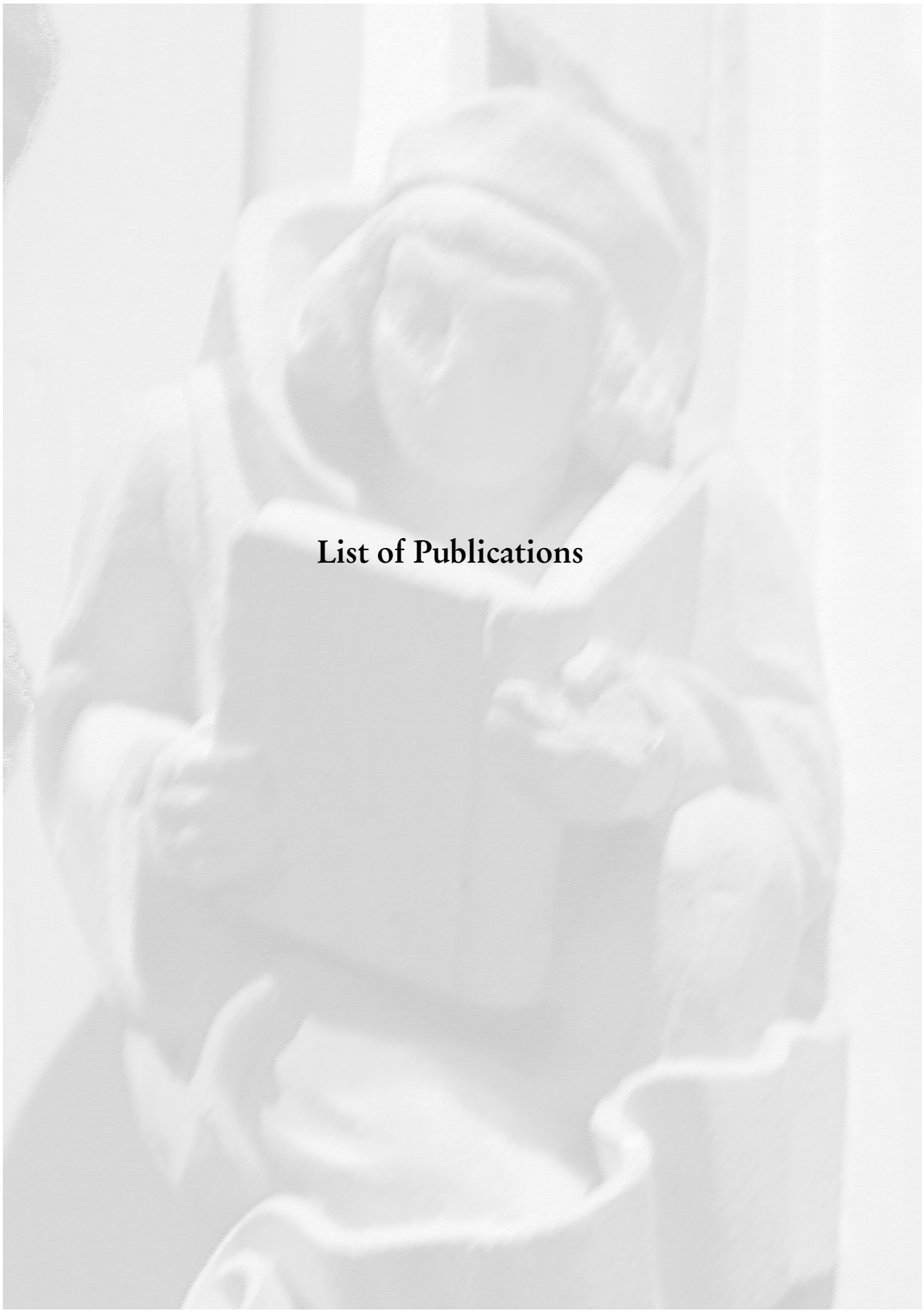
Mijn liefste. Lieska, hoe snel overtuigd waren we van elkaar toen we elkaar in Nijmegen ontmoetten en vanaf die eerste dag ben ik dat nog steeds. Jouw liefde, intelligentie en creativiteit zijn voor mij onmisbaar. Ondanks het verdriet om Alexandra, is ons leven samen iedere dag mooi en goed. Nog vele jaren hoop ik je te zien bloeien. Jouw steun is enorm!



Curriculum Vitae

The author of this thesis was born on the 13th of April 1970 in the North of the Netherlands, in the village of Musselkanaal (Groningen). When he was three years old, he and his family moved to the south of the Netherlands, to the village of Raamsdonksveer (northwest of Brabant). He grew up there with his (little) sister Miranda (who is born in 1971). In Raamsdonksveer, he attended the local primary school (De Vonder), and the Lyceum Atheneum Dongemond College. His leisure time was spent with playing soccer, fishing, visiting the community library and a lot of reading. In 1988, he attended Medical School at the Catholic University Nijmegen. During his university period, the first steps in research were made together with dr Leo Geeraards and later in a project with drs F Nagengast and I van Munster. Student life was celebrated with his friends united in the medical student society SO.DA.NO.GO, which was under his presidency in 1993. Additional income was obtained with giving educational tours in the University museum of anatomy and pathology, and as a ambulance paramedic in the city of 's-Hertogenbosch. For the Federation of International Catholic Universities (FIUC) or Féderation Internationale des Universités Catholiques (IFCU), where he was the student observer in the executive council from 1993-1996, he spent some time in Paris where the secretary of the FIUC is located. He travelled around the world for the FIUC and visited by so doing universities like Shinghanacherry College in India, the Catholic University of Lisboa in Portugal, the Catholic University of Quito in Ecuador, Notre Dame University in Indiana, USA, Georgetown University in Washington DC, USA and the Centre Catholique in Toulouse, France. This period was filled with activities, concerning international politics, catholic university education and administration. To add to his academic development, he was member of the steering committee of the Catholic Study Centre in Nijmegen. After obtaining his medical degree - cum laude - in 1996, he started his clinical career in the Royal District Hospital of Greenwich, in London. After that, he worked as a senior house officer in the departments of Internal Medicine (under supervision of dr A van de Wiel) and Cardiology (under supervision of professor dr W Mosterd) in the Eemland Hospital Amersfoort. In 1998, he became a research fellow in the laboratory of professor dr Willlem Erkelens, in which he started to study lipoprotein metabolism in relation with growth hormone. Together with Lieska H. Ester, who attended law School of Utrecht University, and their beloved cat Cobus, he happily lived in Amersfoort They married in the 24th of September 1999 in Amersfoort. At 2000, he started in Gooi-Noord Hospital, Blaricum, with his residency in Internal Medicine (under supervision of dr P Niermeyer) in the Utrecht region (director of the program professor dr DW Erkelens). This period finished in a tragedy with the first pregnancy of his wife (in July 2001). His daughter Alexandra died on the 14th of july and his wife Lieska nearly survived the HELLP syndrome that had complicated her pregnancy. He restarted in the University Medical Centre Utrecht with teaching medical students. In February 2002, he left with Lieska for Paris, were he could do a cotutelle PhD program in John Chapman's research unit INSERM 551. With fellowships of the Institut de Santé et de la Récherche Médicale (INSERM; Post Verte) and the International Atherosclerosis Society, the physico-chemical characterisation of lipoprotein remnants and their property to induce foam cell formation was investigated in the stimulating academic environment in the Pitié Salpétrière Hospital Paris. During this period, the concept of Cardiovascular Endocrinology was further developed. In March 2003, the author of this thesis will return to his residency program of internal medicine in the UMC Utrecht. He intents to defend this thesis at the 29th of April 2003 in the University Utrecht, and the 5th of May 2003 in Université Paris VI.



List of Publications

Published or In Press

1. **Twickler ThB, Cramer MJM, Senden PJ, de Vries WR, Erkelens DW, Koppeschaar HPF** Hormones and the Heart (focus on growth hormone) Does the Cardiovascular System Need Growth Hormone in Adult Life? Editorial Neth Heart J (2003, In Press)
2. **Twickler ThB, Cramer MJM, Koppeschaar HPF, de Vries WR, Erkelens DW.** Cardiovascular Endocrinology: a new dimension in medicine. Lancet 2002 359:799.
3. **Twickler ThB, de Sain-vander Velden MG, van Doorn J, van Haeften TW.** Insulin-like growth factor-I genotype and birthweight. Lancet 2002 21;360(9337):946.
4. **Twickler ThB, Cramer MJM, Dallinga-Thie GM, Erkelens DW.** The continuous postprandial state of man and its influence on atherosclerosis (editorial). Neth Heart J. 2002
5. **DeSauvage Nolting PRW, Twickler ThB, Dallinga-Thie GM, Buirma RJA, Hutten BA, Kastelein JJP, for the ExPRESS study group.** Elevated remnant-like particles in heterozygous familial hypercholesterolemia and response to statin therapy. Circulation 2002 ;106:788-92
6. **TwicklerThB, Bruckert E, Cramer MJM, Erkelens DW, Koppeschaar HPF.** L'endocrinologie cardiovasculaire: un rôle pour l'axe hormone de croissance/insulin-like growth factor dans le processus athéroscléreux ? La Presse Médicale (2003, in Press)
7. **Twickler ThB, Bruckert E.** De bon usage de .. Simvastatin (2003, in Press, Association Nationale de Médicaments)
8. **Twickler ThB, Cramer MJM, Koppeschaar HPF.** Unraveling Reaven's syndrom X. Circulation (2003, in press)
9. **Twickler ThB, Cramer MJM, Erkelens DW.** The assessment of a postprandial atherogenic phenotype. Am J Cardiol 2003;91: 258.
10. **Twickler ThB, Dallinga-Thie GM, Visseren FLJ, de Vries WR, Erkelens DW, Koppeschaar HPF.** Induction of postprandial inflammatory response in adult-onset growth hormone deficiency is related to plasma remnant-like particle cholesterol (RLP-C) concentration. J Clin Endocrinol Metab; 2003 88:1228-1233.
11. **Twickler ThB, Prinsen HCMT, de Vries WR, Koppeschaar HPF, deSainvan der Velden MGM.** Analysis of the separate secretion of VLDL-1 and VLDL-2 by the liver will be a principal factor in resolving the proatherogenic lipoprotein profile in hypopituitarism (Letter). J Clin Endocrinol Metab. 2002 ; 87(4):1907
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14. **Wilmink HW, Twickler ThB, Banga JD, Dallinga-Thie GM, Eeltink H, Erkelens DW, Rabelink TJ, Stroes ES.** Effect of statin versus fibrate on postprandial endothelial dysfunction: role of remnant-like particles. Cardiovasc Res. 2001; 50(3):577-82.
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18. **Twickler ThB, Dallinga-Thie GM, de Valk HW, Schreuder PCNJ, Jansen H, Castro Cabezas M, Erkelens DW.** High dose of simvastatin normalizes postprandial like particle response in patients with heterozygous familial hypercholesterolemia. Arterioscler Thromb Vasc Biol 2000; 20:2422-2427.
19. **Twickler ThB, Wilmink HW, Schreuder PCNJ, Castro Cabezas M, van Dam PS, Koppeschaar HPF, Erkelens DW, Dallinga-Thie GM.** Growth hormone (GH) treatment decreases postprandial-like particle cholesterol concentration and improves

endothelial function in adult-onset GH deficiency. J Clin Endocrinol Metab 2000; 85:4683-4689.

20. **Van Oostrom AJHHM, Castro Cabezas M, Ribalta J, Masana L, Twickler ThB, Remijnse TA, Erkelens DW.** Diurnal triglyceride profiles in healthy normolipidemic subjects are associated to insulin sensitivity, body composition and diet. Eur J Clin Invest 2000; 30 (11) : 964-971.
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23. **van Deuren M., Th.B. Twickler, Mc de Waal Malefyt, H van Beem, J. van der Ven-Jongekrijg, C.M.M. Verscheuren, J.W.M. van der Meer.** Elective orthopedic surgery, a model for the sudy of cytokine activation and regulation. Cytokine. 1998, 10 ; vol 11:897-903.
24. **Oostrom van AJHHM, Castro Cabezas MC, Harmelink MJTH, Twickler ThB, Remijnse TA, Erkelens DW.** Triglyceridedagprofielen bij 30 jonge gezonde mannen als functie van voeding, nuchtere triglycerideconcentraties, lichaamssamenstelling en insuline gevoeligheid. Ned Tijdschr Geneeskd 1999; 143:1868-1872.
25. **Twickler ThB, Gelderman WAH.** Vijf jaar follow-up na een curatieve resectie van het colon carcinoom in een perifere opleidingskliniek. Ned tijdschr Heelk 1997, 6, nr 2,; 44-45.
26. **Twickler ThB.** Le point de vue d'un étudiant néerlandais, blz 60-63 dans "La formation des personnes au sein des universités européennes aujourd'hui" Eds M Falise/V Hanssens, presses interuniversitaires européennes, Bruxelles.
27. **Twickler ThB, MH Otten.** De keten van de spijsvertering; zwakke schakels op oudere leeftijd. Denkbeeld 1997; 9i:12-15.
28. **Twickler ThB, MJM Cramer, A. Hovestadt.** Het knarsende wiel aan de zwaar beladen wagen; de relatie tussen dementie en hypertensie. Ned Tijdschr Chron Zktn. 1997;7: 97-100.
29. **Van der Wielen MLJ, Twickler ThB, MJM Cramer.** Pericarditis; strak in het harnas? Cardiogram 1997 Jaargang 13; 4163-165.
30. **Munster van I.P., Adamo N., Twickler ThB, et al.** Fermentation of lactulose and resistant starch by high and low methane producing faecal inocula. 1994; 81-93. Hoofdstuk 6 Proefschrift Dr. I.P. van Munster, Fermentation and coloncarcinogenesis, 29.4.1994 ; Drukkerij van Gerwen B.V..

Submitted

1. **Wilmink HW, Twickler Th B, Dallinga-Thie GM, Banga JD, Erkelens DW, Rabelink TJ, Stroes ESG.** Effect of dietary saturated and polyunsaturated fatty acids on endothelial function and lipid profile. (after revision)
2. **Twickler ThB, Koppeschaar HPF, de Vries W, Erkelens DW, Berger R, de Sain-van der Velden M.** Fasting plasma IGF-1 levels in AGHD predicts the level of insulin resistance after rhGH substitution; short communication.
3. **Twickler ThB, van Dam PS, Cramer MJM, Koppeschaar HPF.** Dilated cardiomyopathy is a clinical feature in acromegaly.
4. **Twickler ThB, van Haeften TW.** Insulin secretion and the IGF system (a review and hypothesis).
5. **Twickler ThB, de Sain-van der Velden MGM, van Doorn J, van Haeften TW.** Relationships between the Insulin-like Growth Factor system, pancreas B-cell function and peripheral insulin sensitivity in healthy subjects: studies with hyperglycemic glucose clamps.
6. **Twickler ThB, de Barse MMJ, Dallinga-Thie GM, Koppeschaar HPF, de Vries WR, Erkelens DW, Berger R and de Sain-van der Velden MGM.** Is Adequate Growth Hormone Substitution Sufficient to Ameliorate the atherogenic lipid profiles in adult-onset GH deficient patients?
7. **Moschetta A, Twickler Th B, Rehfeld JF,. van Ooteghem NAM, Castro Cabezas M, Portincasa P,. vanBerge-Henegouwen GP, van Erpecum KJ.** Effects of growth hormone defiency and recombinant growth hormone therapy on postprandial gallbladder motility and cholecystokinin release.
8. **Twickler ThB, Cramer MJM, Dallinga-Thie GM, Chapman MJ, Erkelens DW, Koppeschaar HPF.** Adult-onset Growth Hormone Deficiency: Relation of Postprandial Dyslipidemia to Premature Atherosclerosis.

9. **Marcel ThB Twickler, Pernette R.W. Sauvage-Nolting; Koos Zwinderman; John J.P. Kastelein; Geesje M. Dallinga-Thie; for the ExPRESS Study Group.** Remnant lipoprotein levels and the association with carotid intima media thickness in patients with heterozygous familial hypercholesterolemia (FH); independent of plasma LDL-cholesterol level.

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Notes