From the Department of Women and Child Health and
the Department of Molecular Medicine,
Karolinska Institutet, Stockholm, Sweden and
the Department of Clinical Science, Pediatrics, Umeå University, Sweden

Diabetes and Puberty.

Studies on hormonal factors of importance to the blood glucose control.

Ingmar Zachrisson

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Abstract

It is well known that, in children with type 1 diabetes, metabolic control often deteriorates during puberty. Better understanding of the mechanisms that cause deterioration of blood glucose control can lead to improved therapeutic approaches. Preliminary observations suggest that one component of the impaired glycemic control is a large variation in blood glucose. Several of the hormonal changes during puberty affect insulin sensitivity and could, in addition to lifestyle factors, also cause blood glucose variation. A marker of hormonal activity during puberty is linear growth, which thus can be affected by the metabolic control.

The aim of this thesis was to study hormonal changes during puberty in children with type 1 diabetes in relation to blood glucose level as well as blood glucose variability, with special focus on anabolic hormones and linear growth as a mirror of hormonal and nutritional status.

In summary, we have demonstrated that in children with type 1 diabetes, there is during puberty increased long-term variability in blood glucose and elevated blood glucose. The long-term blood glucose variability was related to linear growth and mean 24 hours GH level. Linear growth velocity can be used as a biological marker of the GH-IGF-I system. We showed that there was both hepatic growth hormone and insulin resistance during mid-puberty. However, the blood glucose levels during a 24 hour period was not correlated to GH but to IGFBP-1 confirming the importance of free IGF-I in the regulation of the glucose homeostasis. This correlation was dependent on insulin during mid-puberty but was independent of insulin in Tanner stage 5 patients. Neither cortisol, testosterone nor leptin could explain the blood glucose levels. However, we found a correlation between blood glucose and IGFBP-3 protease activity during mid-puberty. We speculate, the increased protease activity is a compensatory mechanism to restore free IGF-I concentrations to normalise glucose homeostasis. Low IGF-I levels and high IGFBP-1 levels were found in diabetic boys both during the mid-pubertal and the post-pubertal stages. These findings were correlated to low insulin concentrations and elevated HbA1c or blood glucose.
Leptin, a hormone reflecting nutritional status, was elevated in diabetic boys with a correlation to BMI ratio but not to insulin. The importance of increased leptin levels is not fully understood but could serve as a marker of incomplete insulin treatment and increased risk of later diabetic complications, e.g. cardiovascular disease.
In conclusion the IGF-I - IGFBP system is important in the regulation of glucose homeostasis in diabetic boys both during and after puberty. Bioavailable IGF-I levels determine blood glucose levels partly independent of insulin. The blood glucose variability measured as standard deviation of blood glucose over a longer period can be an additive marker of metabolic control in diabetic children. To achieve optimal metabolic control the treatment should be aimed to lower the blood glucose variability and to normalise the changes in the GH-IGF-I-IGFBP system.

Keywords: Type 1 diabetes, Puberty, Adolescence, Metabolic Control, IGF-I, IGFBP-1, IGFBP-3, Proteases, Leptin.

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Reading maketh a full man, conference a ready man, and writing an exact man.

Lord Francis Bacon

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Cover: "The Wave". Oil painting on canvas by Barbro Zachrisson.

List of original papers

This thesis is based on the following studies, which will be referred to by their respective Roman numerals:

I: **Zachrisson I**,Wallensteen M.,Dahlqvist G.; Determinants of blood glucose variability in adolescents with insulin-dependent diabetes mellitus.
Acta Paediatrica 1995 Vol84 (1): 70-74

II: **Zachrisson,I**,Brismar,K.,Hall,K.,Wallensteen,M., Dahlqvist,G. Determinants of Growth in Diabetic Pubertal Subjects.
Diabetes Care 1997 Vol20 (8): 1261-1265

III: **Zachrisson I**, Wallensteen M, Dahlquist G, Brismar K: Insulin like growth factor binding protein-1 as glucose regulator in adolescent boys with IDDM.
Acta Paediatrica 2000 Vol89 (9):1044-1049

IV: **Zachrisson I**, Brismar K, Dahlquist G, Wallensteen M, Bang P: Increased 24 hours mean Insulin-Like Growth Factor Binding Protein-3 Proteolytic activity in Pubertal Type 1 Diabetic Boys.
In Press. Growth Hormone & IGF Research. December 2000

V: **Zachrisson I**, Brismar K: Leptin, Cortisone and Testosterone levels are puberty dependent both in Healthy and Diabetic Boys. Only Leptin is impaired in diabetes.
Manuscript

Definitions and abbreviations:

Definitions:

Adolescence	The period of life beginning with the appearance of secondary sex characteristics and terminating with the cessation of somatic growth.
Body Mass Index	weight (kg) / height $(m)^2$
Glucose variability	Fluctuations in blood glucose level measured as the standard deviation of the measured values over a specific time period. See SDbg.
Growth velocity	Height increment measured as mm/year.
Tanner stage	External changes in secondary sex characteristics graded chronologically from stages 1 to 5. Stage 1 is before puberty and 5 after completed puberty.

Abbreviations:

ALS	Acid Labile Subunit
BMI	Body Mass Index
CSII	Continuos Subcutaneous Insulin Infusion
DHEAS	Dehydroepiandrosterone sulphate
GH	Growth Hormone
GHR	Growth Hormone Receptor
GHBP	Growth Hormone Binding Protein
GHRH	Growth Hormone Releasing Hormone
IGF-I	Insulin-like growth factor-1
IGF-IR	IGF-1 Receptor
rhIGF-I	recombinant human IGF-I
tIGF-I	total IGF-I
fdIGF-I	free dissociable IGF-I
IGF-I SDS	IGF-I Standard Deviation Score
IGFBP	Insulin-like growth factor binding protein
IGFBP-3-PA	Insulin-like growth factor binding protein-3 protease activity
IR	Insulin Receptor
RIA	Radio Immuno Assay
SDbg	Standard Deviation of blood glucose
SMBG	Self Monitoring of Blood Glucose
SS	Somatostatin

1 Diabetes and puberty

Working in the clinic as a paediatric diabetologist meeting adolescents with type 1 diabetes many questions have arisen concerning the possibilities of keeping a good metabolic control during puberty. Despite great efforts from the patient it seems an impossible task. In the clinic I also have the impression that the blood glucose values are unstable during puberty. Morning blood glucose levels tend to be high and increased insulin doses in the evenings often cause hypoglycaemia during the night.
Ever since insulin was discovered and the first patient was treated with insulin injections, the growth and development of children acquiring type 1 diabetes before puberty have been of great concern to the children and their families as well as to the treating physicians.
The problem of living through the teen ages with type 1 diabetes touches many scientific fields; behavioural science, philosophy, psychology, but not the least paediatric endocrinology and diabetology. MEDLINE covers 21145 articles on the keywords "diabetes" and "adolescence" since 1966 till today, of which 1115 also include psychological considerations. This shows a great interest in the field and, quoting Joslin, Root et al from JAMA 1925[1]: "Here are opportunities for the paediatrician, the dietician and the endocrinologist". In western societies today we could also add psychologists, social workers, teachers, parents, families and most important the patient him/herself to these categories.
I wanted to study the changes in blood glucose control during puberty further, without blaming the changes on behavioural factors alone. Instead, I wanted to look into possible hormonal changes connected with puberty and linear growth, and study how they affect blood glucose homeostasis. This thesis is the result of the effort to try to understand more about the hormonal changes connected with puberty and type 1 diabetes.

1.1 Normal Puberty

To give a further background to the questions at issue in this thesis, the normal hormonal changes during puberty and the pubertal growth spurt are very briefly reviewed.
Serum levels of dehydroepiandrosterone (DHEA) and its sulphate (DHEAS) begin to rise at approximately 6-8 years of age before the physical changes of puberty. This is called *adrenarche* and typically antedates the onset of gonadal puberty, i.e. *gonadarche,* by a couple of years. In adrenarche a slight increase in linear growth rate (mid-childhood growth spurt) and possibly the appearance of some pubic and/or axillary hair, predominantly in girls, can take place[2].
Gonadarche is initiated by increased amplitude of the gonadotropin-releasing hormone (GnRH) secretion from the hypothalamic region resulting in pulsatile release of the gonadotropins; luteinising hormone (LH) and follicular stimulating hormone (FSH) from the anterior pituitary. This pulsatile secretion of gonadotropins is responsible for the enlargement of the testicles and the ovaries and the secretion of sex steroids, testosterone and estradiol. The sex steroids then cause the development of the secondary sex characteristics. These can be classified in 5 stages according to Tanner[3] evaluating breast development and pubic hair in girls, and genital organ development and pubic hair in boys. Serum levels of testosterone and estradiol, as well as DHEAS, continue to increase throughout puberty.

Important regulators of linear growth are; nutrition, thyroid hormones, insulin, growth hormone (GH), insulin-like growth factors (IGFs) and sex steroids.
The hormonal regulation of the linear growth spurt during puberty which takes place earlier in girls (11-12 years) than in boys (13-14 years) is summarised briefly.

GH is secreted from the anterior pituitary in a pulsatile fashion regulated by two hypothalamic peptides, growth hormone releasing hormone (GHRH) and somatostatin (SS)[4]. GH pulse amplitude increases in puberty[5] peaking during the linear growth spurt[4,6]. After puberty GH levels decreases. The sex steroids, predominantly estradiol, stimulate GH secretion. To cease linear growth estradiol also fuses the epiphyseal growth plate[4,6]. Testosterone can be aromatized to estradiol thereby also mediating these effects. GH predominantly exerts its effect via IGF-I that mainly is produced in the liver[4]. It is however believed that local GH-stimulated production of IGF-I in the growth plate is also important for linear growth. The increase in both GH and IGF-I is temporally associated with the pubertal growth spurt. The GH-IGF-1 system is described in more detail in section 4.2.2.
Most hormonal and environmental factors regulating linear growth also have the potential to affect blood glucose level in type 1 diabetes as discussed in the following.

2 Blood Glucose Level

The blood glucose level is the result of glucose uptake and hepatic glucose production. Insulin is the most important hormonal regulator of these metabolic processes. In type 1 diabetes when the endogenous insulin production is extinguished the normal regulation of the metabolic processes is blunted. Consequently the blood glucose can fluctuate rapidly between high and low values, and also stay high or low over longer periods.

2.1 Why is the blood glucose level important?

The blood glucose level or the HbA1c which integrates the level over several weeks is the main predictor of micro- and macro angiopathic complications in type 1 diabetes[7,8]. It is well known that the metabolic control during puberty is deteriorated with elevated HbA1c levels[9-11]. It has been proposed that the duration of type 1 diabetes before puberty plays a less important role in the development of late complications than the time from puberty and onwards[12]. This is, however opposed by other authors[13,14].

3 Blood Glucose Variability

Blood glucose variability has been of interest to researchers in the context of brittle diabetes. Tattersal, in 1977 proposed a concept defining brittle diabetes as 'insulin dependent diabetics whose lives are constantly disrupted by episodes of hypo- and hyperglycaemia, whatever the cause'[15]. More recent reviews also add to the definition frequent episodes of ketoacidosis with hospitalisation and/or frequent hypoglycaemia[16,17]. There are few follow-up studies of the natural course of brittle diabetes with low numbers of patients[18-20], but these have shown a connection between brittle diabetes and an enhanced risk of late complications compared to patients with more stable glucose control.
Blood glucose variability as a measure of blood glucose control is more frequently used since the introduction of computer programs connected to glucose meters for self-monitoring of blood glucose (SMBG). Different methods to calculate blood glucose variability have been described by Moberg et al.[21], proposing the use of the standard deviation of 5 daily values measured over 4 weeks (SDbg) to be a good estimate of blood glucose variability[22].

3.1 Why is the blood glucose variability important?

A rapid fluctuation in blood glucose level causes unpleasant symptoms not only in connection with hypoglycaemic episodes. Whether enhanced blood glucose fluctuations causes diabetic

complications is not fully studied as no systematic follow up studies have been published. There are studies showing changes in cerebral blood flow in response to a rapid decrease in blood glucose level to values above normal in poorly controlled type 1 diabetic patients[23]. Jones et al.[24] showed increased cell growth, collagen synthesis and cytokine secretion in cultured human renal tubulointerstitial cells with increased glucose concentrations. These effects were enhanced following intermittent exposure to high glucose concentrations showing that short-lived excursions in glycemic control have important pathological effects on the human tubulointerstitium. Blood glucose contributes to the plasma osmolality and fluctuations at high levels consequently affect the plasma osmolality.
Postprandial blood glucose values have been shown to affect HbA1c more than fasting values implicating that variability could enhance the risk of diabetic complications[25].
Consequently there are reasons to aim at diminishing the variability of blood glucose, both for the immediate well being of the patient as well as for reducing the likelihood of late diabetic complications.

4 Factors of importance to the blood glucose homeostasis.

4.1 Psychosocial factors

Psychosocial factors affecting the possibility to successfully adjust to the restrictions in life that living with type 1 diabetes imposes are of importance in all ages. Puberty is a period in life with dramatic biological and psychological changes. Tattersall and Lowe[26] have summarised the interference between the development during normal adolescence and the demands good self treatment of type 1 diabetes puts on the individual. Lernmark B stated recently in her thesis[27] that the relationship between estimates of general psychological functioning and metabolic control is still unclear as results from different studies are contradictory. Some studies have shown more anxiety and depression in children and adolescents in poor metabolic control[28,29]. Others have demonstrated better metabolic control in children with mild depression and psychological difficulties[30-32].
Stressful events, both psychological and physiological have been shown to affect blood glucose level both on short- and on long-term[33,34]. The elevated blood glucose level is probably caused by elevation in epinephrine, glucagon and cortisol release[35].
Eating disorders are both common and persistent in young women with type 1 diabetes[36], the most common purging behaviour is insulin omission[36]. This of course affects blood glucose level but further discussion in this field is beyond the limit of this thesis.
In the studies (Papers I-V) in this thesis the possible impact of psychosocial factors are not taken into consideration as the aim was to disentangle the impact of physiological changes during puberty on the blood glucose homeostasis.

4.2 Hormonal factors

4.2.1 Insulin

Insulin is the main regulator of glucose homeostasis acting on the liver, muscle and adipose tissue, preferentially via the insulin receptor (IR). Insulin inhibits hepatic gluconeogenesis and stimulates glucose uptake by regulating glucose transporters. Insulin receptor knock-out mice do not develop diabetes[37] which is explained by the fact that insulin also acts via the IGF-I receptor (IGF-IR).

4.2.2 GH-IGF-I-IGFBP-system

GH pulse amplitude increases during puberty peaking in Tanner stage III-IV[4] both in boys and girls. GH hypersecretion in young adults with type 1 diabetes has been consistently demonstrated[38], and characterised by both increase in pulse amplitude and baseline concentrations[39,40]. The dawn phenomenon in type 1 diabetes has partly been connected with GH hypersecretion[41,42].
GH induces insulin resistance and reduces glucose tolerance via impaired suppression of hepatic glucose production and impaired stimulation of glucose utilisation as demonstrated by Rizza et al[43]. In addition, GH has been demonstrated to decrease the phosphorylation of the IR and several of its intracellular messengers including insulin receptor substrate-1 (IRS-1) in liver and muscle in rats[44-46]. Increased GH secretion during puberty decreases insulin sensitivity in healthy and diabetic adolescents[10,11,47,48].
GH binds to the GH receptor (GHR) on the hepatocytes and induces IGF-I production (figure 1). Growth hormone binding protein (GHBP) consists of the extra cellular part of the GHR and is decreased in type 1 diabetes[49-51]. The GHR signalling and expression is dependent on insulin[52] and GHBP has been shown to increase after initiation of insulin therapy[53] in newly diagnosed type 1 diabetes. The majority of circulating IGF-I is produced in the liver having an endocrine effect. IGF-I is produced in many cell types and also has peripheral para-, and autocrine effects but in this thesis only the endocrine effects of IGF-I will be discussed.IGF-1 circulates bound to IGF binding proteins, of which six (IGFBP 1-6) are characterised[54,55] leaving less than 1% as free IGF-1[56]. Within the limits of this thesis the impact of IGFBP-1 and IGFBP-3-PA with regard to bioavailable IGF-I is discussed.
IGF-I is predominantly bound to IGFBP-3 and an acid labile subunit (ALS) forming a ternary complex. Both IGFBP-3 and ALS are produced in the liver and are GH dependent [57,58]. The ternary complex is restricted to the circulation and has a half-life of more than 14 hours without any significant diurnal variation[59]. IGFBP-3 proteases cleave circulating IGFBP-3 within the ternary IGF-I-IGFBP-3-ALS complex and are thought to destabilise the complex and increase IGF-I bioavailability[60-62].
IGFBP-1 constitutes only a few percent of the total pool of circulating IGFBPs but is an important determinant of free IGF-I in vivo[63,64]. IGFBP-1 has a high production rate, rapid fluctuations in serum concentration[65] and a marked diurnal variation[66]. IGFBP-1 is synthesised in the liver under the control of insulin[65] that inhibits the transcription of IGFBP-1 in the hepatocytes. Since the half-life of circulating IGFBP-1 is 1-2 hours[67], levels of IGFBP-1 are rapidly suppressed following meals[66,68,69] and increased during fasting[70,71].
IGF-I acts via its own receptor[72], which is structurally and functionally similar to the insulin receptor[73]. Post-receptor signalling is very similar for the IGF-I and insulin receptors, both activating the tyrosine kinase[74] and IRS-1[75] cascade. IGF-I receptors are found on all tissues except the liver and adipose tissue[76,77]. IGF-1, or the free fraction of IGF-I, can mimic all metabolic effects of insulin in vivo as well as in vitro.
In vitro IGF-I has been shown to stimulate glucose uptake in the muscle[78,79].
Since only the free IGF-I is believed to a have a biological effect[61,80] on the blood glucose homeostasis, the importance of IGFBP-1 and the IGF-I-IGFBP-3-ALS complex in this context is evident and will be discussed more in detail in sections 7.2.3, 7.2.4 and 7.2.5.

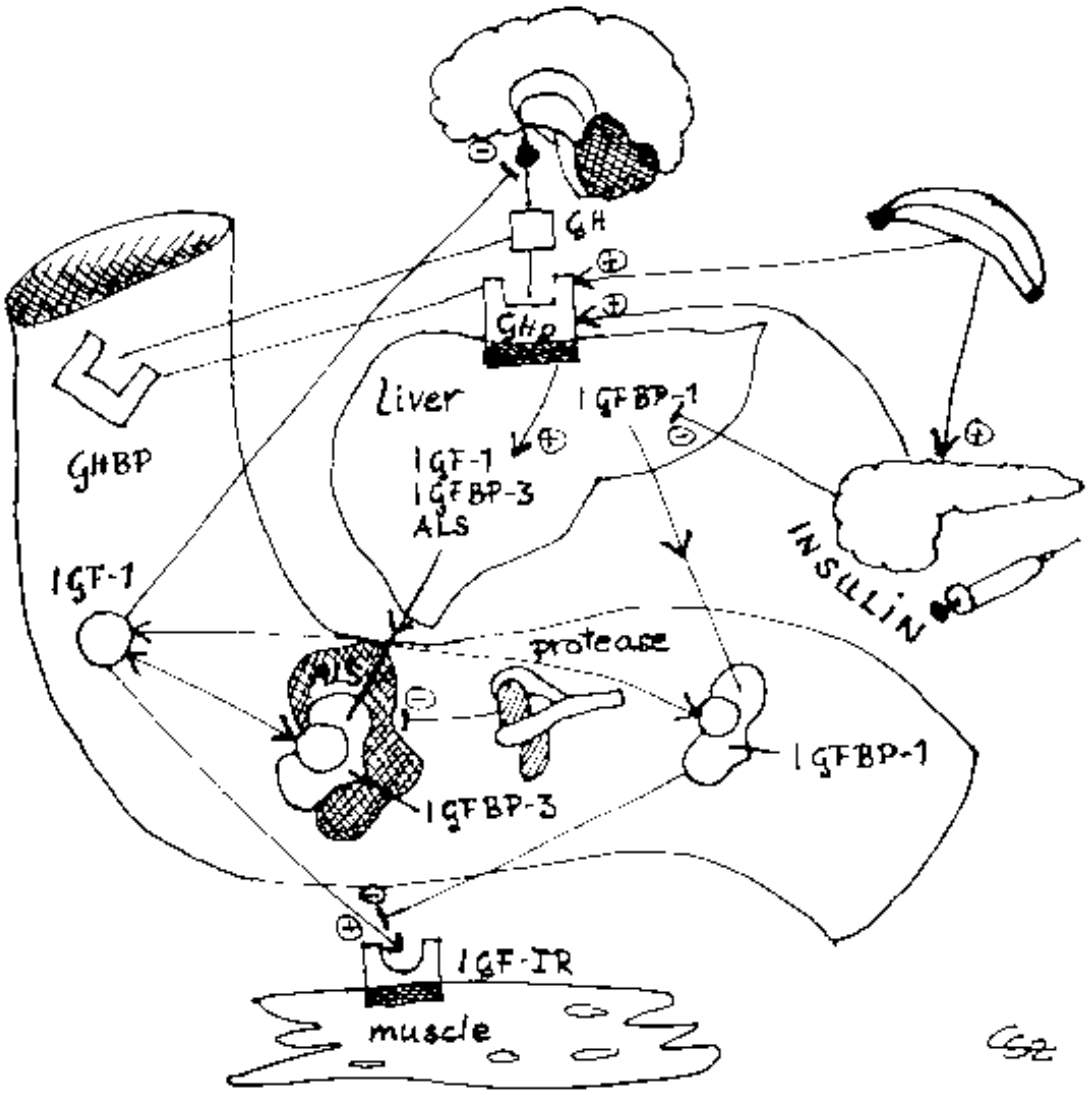

Figure 4. Simplified overview of the relationship between the GH/IGF-I-IGFBP system, insulin and nutrition. For abbreviations and explanation, see text.

In type 1 diabetes the GH-IGF-I-IGFBP system is deranged with high levels of GH, low levels of GHBP, IGF-I and IGFBP-3 and high levels of IGFBP-1[65,67,81]. Both high GH levels and possibly low bioactive IGF-I levels could enhance hyperglycaemia in type 1 diabetes. Adequate insulin treatment inhibits IGFBP-1 production, which increases bioavailable IGF-I and thereby inhibits GH secretion. Treatment with administration of insulin via the umbilical vein in adults[82] has been shown to correct GH levels.
Since injected IGF-I has a blood glucose lowering effect[83] both short term[84,85] and long term trials[86] with subcutaneously administered recombinant IGF-I (rhIGF-I) have been made. The results are promising with decreased HBA1c levels in parallel with decreased insulin dose, decreased GH and increased IGF-I level[87]. Severe side effects, however, have been reported on high doses, and stopped further trials with rhIGF-I. Concerns about future usage of rhIGF-I as an adjuvant treatment in type1 diabetes include the possible risk of IGF-I having a role in the development of microangiopathic complications[87]. On the other hand low IGF-I stimulates growth hormone secretion, which may also be responsible for micro angiopathy[88,89]. To diminish side effects of rhIGF-I treatment Clemmons et al. gave a binary complex of rhIGF-I and IGFBP-3[90] with a sustained effect on the glucose metabolism but no side effects i.e. oedema, head ache, jaw pain, retinal oedema or Bell's palsy.
The possible use of somatostatin in treating maturity onset diabetes of the young (MODY) has been investigated[91,92]. Other possibilities to suppress GH secretion to decrease insulin resistance in girls with pirenzepine after cessation of growth have been explored[93]. The gains from these therapeutic approaches compared to the possible side effects have not brought them into daily clinical practice in the treatment of type 1 diabetes.

4.2.3 Sex Hormones

DHEAS, being the stable form of DHEA, which is produced in the adrenals and constitutes the hormonal cause of adrenarche. It shows only a small diurnal variation and is not believed to be directly involved in blood glucose homeostasis[94,95].

Testosterone has been described to increase insulin resistance[96], while others have reported only minor influence of testosterone on the blood glucose homeostasis[97,98]. Testosterone shows a positive correlation to IGF-I in healthy young men[99] but this correlation has not been studied in type 1 diabetes.
Testosterone has a marked diurnal variation in healthy boys from the late prepubertal stage, followed by increasing levels during both day and night in puberty[100], with a diurnal pattern mimicking IGFBP-1, leptin and cortisol[101].

Estradiol
The effect of estradiol on insulin resistance has been studied both in connection with the use of oral contraceptives and replacement therapy[102,103], as well as in the menstrual cycle[104] and has only showed minor effects. Diamond et al.[104] showed high insulin resistance during the luteal phase (high progesterone) compared to the follicular phase (high oestrogen). Progesterone has consistently been shown to cause insulin resistance[103] and explains the deterioration in blood glucose control the days before menstruation that many women with type 1 diabetes experience[105,106].

Sex Hormone Binding Globulin (SHBG)
SHBG is the binding protein of testosterone and estradiol and regulates the bioavailability of these hormones[107,108] and the level normally decrease through puberty. SHBG level has been shown to be regulated by insulin[109] in that the pubertal fall in SHBG is related to the pubertal rise in insulin in both sexes. Holly et al.[110] found no correlation between SHBG level and metabolic control in 80 type 1 diabetic adolescents.

4.2.4 Cortisol

Cortisol limits glucose utilisation and stimulates hepatic glucose production, causing an increase in plasma glucose level 2-3 hours after injection[35]. Cortisol production is increased by physical stress, including hypoglycaemia, and is, in combination with GH, responsible for the late rebound glucose increase after an hypoglycaemic episode[35]. Recently a diminished release of cortisol during nighttime hypoglycaemia was demonstrated in prepubertal type 1 diabetic children[111]. Cortisol shows the same circadian variation during physiologic conditions as IGFBP-1[112] and leptin[113,114] with the peak in the morning.

4.2.5 Leptin

Leptin, a 146 amino acid circulating protein produced in the adipocytes[115] with hypothalamus[116] as one target regulating food intake and energy expenditure, has a multiplicity of effects potentially affecting the blood glucose homeostasis. High leptin levels inhibit appetite and energy expenditure, while low levels have the opposite effect. Insulin increases the expression of leptin[115,117], and leptin possibly exerts a negative influence on the ß-cell function[118] constituting a feed-back system[119]. Measures of body fat mass are the most significant determinants of leptin through childhood and body mass index (BMI) standard deviation score (SDS) is positively correlated with serum leptin concentrations[120]. Leptin levels are high in conditions with high insulin resistance, but high BMI and high insulin levels are simultaneously high and the independent role of leptin in insulin resistance is not settled. Leptin interacts with the IGF-IGFBP-system with an inverse correlation to IGFBP-1 in healthy non-obese men and women[121] but not independent of insulin[122]. In children with type 1 diabetes leptin levels have been reported both to be elevated[123] and to be unaffected[124] in comparison to healthy children.
Plasma leptin levels have been shown to vary with the cortisol level in the physiologic range in healthy male subjects[125]. There is a gender difference in leptin levels in healthy children, with boys having lower leptin levels than girls[126], the difference increasing towards the end of puberty paralleling the change in sex steroids[120]. In boys the rise in testosterone level during puberty parallels a decline in leptin level[127].
Circulating levels of leptin follow a diurnal rhythm with the highest level between midnight and early morning hours and lowest around noon to mid-afternoon[113,114] possibly as a result of insulin or glucose induced changes in leptin secretion[128].

5 Aims

Better understanding of the mechanisms that lead to deterioration of blood glucose control during puberty can lead to improved therapeutic approaches. The overall aim of this thesis was to study the hormonal changes during puberty in relation to blood glucose level and

blood glucose variability in children with type 1 diabetes, with special focus on anabolic hormones and linear growth as a mirror of hormonal and nutritional status.

Specific aims were to answer the following questions:

- Do pubertal children with type 1 diabetes compared to postpubertal subjects have increased blood glucose variability (SDbg) measured over 4 weeks and, in that case, which are the possible determinants of SDbg including sex, sex hormones, pubertal stage, linear growth velocity, duration of diabetes, insulin dose and HbA1C ? (paper I)

- Which are the factors determining linear growth in adolescents with type 1 diabetes including metabolic control, total IGF-I, IGF-I standard deviation score (IGF-I SDS), IGFBP-1, sex hormones and/or insulin level? (Paper II)

- Is day to day blood glucose variability in pubertal type 1 diabetic boys over a longer period (weeks) correlated to within the day variability (24 hours)? (Paper III)

- Are changes in the blood glucose levels in type 1 diabetic adolescent boys dependent on the circadian rhythms and/or levels of insulin, GH, tIGF-I, fdIGF-I, IGFBP-1, IGFBP-3-PA, cortisol, testosterone or leptin? (Papers III-V)

- Are the interactions between the GH ↔ IGF-I ↔ IGFBP-I system and insulin, testosterone or leptin different in type I diabetes compared to controls and/or between pubertal and postpubertal boys? (Paper III-V)

6 Material and methods

6.1 Subjects

The local Ethics Committee at Karolinska Sjukhuset have approved the studies (Papers I - V) and informed consent to participate in the studies was obtained from all children and their parents.

6.1.1 Study group 1 (Paper I and II)

Children 10-18 years of age and with a duration of type 1 diabetes > two years attending the outpatient paediatric diabetes clinic at Danderyds Hospital, were asked to participate (n=103) in the studies. Forty-three boys and 39 girls accepted. The distributions in sex, age and Tanner stages are shown below (table 1).

Tanner stage	No.		Age ± SEM (years)	
	Male	Female	Male	Female
1	12	2	11.3 (±1.0)	10.1 11.5
2	6	2	11.9 (±1.2)	11.9 12.5
3	4	5	14.2 (±0.8)	13.3 (±1.1)
4	5	5	15.4 (±2.0)	13.8 (±0.5)
5	16	15	16.7 (±1.4)	16.6 (±1.5)

Table 1. Number, Tanner stage and age of participants Paper I-II.

The majority, 59 patients, was on multiple insulin injections, 12 were on three and one patient was on two daily injections. The patients participated in the study in groups of 4-7 persons. On day 1 instructions on self-monitoring of blood glucose levels (SMBG) were given, a clinical investigation was made and blood samples were drawn. Pubertal stage was assessed using Tanner´s method by two experienced paediatricians (IZ and MW). The participants performed self-monitoring of blood glucose (SMBG) 5 times daily, every second day during four weeks. Values were monitored before breakfast, before lunch, before dinner, 1.5 hours after dinner and at 21.00-22.00. On day 14 and day 28 the group visited the outpatient diabetes clinic where protocols were checked and collected. On day 90 a blood sample for a second HbA1c was drawn and weight and height were measured. Growth velocity was measured with a Harpenden stadiometer over a period of 3 months from the start of the study and was expressed as mm/year.
Relative weight was calculated as weight/length index = [(height/weight)/(mean weight for age/mean height for age)] x 100 according to Durant and Linder[129].
The age-, sex- and puberty corrected IGF-I values and IGF-SD-score respectively were calculated from: IGF-I SDS = $\sqrt{}$(IGF-I -γ)/SD) where γ = predicted value of IGF-I = $\alpha + \beta$ x age according to Juul et al. [130]

6.1.2 Study group 2 (Paper III-V)

The study group consisted of 11 boys with type 1 diabetes mellitus, randomly selected from the outpatient paediatric diabetes clinic at Danderyds Hospital, and nine age and pubertal stage matched healthy boys. The boys were either in pubertal stage Tanner 3 or Tanner 5. For details of the study groups see below (table 2).

GROUP	Tanner Stage	Number (n=)	Control/Diabetes	Age (years)	HbA1c (%)
C 3	3	4	Control	13.15 ±0.52	
C 5	5	4	Control	17.83 ± 0.77	
D 3	3	5	Diabetes	13.82 ± 0.51	7.0 ± 1.0
D 5	5	6	Diabetes	19.07 ± 0.32	7.7 ± 1.7

	Weight (kg)	Length (cm)	BMI	BMI ratio
C 3	57.1±6.1	164.9±6.2	20.85±1.48	1.14±0.09
C 5	68.8±5.4	179.8±0.8	21.25±1.48	1.01±0.07
D 3	55.0±6.9	163.7±3.1	20.23±1.89	1.08±0.09
D 5	69.7±3.1	173.6±3.0	23.13±0.94	1.08±0.04

Table 2 Participants in the Papers III-V, study group 2. Values ± SEM.

Starting in the morning, blood was continuously collected from a cubital vein with a Con-Flo-pump every 30 min for 24 hours. The samples were centrifuged and stored at -20° C until analysis. Standardised regular meals were served as breakfast (08.00) lunch (12.00) and dinner (17.00) as well as snacks at 10.00, 15.00 and 22.00.
In the diabetic boys insulin was given in four daily doses (0.7 - 1.8 U/kg BW/24 hrs) at 07.30, 11.30, 16.30 and 22.00, in all but 1 patient who was treated with continuos subcutaneous insulin infusion (CSII).
During one week before and one week after the 24-hour study period the diabetic participants performed self-monitoring of blood glucose (SMBG), before breakfast, lunch, dinner, 1.5 hours after dinner and at 21.00-22.00.
BMI ratio was calculated from a British normal material[131] as actual BMI / expected BMI for age and sex.
One healthy control was omitted from the study since he acquired a viral infection with high temperature during the 24 hours study period. His level of growth hormone (35,9 mU/L mean 24 hours level) was extremely high and he is presently under further investigation.

6.2 Assays

Blood glucose was measured using a Reflolux 2 (Boehringer Mannheim GmbH, Germany). The total system error of Reflolux 2 compared to a laboratory method is $\pm$ 15 %[132].
HbA1c was analysed with high-performance liquid chromatography (HPLC) Pharmacia,Uppsala, Sweden, reference value <5.2% (mean 4.4 + 2SD).
As an indirect biochemical measure of growth velocity, **alkaline phosphatase (AP)** in serum[133] was analysed using a routine photometric method (Paramax Analytical System®, Baxter, Santa Ana, CA, USA).
Testosterone (Paper I) levels in serum were analysed[134] using commercial RIA techniques (Coat-A-Count®, DPC, Los Angeles, CA, USA).
Dehydroepiandrosterone sulphate (DHEAS) levels in serum were analysed according to[134], using commercial RIA techniques (Coat-A-Count®, DPC, Los Angeles, CA, USA).
Estradiol levels in serum were analysed by a RIA technique[135] (Clinical Assays TM, Sorin Biomedica, Sallugia, Italy).

Sex hormone-binding globulin (SHBG) levels in serum were analysed by a fluoroimmunoassay technique [136](Delfia® SHBG, Pharmacia, Uppsala, Sweden).
Insulin was measured using a RIA technique using guinea-pig antiserum[137]and charcoal addition was used to separate bound and free insulin. The intraassay coefficient of variation (CV) was 5.0% and interassay CV 9.7%. The detection limit was 57 pmol/L.
The concentrations of **IGFBP-1** were determined using a RIA technique according to Póvoa et al.[138]. The sensitivity of the RIA was 3μg/L and the intra- and interassay coefficients of variations were 3 and 10% respectively.
Serum samples for the determination of **total IGF-l** were acid ethanol extracted and cryoprecipitated prior to the RIA, and to further eliminate major interactions of IGFBPs, des (1-3) IGF-l was used as ligand[139]. The intra- and interasay coefficients of variations were 4 and 8 % respectively.
GH was analysed using Delfia® fluoroimmunoassay with a detection limit of 0.03 μg/L, intra-assay CV 5.0 % and inter-assay CV 6.3 %.
IGFBP-3-PA was measured as the ability of a sample to degrade radiolabelled [^{125}I] IGFBP-3 as originally described by Lamson et al.[140] with some modifications[141]. [^{125}I] IGFBP-3 was prepared by iodination of recombinant human glycosylated IGFBP-3 (provided by Genentec Inc, South San Fransisco, CA, USA) by the cloramine-T method resulting in a specific activity of approximately 2000 Ci/mmol after purification of the tracer on Sephadex G75. Patient sera (4 μl) was incubated with approximately 30.000 cpm [^{125}I] IGFBP-3 for 5 h. at 37° C in a total volume of 50 μL 25 mmol/L HEPES (pH 7.4), 2.5 mmol/L Ca2+, and 0.1 % BSA. The reaction was terminated by the addition of SDS sample buffer, and the reaction buffer mixture was separated by SDS-PAGE (12% gels) at 45 V overnight[142]. Gels were dried and analysed by PhoshporImager (Fujifilm BAS-1000 System, Fuji, Tokyo, Japan) displaying linear detection of ^{125}I-labeled IGFBP-3. IGFBP-3-PA was determined as the sum of the intensity of the major fragment bands at 30-, 18- and 15-kDa, respectively, relative to the total intensity of the sum of these bands and the intact IGFBP-3 bands at approximately 39 and 41 kDa. All samples from each patient where assayed within the same SDS-PAGE gel. The inter-assay (among gels) coefficient of variation was 5.6 % and 1.3 % (n = 20) for non-pregnant and pregnant human reference serum, respectively.
Free dissociable IGF-I was measured by a commercial two site IRMA Kit (DSL-9400 ACTIVE™ Free IGF-I IRMA Kit, Diagnostic System Laboratories, Webster, Texas, USA) according to the manufacturers suggestions. The detection limit was 0.03 μg/L, the intra-assay CV 5.1 % and the inter-assay CV 3.6 %.
Leptin was analysed with a commercial RIA kit (Human leptin RIA kit Cat. # HL-81K, LINCO research, Inc. St Charles, Missouri, USA). The patient sera were incubated with [^{125}I] Human Leptin as tracer and rabbit anti human leptin serum as antiserum. As precipitating reagent goat anti rabbit IgG was used. The detection limit was 0.5 μg/L, the intra-assay coefficient of variation (CV) 4.6% and the interassay CV 5.0%.
Cortisol was measured by a commercial solid phase fluoroimmunoassay (AutoDELFIA™ Cortisol, Wallac Oy, Turku, Finland) according to the manufacturers suggestions. The detection limit was 15 nmol/L, the intra-assay CV 2.9 % and the inter-assay CV 0.8 %.
Testosterone (Paper V) was measured by a commercial solid phase fluoroimmunoassay (AutoDELFIA™ Testosterone, Wallac Oy, Turku, Finland) according to the manufacturers suggestions. The detection limit was 0.4 nmol/L, the intra-assay CV 5.5 % and the inter-assay CV 3.5 %.

6.3 Statistical methods

6.3.1 Paper I-II

Data are presented as means ± 1 SD.
To assess the blood glucose variability, we calculated the standard deviation (SDbg) of all blood glucose values obtained from each patient, since this provides a good estimate of blood glucose variability[21,143].

IGFBP-1 was log-transformed to achieve near normal distribution. The other variables investigated were normally distributed.
Mean values, student´s t-test, one way analysis of variances with ANOVA test, Pearson`s single correlations and stepwise multiple linear regression were used for analysis of the data. All calculations were made using QUEST database, statistical and epidemiological software package. All values were presented as mean ± SD. P-values <0.05 were considered significant.

6.3.2 Paper III-V

Data are presented as means ± 1 SD or ± 1 SEM.
IGFBP-1 and GH were log-transformed to achieve near normal distribution. The other variables investigated were normally distributed. Mean values, Pearson`s single correlations, stepwise multiple linear regression, two-way ANOVA, Student's t-test and Mann Whitney U-test were used for analysis of the data. As a measure of the magnitude of the fluctuations in a variable over a certain time in an individual we used the standard deviation (SD) according to Moberg et al. [21]. Daytime is defined as 07.00 until 18.30 and nighttime as 19.00 until 06.30. All calculations were made using STATISTICA database, statistical and epidemiological software package. P-values <0.05 were considered significant.

7 Results and Conclusions

7.1 Controls (Papers III-V, Study Group 2)

*In this section results are given in the controls in Tanner stages 3+5 (**C3+C5** see page 16) thus giving background data and pointing out some data unconnected to the type 1 diabetic condition:*

Mean blood glucose level (4.4±0.1 mmol/L) was equal in Tanner stage 3 (4.6±0.24 mmol/L) and Tanner stage 5 (4.3±0.1 mmol/L)(figure 5a). Nighttime mean value (4.65±0.15 mmol/L) was higher (p=0.03) than the daytime mean value (4.11±0.14 mmol/l) in the Tanner stages combined.

Insulin, diurnal variation showed decreasing levels after midnight (figure 5a) and mean 24 hours level (18.6± 2.2 mU/L) was higher (p<0.001) in Tanner 3 (22.2± 0.7 mU/L) than in Tanner stage 5 (15.0±0.6 mU/L). The daytime mean value (21.1± 0.9 mU/L) was higher (p<0.001) than the nighttime mean value (16.1± 0.8 mU/L) in the Tanner stages combined.

IGFBP-1 showed a diurnal variation with higher levels in the morning with peak level at 06.30 (28 µg/L) and nadir 22.30 (3 µg/L) in Tanner stage 3 and peak level at 07.00 (10 µg/L) and nadir 23.30 (3 µg/L) in Tanner stage 5 (figure 2). Mean 24 hours value (6.7± 1.0 µg/L) was equal in Tanner stage 3 (7.3±1.8 µg/L) and Tanner stage 5 (8.3±1.1 µg/L). Daytime and nighttime mean values were equal in both Tanner stages.

GH mean 24 hours level (5.7±0.6 µg/L) was similar in Tanner stage 3 (5.1±0.7µg/L) and Tanner stage 5 (6.3±0.9 µg/L). In Tanner stage 3 the nighttime peaks were higher (figure 2) while the secretion pattern was turning more adult in Tanner stage 5 with a few high peaks during the day.

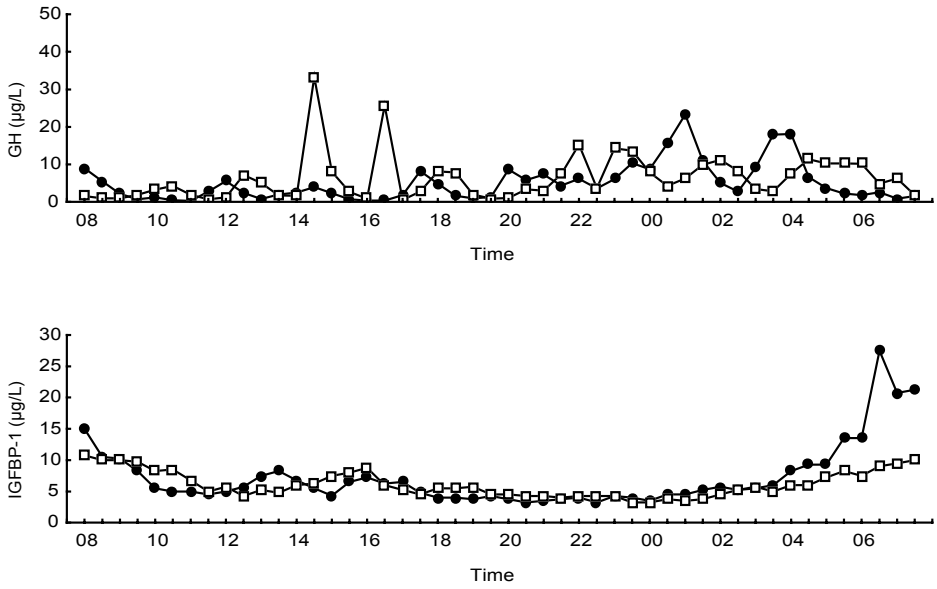

Figure 2. Growth hormone (upper panel) and IGFBP-1 (lower panel) mean value in each time point in the controls. ● = Tanner stage 3. □ = Tanner stage 5.

Total IGF-I mean level (433± 59µg/L) was equal in Tanner stage 3 (442±122 µg/L) and Tanner stage 5 (423±30 µg/L)

Free dissociable IGF-I showed only small variations and the 24 hours mean value (14.3±1.1 µg/L) was equal in Tanner stage 3 (14.1±2.2 µg/L) and Tanner stage 5 (14.5±0.8 µg/L).

Mean IGFBP-3-PA (22.1±0.7 %) was equal in Tanner stage 3 (21.2±0.9%) and Tanner stage 5 (23.0±1.1 %). The daytime mean value (23.3±0.8 %) was higher (p=0.01) than nighttime (20,9± 0,7 %) in the two Tanner stages combined.

Testosterone diurnal rhythm showed a pattern with low values in late evening-night and peak values in the morning and the mean 24 hours level was 14.7±2.2 nmol/L. As expected the 24 hours mean value was higher ($p<0.001$) in Tanner stage 5 (17.9±1.1 nmol/L) than in Tanner stage 3 (11.5± 1.07 nmol/L).

Cortisol showed a diurnal rhythm in similarity with testosterone with high levels in the morning and nadir around midnight. Mean 24 hours level (211.7±32.4 nmol/L) was higher (p=0.004) in Tanner stage 5 (270.2± 22,8 nmol/L) compared to Tanner stage 3 (183,3± 19,9 nmol/L).

Leptin diurnal rhythm was similar to that of testosterone and cortisol. The mean 24 hours leptin level (4,9± 0.3 µg/L) was higher (p<0.001) in Tanner stage 3 (6.6± 0.4 µg/L) than in Tanner stage 5 (3.4± 0.3 µg/L). There was a close correlation between the fasting morning levels (06.00) of leptin and mean 24 hours level (r=0.95 p<0.001).
There was an inverse correlation between mean 24 hours leptin and testosterone levels (r=-0.74 p=0.037) in accordance with earlier studies[127] but no correlation to cortisol as earlier described[125]. Mean 24 hours leptin level was found to correlate to mean 24 hours insulin level (r=0.81 p=0.003) as well as BMI ratio (r=0.73 p=0.037), and inversely to mean 24 hours IGFBP-1 level (r = -0.41 p<0.001). In multiple regression analysis in healthy boys 97% of the variation in leptin was explained by insulin, IGFBP-1, and BMI ratio (Table 3).

$R^2 = 0.976$ $p < 0.001$

	Two tailed p-value	**Stand. regression coeff.**	**R^2 stepwise**
Mean Insulin	0.002	0.55	0.872
Mean IGFBP-1	0.02	-0.26	0.932
BMI ratio	0.06	-0.21	0.976

Table 3. Forward stepwise linear regression model with mean 24 hours leptin level as dependent variable.

The high R^2 with almost the total variation of leptin explained by insulin, IGFBP-1 and BMI ratio has not been reported in earlier studies.

The healthy controls had higher insulin levels in Tanner stage 3 than in Tanner stage 5, higher during the day than during the night, and normal blood glucose level indicating insulin resistance. In our study the mean level of GH was equal in the Tanner stages but the secretion pattern was different possibly affecting insulin resistance.
There was no correlation between mean 24 hours IGFBP-3-PA and mean 24 hours insulin level in the healthy boys, in contrast to that described in healthy adult patients postoperatively[142].
Leptin is involved in puberty onset[144,145] and the elevated leptin level in Tanner stage 3 could be related to the insulin resistance, increased insulin levels[115] and low testosterone level[127] compared to Tanner stage 5. This study also supports the importance of food intake and fat mass (BMI) for determination of leptin levels. The finding of an inverse correlation to BMI ratio in a multiple linear regression model (table 3) is in contrast to that found in adult subjects. The findings, however, suggest that in children high leptin will reduce food intake and thereby regulate body fat mass, which have been reported in animal models[146]. This counteracts the effect of increased insulin levels.
In summary healthy boys have during mid puberty insulin resistance, increased GH and leptin levels and decreased cortisol levels. The latter may be due to decreased cortisol binding globulin (CBG) by insulin. The hormonal changes optimise substrate availability for growth and development.

7.2 Type 1 diabetes.

7.2.1 Blood glucose variability (Papers I and III)

Blood glucose variability calculated as the standard deviation of the blood glucose values (SDbg) measured over a period of 4 weeks in 72 children 10-19 years old (Paper I) are shown below (figure 3), in sexes and Tanner stages, separately. As there were only two girls participating in Tanner stage 1 and 2 respectively there are no SD-bars shown on the two columns representing those individuals.
No correlation to HbA1c was found. SDbg was not associated with hypoglycaemia as there was no correlation between subjectively experienced hypoglycaemic episodes or number of blood glucose values <3 mmol/L. The patients with mean blood glucose values in the lowest quartile (3.3 mmol/L) had lower SDbg than those in the upper three quartiles (4.7 mmol/L), respectively ($p<0.001$).
There was no difference in SDbg between the sexes for all the participants pooled ($p=0.1$), or within the Tanner stages.

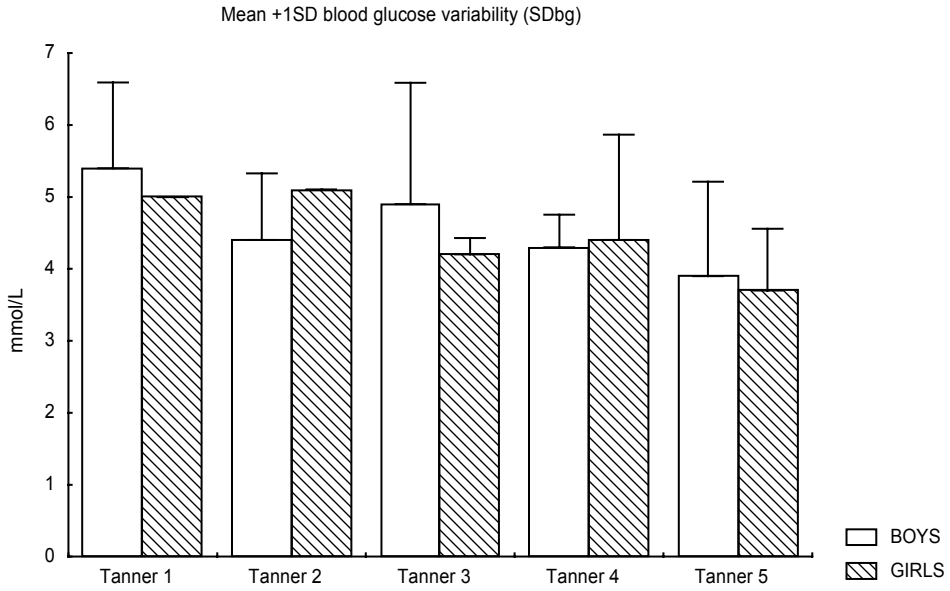

Figure 3. Blood glucose variability (SDbg) measured over 4 weeks.

In postpubertal adolescents (Tanner stage 5) SDbg was significantly lower than in Tanner stage 2-4 pooled ($p=0.02$) and Tanner stage 1 ($p=0.01$). Tanner stage 1 had the highest SDbg compared to Tanner 2-4 pooled ($p=0.02$) and Tanner stage 5 ($p=0.01$). Linear growth rate was higher in Tanner stage 1 than in Tanner stage 5 (figure 4).

Looking at SDbg over a shorter time period i.e. 24 hours with hourly measurements of blood glucose (Paper III) in boys, we could not find any difference between boys in Tanner stage 3 and Tanner stage 5 ($p=0.84$). But, looking in the same boys ($n=11$) over a longer period with five daily blood glucose values during two weeks a difference was found with higher SDbg in Tanner stage 3 than in Tanner stage 5 ($p=0.02$).
The increased long-term blood glucose variability during normal daily conditions in pubertal compared to postpubertal type 1 diabetic individuals was correlated to linear growth rate in the 72 children in study group 1 ($r=0.28$ $p=0.02$) and to alkaline phosphatase, a biochemical

marker of linear growth (r=0.35 p=0.003) but not in the sexes separately. Selecting only boys in Tanner stage 3 and 5 (n=19) there was a correlation between SDbg and linear growth (r=0.45 p=0.05). This is of interest, since we in the 11 diabetic boys in study group 2 found a correlation between long term SDbg over 2 weeks and the mean 24 hours GH level (r=0,75 p=0.033) suggesting a relation between growth and long term SDbg in midpubertal and postpubertal diabetic boys.
In study group 1 the long term SDbg correlated not only to linear growth but also inversely to DHEAS level in the sexes pooled (r=-0.26 p=0.03), to Testosterone/SHBG ratio in the boys (r=-0.31 p=0.04) and Estradiol/SHBG ratio in the girls (r=-0.49 p=0.01). However, in study group 2 we could not find any determinants of the short term SDbg (24 hours).
In summary higher SDbg in Tanner stage 3 was, in a forward stepwise multiple linear regression model (Table 4) explained to 18.6 % by alkaline phosphatase, weight/length index, insulin dose and DHEAS. Alkaline phosphatase, was the strongest determinant. The R^2 for each step is given in the last column.

Total R^2=0.186 p=0.008

	Two tailed p-value	Stand. regression coeff.	R^2 stepwise
Alkaline Phosphatase	0.05	0.26	0.123
Weight/length index	0.09	0.20	0.153
Insulin dose	0.16	0.17	0.175
DHEAS	0.35	-0.12	0.186

Table 4. Forward stepwise linear regression model with SDbg as dependent variable.

The increased long term variability in blood glucose levels in Tanner stage 3 could be explained mainly by growth stimulating hormones, but also by nutrition (i.e. weight/length index) and insulin doses. However this model could not explain 80 % of the long term SDbg variation. In addition during standardised conditions (study group 2) regarding food intake and physical activity none of the hormones studied could explain short term blood glucose variability.
Since we found that blood glucose variability was correlated to linear growth we wanted to continue the study of blood glucose in relation to growth, using growth as a projection of nutritional and hormonal factors possibly affecting blood glucose.

7.2.2 Linear growth. (Paper II)

The linear growth rate was as expected lower (p<0.001) in Tanner stage 5 than in the other pubertal stages (figure 4) (Paper II). There was a difference in linear growth rate between Tanner stage 1 and 5 in the sexes pooled (p=0.01), as well as in boys (p=0.01) and girls (p=0.01), separately.

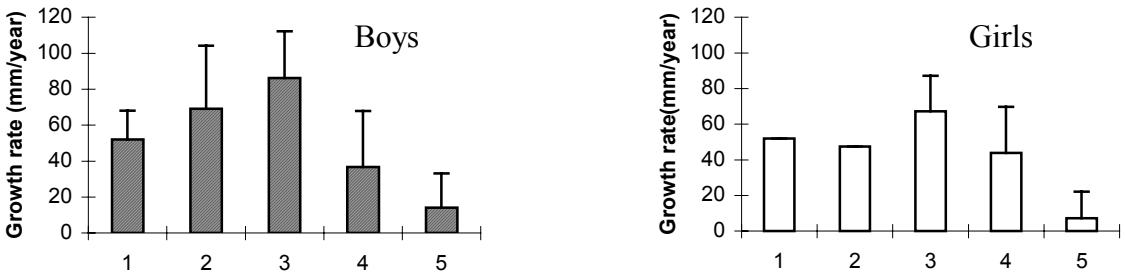

Figure 4. Growth rate in different Tanner stages. Papers I-II.

In study group 1 linear growth was measured over a period of 12 months. In single correlations, insulin dose (r=0.25 p=0.003), IGF-I SD score (r=0.37 p=0.002) and DHEAS (r= -0.53 p<0.001) were associated with growth rate. We found a tendency of relation between growth rate and HbA1c (r=-0.06 p=0.64) but not to weight-length index (r=-0.15 p=0.21) or sex (r= -0.19 p=0.10). Since DHEAS level increases through puberty with the lowest level in Tanner 1 and the highest in Tanner 5 in both sexes it can be used as a marker of pubertal progression. The levels were dependent on pubertal stage in boys (p=0.001), girls (p=0.04) and the sexes pooled (p<0.001) in an analysis of variance. In a forward stepwise multiple regression analysis with growth rate as the dependent variable entering IGF-I SDS, DHEAS, insulin dose, HbA1c and sex as independent variables only IGF-I-SDS and DHEAS were significant determinants of linear growth rate (table 5).

Total R^2=0.383 p<0.001

	Two-Tailed P-value	Stand. Regr. Coeff.	R^2 stepwise
DHEAS	<0.005	-0.468	0.283
IGF-I-SD score	0.02	0.241	0.350
Insulin dose	0.21	0.143	0.370
Sex	0.25	0.11	0.382
HbA1c	0.77	0.03	0.383

Table 5. Forward stepwise linear regression model with linear growth rate as dependent variable.

HbA1c was not a significant determinant of linear growth in this model but was significantly correlated to IGF-I SDS (r=-0.35 p = 0.003) in single correlation suggesting that metabolic control could be of importance to the linear growth.
Final height in the 8 boys and 10 girls who had terminated linear growth did not deviate from their expected final height (0.09 ±0.45 SD) as projected from prepubertal height and age of pubertal onset in the ICP growth chart[147].
We found no sign of a retarded growth or impaired final height in the studied children with diabetes as they followed their prediabetic growth centiles, as recorded consecutively. Whether they had a taller stature compared to controls at the diagnosis of diabetes [148] was not validated in our study. However the duration of diabetes seemed to influence the negative deviation from projected final height (r=-0.47 p =0.049). In poorly controlled diabetes with the extremes untreated type 1 diabetes[1] and Mauriac´s syndrome[149] the stature is severely

affected. In a follow up study[150] 1962 in a Swedish group of type 1 diabetic patients with more than 15 years duration final height was severely affected. Other authors have demonstrated a blunted growth spurt but normal final height in diabetic children [151-153] partly attributed to delayed puberty. Growth failure is not a common finding in paediatric diabetes clinics today[154], probably due to a better insulin regime and better metabolic control. Since we found a relation between growth velocity and IGF-I-SD score, which in turn was determined by metabolic control, the importance of metabolic control to growth in our study can not be ruled out. The hypothesis of a blunted linear growth in diabetes caused by low levels of IGF-I as an effect of hepatic GH resistance is today challenged by the finding in mice with postnatal liver production of IGF-I selectively knocked out resulting in a 80 % decrease in circulating IGF-I[155] but preserved growth due to a direct effect of GH on local IGF-I production.

7.2.3 Tanner stage 3 (Papers III-V)

*In this section results are given in diabetic boys in Tanner stage 3 (**D3** see page 16) in comparison to the results in diabetic boys in Tanner stage 5 (**D5** see page 16) thus exploring the impact of puberty within the type 1 diabetic group:*

HbA1c (7.0±1.0 %) was equal to Tanner stage 5 diabetic boys.

Blood glucose variability over a longer period (SDbg) (5.49±0.66 mmol/L) was equal to that in Tanner stage 3 boys in study group 1 and was increased ($p=0.02$) compared to Tanner stage 5 in study group 2.

Mean 24 h blood glucose level (13.0±2.8 mmol/L) was increased ($p<0.001$) compared to Tanner stage 5 (figure 5a) while SDbg over 24 hours was equal to Tanner stage 5 and did not correlate to other variables.

Insulin, diurnal variation showed meal related peaks and decreasing levels after midnight. The mean 24 hours value (27.1±11.1 mU/L) were equal to Tanner stage 5 (figure 5a). Day time mean value (32±2 mU/L) was higher ($p=0.001$) than night time mean value (21.6±1.2 mU/L).

IGFBP-1 diurnal rhythm showed low values during the day with nadir in the afternoon rapidly increasing after midnight with peak levels in the morning. The mean 24 hours value (27.7±5.8 µg/L) were similar to Tanner stage 5. LogIGFBP-1 correlated to simultaneously measured blood glucose values ($r=0.52$ $p=0.004$) independently of GH but not of insulin (Figure 5b).

GH showed broader and higher peaks with maximum after midnight and mean 24 hours level (12.7 $\pm$ 13.2 µg/L) was increased ($p=0.001$) compared to Tanner stage 5 (figure 5b).

Total IGF-I (313± 67 µg/L) was slightly, but not significantly elevated ($p=0.27$) compared to Tanner stage 5.

Free dissociable IGF-I diurnal rhythm showed decreasing values after midnight similar to Tanner stage 5 and the 24 hours mean value (9.04±1.89µg/L) was equal to Tanner stage 5.

IGFBP-3-PA diurnal rhythm showed higher peaks with increased variability (5,93±0.63 %) ($p<0.001$) and increased mean 24 hours value (27.01±1.96 %) ($p= 0.003$) compared to Tanner stage 5. The day time mean value was higher than night time mean value ($p=0.01$).

Testosterone diurnal rhythm showed low values in late evening-night and peak values in the morning similar to Tanner stage 5. Mean 24 hours level (10.0±3.9nmol/L) was lower ($p<0.001$).

Cortisol diurnal rhythm was similar to that of testosterone with high levels in the morning and nadir around midnight. Mean 24 hours level (198.9± 19.5nmol/L) was lower but not statistically significant different from Tanner stage 5.

Leptin diurnal rhythm showed a more distinct fall from early morning to lunch than in Tanner stage 5 and the 24 hours mean value (6.7±1.5µg/L) was higher ($p<0.001$).

In the present study the mid pubertal diabetic boys had a well controlled diabetes with HbA1c and 24 h SDbg not different from postpubertal boys.
In accordance with other authors[10,11] we found however that both the mean 24 hours blood glucose level and mean 24 hours GH level was higher in Tanner stage 3 compared to Tanner stage 5 despite comparable insulin levels, food intake and physical activity. This confirms insulin resistance with impaired insulin induced suppression of glucose production and uptake during the period of rapid linear growth. The SDbg over a longer time period was higher in Tanner stage 3 than in Tanner stage 5 and correlated to mean 24 hours GH level. No correlation was found between mean 24 hours blood glucose level and mean 24 hours GH level in accordance with Batch et al.[40]. This implicates an association between SDbg over a longer period and linear growth. The fluctuations in blood glucose measured every hour during 24 hours did not co-variate with GH but with insulin and IGFBP-1 that will be further commented in section 7.2.4. Neither did blood glucose correlate to leptin, cortisol, fdIGF-I or testosterone.
IGFBP-3-PA mean 24 hours level and variability were elevated both compared to Tanner stage 5 diabetic boys as well as to controls. This indicates an activation of the IGFBP-3-PA in this situation with increased blood glucose and increased GH despite similar insulin levels i.e. insulin resistance. We found higher IGFBP-3-PA during the day than during the night in congruence with the finding by Cheetham et al.[156] of a higher fragmentation during the day than during the night of endogenous IGFBP-3 in patients with type 1 diabetes. We speculate the increased IGFBP-3-PA in Tanner stage 3 in diabetic boys to be a compensatory mechanism to restore free IGF-I concentrations and thereby stimulate glucose uptake in muscles. Insulin has been proposed to inhibit IGFBP-3-PA[157] but in the present study insulin levels were higher during the day when IGFBP-3-PA was increased. In addition our unpublished data from nondiabetic patients propose a stimulatory effect of insulin on IGFBP-3-PA postoperatively. IGFBP-3-PA is elevated in other insulin resistant states with hyperinsulinemia; type 2 diabetes[141], after surgery[142,158], in pregnancy[62] or in severe illness[159]. However we didn't find, in the diabetic children, a correlation between insulin and IGFBP-3-PA but between mean 24 hours blood glucose level and mean 24 hours IGFBP-3-PA. In a forward stepwise linear regression model including all the diabetic patients (n=11) mean 24 hours blood glucose was the only significant determinant of IGFBP-3-PA ($R^2=0.728$ $p=0.007$) including mean 24 hours fdIGF-I and mean 24 hours IGFBP-I (table 6). However, fdIGF-I and IGFBP-1 explained 13.8 % of the variation in IGFBP-3-PA. This suggests that low free IGF-I stimulates IGFBP-3 protease activity.

Total R^2=0.728 p=0.007

	Two tailed p-value	Stand. regression coeff.	R^2 stepwise
Mean blood glucose	0.007	0.79	0.590
Mean fdIGF-I	0.11	-0.39	0.678
Mean IGFBP-1	0.30	-0.25	0.728

Table 6. Forward stepwise linear regression model with mean 24 hours IGFBP-3-PA as dependent variable.

The increased IGFBP-3-PA could not normalise fdIGF-1, may be due to other IGFBPs, i.e. IGFBP-1.
The diurnal rhythm of fdIGF-I with decreasing level after midnight could partly be an effect of lower IGFBP-3-PA during the night compared to the day.

24 hours mean level of cortisol was lower but not significantly separated from Tanner stage 5.

Mean 24 hours leptin level was higher in type 1 diabetic boys compared to controls but also in Tanner stage 3 compared to Tanner stage 5. Leptin will be further discussed in section 7.2.5. Leptin, cortisol and testosterone could not explain the increased B-Glucose in Tanner stage 3.

7.2.4 Tanner stage 5 (Papers III-V)

*In this section results are given in diabetic boys in Tanner stage 5 (**D5** see page16) compared to results in diabetic boys in Tanner stage 3 (**D3** see page 16) thus further exploring the impact of puberty within the type 1 diabetic boys group.*

HbA1c (7.7±1.7 %), SDbg over 24 hours and insulin diurnal variation (figure 5a) with decreasing levels after midnight and mean 24 hours value (25.9±8.3 mU/L), were equal to Tanner stage 3 diabetic boys. The insulin levels were higher than in the controls.

Mean 24 hours blood glucose level (8.4±2.4 mmol/L) was decreased ($p<0.001$) compared to Tanner stage 3 (figure 5a).

Blood glucose variability over a longer period (SDbg) (3.65± 0.18 mmol/L) was decreased ($p=0.02$) compared to Tanner stage 3.

IGFBP-1, diurnal rhythm showed low values during the day with nadir in the afternoon increasing after midnight. The mean 24 hours value (25.9±8.3 µg/L) were similar to Tanner stage 3 but higher than in the controls. LogIGFBP-1 correlated to simultaneously measured blood glucose values ($r=0.74$ $p<0.001$) independently of insulin and of GH (figure 5b).

GH showed more frequent small peaks and mean 24 hours level (6.0±4.6 µg/L) was decreased ($p=0.001$) compared to Tanner stage 5 (figure 5b).

Total IGF-I (235±25 µg/L) was slightly, but not significantly lowered ($p=0.27$) compared to Tanner stage 3.

fdIGF-I diurnal rhythm with decreasing values after midnight and 24 hours mean value (8.77±1.27 µg/L) was equal to Tanner stage 3.

IGFBP-3-PA diurnal rhythm showed lower peaks with lower variability (2.64± 0,48 %) ($p<0.001$) and decreased mean 24 hours value (22.97±0.80 %) ($p= 0.003$) compared to Tanner stage 3, which suggest higher available IGFBP-3 in Tanner stage 5. The changes in bioavailable IGFBP-3 for IGF-I binding can be compensated by the presence of other binding proteins e.g. IGFBP-1, which may explain the unchanged levels of fdIGF-I in Tanner stage 5. Daytime mean value was higher than night-time mean value ($p=0.01$).

Testosterone was similar to Tanner stage 3. Mean 24 hours level (17.07± 1.58 nmol/L) was higher than in Tanner stage 3 ($p<0.001$).

Cortisol diurnal rhythm was similar to Tanner stage 3. Mean 24 hours level (224.4± 16.1 nmol/L) was higher but not statistically significant separated from Tanner stage 3.

Leptin diurnal rhythm did not show such a distinct fall from early morning to lunch as in Tanner stage 3 and the 24 hours mean value (4.6±0.5 µg/L) was lower ($p<0.001$) than in Tanner stage 5.

The mean IGFBP-1 value in each time point in the 24 hour curve (n=48) for the 5 boys in Tanner stage 5 correlated to the simultaneously measured mean blood glucose value. In a multiple regression analysis with blood glucose as a function of logIGFBP-1, insulin and log GH ($R^2=0.63$ $p<0.001$) logIGFBP-1 was the strongest ($r=0.66$ $p<0.001$) determinant of blood glucose. We found an inverse correlation between mean 24 hours IGFBP-1 level and mean 24 hours fdIGF-1 level ($r=-0.59$ $p=0.008$) when pooling data from all the participants (n=19). This is in agreement with other reports[56].

Earlier data indicate that the IGF-system, especially IGF-I and IGFBP-1 may be involved in the glucose homeostasis. In vitro IGFBP-1 inhibits the glucose uptake in muscle[78,79]. In rats IGFBP-1 injected alone has been shown to increase blood glucose[160] and to blunt IGF-I induced hypoglycaemia[160]. Transgenic mice, with normal IGF-I overexpressing IGFBP-1 have elevated blood glucose level [161]. Recently, in Cre/loxP recombination system mice where the hepatic production of IGF-I was abolished[155] the concentration of IGF-I in the serum was reduced by 75% without any discernible effect on postnatal growth[155]. But, the carbohydrate and lipid metabolism was affected with increased leptin and insulin levels and decreased glucose tolerance (personal communication). In a patient with IGF-I gene deletion who had high insulin resistance, rhIGF-I treatment resulted in substantially decreased insulin resistance[162]. In vivo IGFBP-1 has been shown to be an important determinant of free IGF-I[63,64]. In healthy adults IGFBP-I decreasing fdIGF-I have been demonstrated to be of importance to the glucose metabolism in the fasting state[63,163,164]. In type 1 diabetic patients IGFBP-1 has been associated with the dawn phenomenon[165-167] with increasing levels of IGFBP-1 and decreasing insulin levels from midnight till morning. Insulin is the main regulator of circulating IGFBP-1 where low insulin activity at the hepatic level increases IGFBP-1[65]. Other stress hormones can also stimulate IGFBP-1 production[171,172]. However we found no correlation between IGFBP-1 and cortisol or GH.
The data in this thesis support the impact of IGFBP-1 on glucose homeostasis not only to be restricted to the dawn phenomenon but also as being a factor partly independent of insulin and GH regulating the blood glucose level, possibly via regulation of the free fraction of IGF-I.

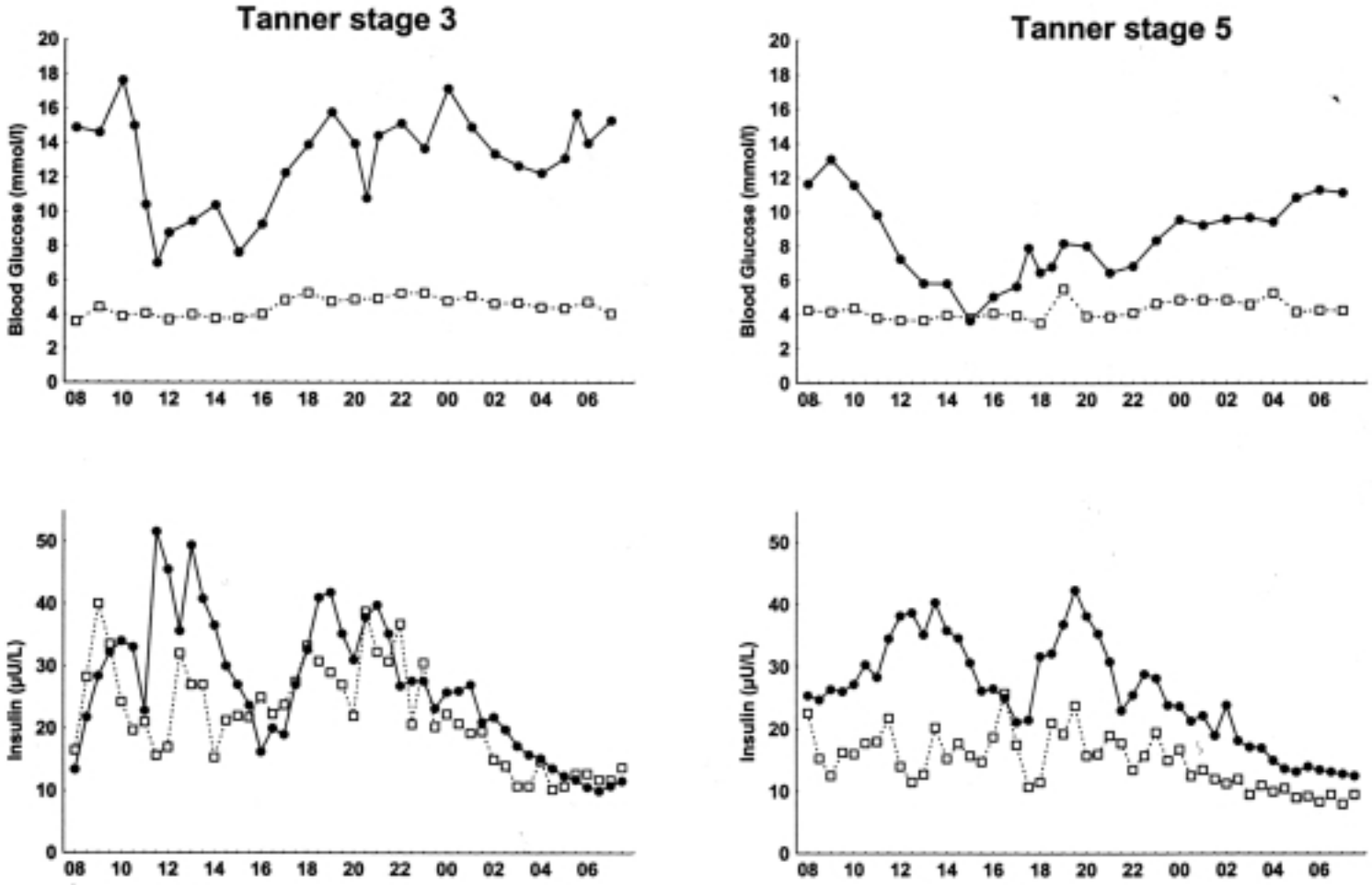

Figure 5a.
Mean level in each time point of blood glucose and insulin in Tanner stage 3(left) and Tanner stage 5(right) ● = type 1 diabetes. □ = controls

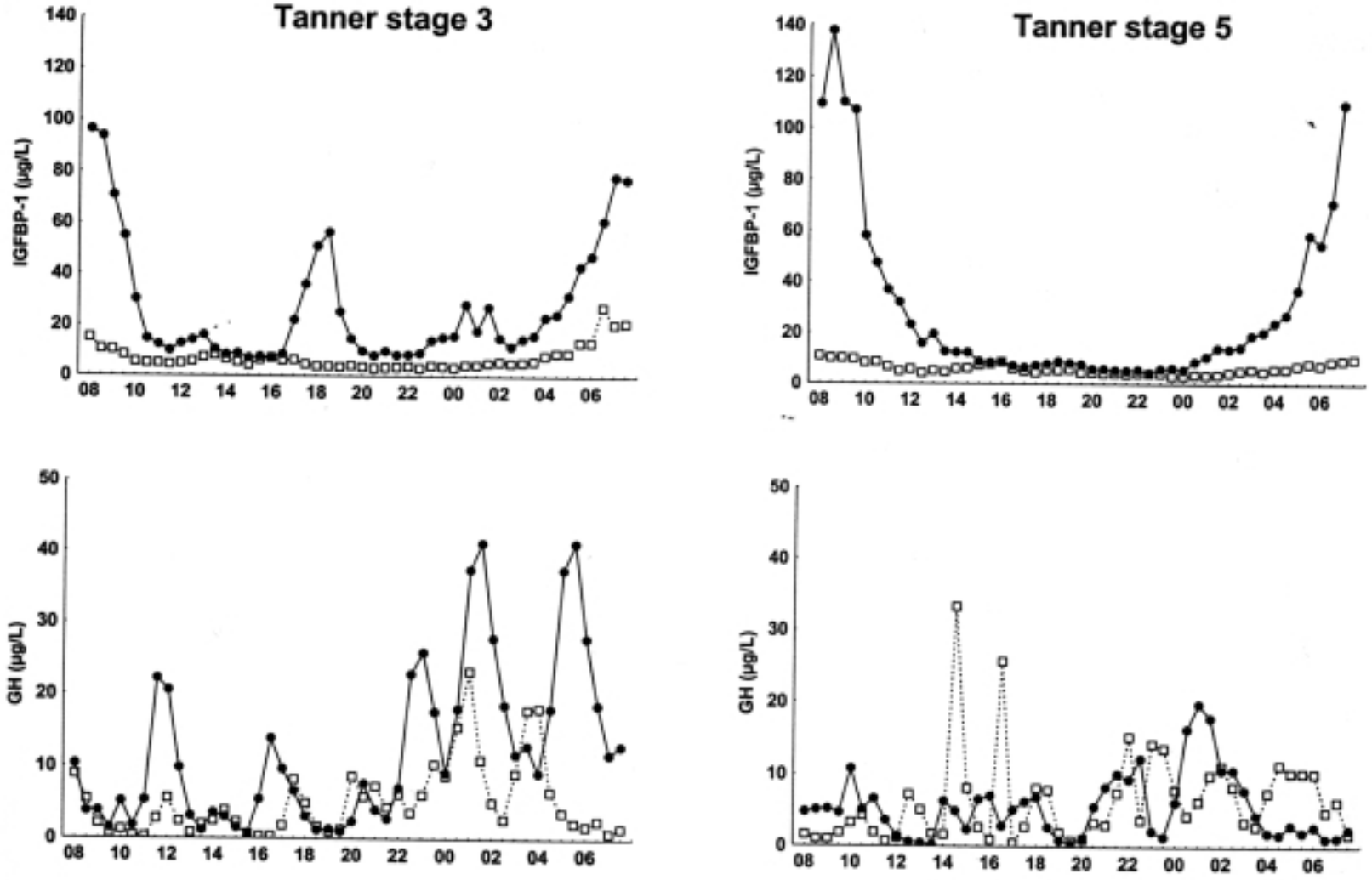

Figure 5b.
Mean level in each time point of IGFBP-1 and GH in Tanner stage 3(left) and Tanner stage 5(right) ● = type 1 diabetes. □ = controls

Diabetic condition per se (Papers III-V)
*In this section results are given in diabetic boys in Tanner stages 3+5 (**D3+D5** see page16) compared to results in the controls in Tanner stages 3+5 (**C3+C5** see page16) thus exploring the impact of the type 1 diabetic condition per se:*

Blood glucose variability over a longer period (SDbg) was similar in the 2 study groups and correlated to mean 24 h GH level (r=0.75 p=0.033) but not to HbA1c.

SDbg over 24 hours when controlled for food intake and physical activity did not correlate to the hormones studied.

Mean blood glucose level (10.6±1.2 mmol/L) was increased during the night (11.7±1.6 mmol/L) compared to the day (9.7±1.1 mmol/L) (p=0.009), which was also the case in the controls.

Insulin, diurnal variation showed decreasing levels after midnight (figure 5a) and mean 24 hours value (26.5±2.5 mU/L) was higher (p=0.04) than in the controls (18.6± 2.08mU/L). Subcutaneous injection of insulin result in higher peripheral level than in the controls but the higher blood glucose level could be explained by a relative hepatic insulin resistance depending either on high GH level or low portal insulin level in type 1 diabetes.

IGFBP-1 diurnal rhythm with low values during the day rapidly increasing after midnight was more pronounced than in the controls (figure 5b) with a 5-10 fold greater increase after midnight. Mean 24 hours value (27.7± 4.8 µg/L) was higher (p<0.001) than in the controls (6,7± 1.0 µg/L). Also the variability in IGFBP-1 was higher (p=0.01) in the diabetic boys (40,0± 10,2 µg/L) than in the controls (4.9± 1.2 µg/L). LogIGFBP-1 correlated to simultaneously measured blood glucose values (r=0.496 p=0.005) partly dependent of GH and insulin.

GH mean 24 hours level (7.41± 1.32 µg/L) was increased (p=0.01) compared to controls (5,22±0.89 µg/L), this was dependent on the increase seen in Tanner stage 3 (D3; 9.79±2.28 µg/L versus C3; 5.13± 1.41 µg/L p=0.001) (figure 5b).
It is to be noted that GH was only increased in the diabetic boys in Tanner stage 3 and not in the controls. One possible explanation to this could be the negative feed back effect of low bioactive IGF-I level in the diabetic boys.

Total IGF-I (270±34 µg/L) was decreased (p=0.02) compared to controls (433±59µg/L).

Free dissociable IGF-I diurnal rhythm showed decreasing values after midnight in all boys with diabetes but two, and the 24 hours mean value (8.9±1.1µg/L) was decreased (p=0.003) compared to controls (14.3±1.1 µg/L).

IGFBP-3-PA diurnal rhythm showed higher peaks with increased variability and increased mean 24 hours value but did not reach statistical significance compared to the control group. Mean 24 hours IGFBP-3-PA was strongly correlated to mean 24 hours blood glucose value (r=0.768 p=0.006). Daytime mean value was higher than nighttime mean value (p=0.01).

Testosterone diurnal rhythm and mean 24 hours level (13.9±2.2 nmol/L) were similar to the controls (14.7±2.2 nmol/L).

Cortisol diurnal rhythm and mean 24 hours level (212.5±12.1 nmol/L) were similar to the controls (211.7±32.4 nmol/L).

Leptin diurnal rhythm showed a more distinct fall from early morning to lunch than in the controls and the 24 hours mean value (5.6±0.2 µg/L) was higher (p=0.02) than in the controls (4.9±0.3 µg/L). There was no correlation between mean 24 hours leptin and testosterone levels, or any other hormones studied.
The mean 24 hours leptin level did not, in contrast to the controls, correlate to mean 24 hours insulin or IGFBP-1 level but to BMI ratio (r=0.81 p=0.002).

In the diabetic boys we found an elevated GH level, increased IGFBP-1 level and increased IGFBP-3-PA that have been discussed in sections 7.2.3 and 7.2.4. We also found a higher peripheral insulin level in the diabetic children especially during the day compared to the controls, while the nighttime level was similar. The increased IGFBP-1 level could be dependent either on lower portal insulin level in the diabetic children or an altered relationship between insulin level and IGFBP-1 in type 1 diabetic patients as proposed by Hilding et al[168]. We collected blood samples in the afternoon 3-4 hours post prandially for analysis of IGFBP-1 in study group 1 and evaluated them as being normal, which is confirmed in study group 2 (figure 5b). The controls and the type 1 diabetic children in study group 2 had similar levels.
Both total and free IGF-I were decreased compared to controls. We found a close relation between total and free IGF-I both in the diabetic children (r=0.76 p=0.006) and in the controls (r=0.77 p=0.025). The possible impact of decreased level of fdIGF-I in diabetic children is discussed in section 7.2.3 and 7.2.4.
Cortisol and testosterone showed no differences in the diurnal rhythms or 24 hours mean levels between healthy and diabetic boys. The 24 hours mean level of both testosterone and cortisol was higher in Tanner stage 5 compared to Tanner stage 3 in the controls. In the diabetic boys 24 hours mean testosterone level was higher in Tanner stage 5 than in Tanner stage 3 while the somewhat elevated mean 24 hours cortisol level did not reach statistical significance. The deteriorated blood glucose control during puberty could not be explained by the changes in these two hormones.
Leptin was increased in pubertal type 1 diabetic boys in comparison both to postpubertal type 1 diabetic boys and to healthy controls. This is in contrast to Verroti et al.[124] who reported that type 1 diabetes did not modify the serum leptin level in prepubertal, pubertal and postpubertal boys and girls. Our findings support the results from Luna et al.[123] who found that both diabetic boys and girls showed higher leptin levels than a group of age-, sex-, and BMI-matched normal children. The normally positive correlation to insulin[115,117] and inverse correlation to IGFBP-1[121] was not present in the diabetic boys in our study, but in the controls. In the diabetic boys mean 24 hours leptin level correlated to BMI ratio that was also the case for the controls. The decrease in leptin level paralleling an increase in testosterone[120,127] was not present in the diabetic boys, but in the healthy controls in our study. These changes in the levels of, and absence of the relations of leptin to insulin, IGFBP-1 and testosterone indicate an impaired regulation of leptin in type 1 diabetic boys. High leptin levels lead to low energy expenditure, which may partly explain the high blood glucose levels. This could be of importance since leptin has been reported to be an independent risk marker for cardiovascular disease (CVD) in adults[169,170].

8 General Discussion

The long-term variability (weeks) in blood glucose level (SDbg) was found to be larger during than after puberty and related to linear growth rate, its biochemical marker alkaline phosphatase (ALP), or GH in adolescents with type 1 diabetes. This suggest that the hormones that regulate growth could affect blood glucose level and blood glucose variability
The short term (24 hours) SDbg during controlled conditions, regarding food intake and physical activity, did not correlate to the long term SDbg. The diabetic children had a linear growth rate and final height that did not differ from that of the healthy children, which most probably is dependent on the insulin regime and doses, as well as metabolic control.
The diabetic children had higher GH and lower tIGF-I and fdIGF-I than the controls suggesting a relative hepatic GH resistance. Insulin affects the hepatic GH receptor activity. The peripheral insulin level in the diabetic children was elevated compared to the controls indicating increased insulin resistance. It could also indicate decreased portal insulin level in the diabetic children. Also the IGFBP-1 levels were increased despite high insulin level in the diabetic children suggesting decreased portal insulin level or impaired hepatic insulin extraction. These findings emphasise the importance of further optimising the insulin treatment to restore the changes in the GH-IGF-I-IGFBP system.
In Tanner stage 3 the IGFBP-3-PA was increased in the situation with high GH, insulin resistance and low fdIGF-I suggesting a compensatory mechanism to increase the low fdIGF-I thereby increasing the glucose uptake in muscles, which could result in a decreased blood glucose level.
In Tanner stage 5 the blood glucose level was related to changes in the IGFBP-1 level independent of insulin and GH. This can possibly be explained by IGFBP-1 regulating the free biologically available IGF-I.
To summarise the effects of the GH-IGF-I- IGFBP system on the blood glucose it seems that during puberty the elevated GH and lowered bioavailable IGF-I level could contribute to the elevated blood glucose level. After puberty, when GH is lower, IGFBP-1 has a greater impact on the blood glucose level.
Some interpretations of possible psychosocial factors influencing the blood glucose level can be made from our data. Stress is a recognised etiological factor in hyperglycaemia. However, against its role during puberty, we observed that cortisol levels, a marker of stress, are somewhat higher in Tanner stage 5 compared to Tanner stage 3. This is in contrast to higher blood glucose level in Tanner stage 3. The low IGFBP-1 level during the day in the diabetic children, that was equal to the controls, also indicates less stress and good compliance with the insulin treatment, since IGFBP-1 is a good marker of the insulin activity and is increased by stress hormones[171,172]. In patients with poor metabolic control IGFBP-1 and IGF-I could be diagnostic tools to evaluate the cause, since low values of IGF-I and high IGFBP-1 indicate lack of insulin while low or normal levels may indicate overeating.
The best way to correct the derangement in the GH-IGF-I-IGFBP system in type 1 diabetes is to optimise the insulin treatment, particularly by elevating the overnight insulin concentrations. This could be achieved via the usage of more long acting insulin analogues in the evening or via the usage of continuous subcutaneous insulin infusion (CSSI).
Treatment with rhIGF-I is also under development and the binary complex rhIGF-I and IGFBP-3 has shown promising results in type 1 diabetes.
Leptin is a hormone that recently has been described to be of importance in metabolic control as well as a risk factor for development of cardiovascular disease. We could demonstrate elevated leptin levels in diabetic boys with a blunted correlation to insulin. The importance of increased leptin levels are not fully understood but could be a marker of incomplete insulin treatment and increased risk of later diabetic complications, and be of importance for the increased blood glucose levels.

In conclusion the GH- IGF-I - IGFBP system seems to be of great importance in the regulation of glucose homeostasis during and after puberty in diabetic boys. Linear growth velocity can be used as a biological marker of the GH-IGF-I-IGFBP system and the blood glucose variability measured as standard deviation of blood glucose over a longer period is suggested to be an additive marker of metabolic control in diabetic children. The treatment should be aimed to lower the SDbg and to normalise the changes in the GH-IGF-I-IGFBP system.

9 Speculations and future

The use of blood glucose variability as a marker of blood glucose control in addition to HbA1c should be recorded and longitudinally evaluated in type 1 diabetic patients in the same way as HbA1c is used today. This will give us knowledge if increased blood glucose variability is of importance to the development of complications. To further disentangle the reasons for increased blood glucose variability, longitudinal studies through puberty following changes in circadian hormonal rhythms, as well as improving the tools for the analysis of these rhythms are needed. Also psychosocial factors should be evaluated in such studies.
Future treatment approaches, apart from improved insulin treatment, include intervention in the GH-IGF-I-IGFBP system at the level of directly affecting the IGFBP levels, e.g. treatments aiming at lowering the IGFBP-1 level or induction of proteolysis of the IGFBP-3-ALS complex.

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