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% CI 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.

<table>
<thead>
<tr>
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<th>Plasma RLP-C (mmol/L)</th>
<th>Area under the incremental postprandial RLP-C curve (mmol/l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control subjects</td>
<td>0.18 ± 0.06</td>
<td>1.14 ± 0.61</td>
</tr>
<tr>
<td>Patients</td>
<td></td>
<td></td>
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<tr>
<td>Familial Hypercholesterolemia (FH)</td>
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<tr>
<td>Before Statin therapy</td>
<td>1.09 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.14 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>During Statin therapy</td>
<td>0.26 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.34 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Adult-onset GH deficiency (AGHD)</td>
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<tr>
<td>Before rhGH therapy</td>
<td>0.29 ± 0.14</td>
<td>2.13 ± 1.60&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>During rhGH therapy</td>
<td>0.32 ± 0.09</td>
<td>0.73 ± 0.34</td>
</tr>
<tr>
<td>Acromegaly</td>
<td>0.40 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.14 ± 1.19&lt;sup&gt;a&lt;/sup&gt;</td>
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All values are expressed as mean ± SD. <sup>a</sup>p<0.05, compared to control subjects, <sup>b</sup>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 atherosclerotic disease. 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-κB, 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-fract-ion (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.

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 acromegals (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 metabolism. 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. 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 hypoglycaemia 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 cycle (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 phosphoenolpyruvate (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 β-cells. An increased apoptosis of β-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 prodiahesive phenotype. In dorsal pancreas agenesis, a decrease in β-cell mass results in a modest increase in GNG, also a feature in the syndrome of insulin resistance. Insufficiencies in β-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 β-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).