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Human chorionic gonadotropin beta genes and recurrent miscarriage: expression and variation study

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To my family

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LIST OF ORIGINAL PUBLICATIONS

I **Rull K, Laan M.** 2005 Expression of beta-subunit of HCG genes during normal and failed pregnancy. Hum Reprod. 20(12):3360–3368. Epub 2005 Aug 25.

II **Rull K, Hallast P, Uusküla L, Jackson J, Punab M, Salumets A, Campbell RK, Laan M.** 2008 Fine-scale quantification of HCG beta gene transcription in human trophoblastic and non-malignant non-trophoblastic tissues. Mol Hum Reprod. 14(1):23–31. Epub 2007 Nov 29.

III **Rull K, Nagirnaja L, Ulander VM, Kelgo P, Margus T, Kaare M, Aittomäki K, Laan M.** 2008 Chorionic Gonadotropin Beta gene variants are associated with recurrent miscarriage in two European populations. J Clin Endocrinol Metab. 93(12):4697–4706. Epub 2008 Sep 9.

My contribution to the articles in the current thesis is as follows:

Paper I: study design, collecting clinical data and tissue samples, conducting the experiments, data analysis, writing the paper.

Paper II: study design, handling the tissue samples (extraction of RNA, DNA), cloning the plasmids, performing the experiments on gene expression, data analysis, writing the paper.

Paper III: clinical evaluation of patients and controls, collecting the clinical material, participating in the design of primers, conducting the re-sequencing work, data analysis, writing the paper.

ABBREVIATIONS

AP-2	activating protein 2
APC	activated protein C
APS	antiphospholipid syndrome
bp	base pair
cAMP	adenosine 3',5'-cyclic monophosphate
CGB	chorionic gonadotropin beta gene
CI	confidence interval
D&C	cervical dilatation and uterine curettage
dNTP	deoxyribonucleotide triphosphate
e.g.	*exempli gratia* (from the Latin phrase), for example
EP	extrauterine pregnancy
ERK1/2	extracellular signal regulated protein kinases 1 and 2
FAM	6-carboxyfluorescein
FSH	follicle stimulating hormone
FSHB	follicle stimulating hormone gene
GAPDH	glyceraldehyde-3-phosphate dehydrogenase gene
GST	gluthatione S-transferase
HCG	human chorionic gonadotropin
HCGβ genes	the genes encoding β-subunit of human chorionic gonadotropin
HCG-H	hyperglycosylated human chorionic gonadotropin
HEX	hexachloro-6-carboxyfluorescein
HLA	human leucocyte antigen
HPRT1	hypoxanthine phosphoribosyltransferase 1 gene
IFN-γ	interferon gamma
IL	interleukin
IVF	*in vitro* fertilization
LD	linkage disequilibrium
LH	luteinizing hormone
LHB	luteinizing hormone gene
MGB	minor groove binder
MHC	major histocompatibility complex
MTHFR	methylenetetrahydrofolate reductase
NK cell	natural killer cell
NOS	nitric oxide synthase
OR	odds ratio
ORF	open reading frame
RM	recurrent miscarriage
RPII	RNA polymerase II largest subunit gene
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SE	standard error

SNP	single nucleotide polymorphism
Sp1	selective promoter factor 1
TAMRA	tetramethyl-6-carboxyrhodamine
TET	tetrachloro-6-carboxyfluorescein
TGFβ	transforming growth factor-beta
Th-cell	T helper lymphocyte
TNF-α	tumor necrosis factor alpha
TSH	thyroid stimulating hormone
TSHB	*thyroid stimulating hormone gene*
UTR	untranslated region
VEGR	vascular endothelial growth factor
v-LHβ	variant luteinizing hormone β

1. INTRODUCTION

Miscarriage is the most frequent complication of the first trimester of the pregnancy. A total of 70% of all conceptions are lost prior the live birth, majority of the losses remain undiagnosed as they occur before the time of the missed menstrual period (Macklon et al. 2002). When clinical pregnancy is established, the risk of spontaneous pregnancy loss is ~10–15% (Wilcox et al. 1988; Zinaman et al. 1996; Cramer and Wise 2000).

Recurrent miscarriage (RM), defined as three miscarriages in a row, affects ~1–2% of the couples who aim a childbirth (Berry et al. 1995; Bricker and Farquharson 2002). The prevalence of RM is higher than it would be expected if three miscarriages happen consecutively only by change (Christiansen et al. 2008). The discrepancy between the observed and expected occurrence of RM implies that there is an underlying pathological explanation. Although the patients of RM undergo multiple tests for detecting parental chromosomal anomalies, maternal thrombophilic, endocrine and immunological disorders, ~50% of the RM cases are classified as having idiopathic, unexplained origin (Christiansen 1996; Bricker and Farquharson 2002; Christiansen et al. 2008).

The studies focused on the familiar predisposition to RM have shown 2–7 fold increased prevalence of miscarriage among first-degree blood relatives of a women suffering from RM (Christiansen 1996). So far, major interest has focused on the mother's physiological response to the pregnancy. Genes involved in the development of immunotolerance, angiogenesis, apoptosis and blood coagulation have been targeted most frequently (Dosiou and Giudice 2005; Goodman et al. 2006; Hviid 2006). As these genes also contribute to complex diseases, the role of their variants in susceptibility to RM may not be specific. The placental proteins coded by the fetal genome certainly have a direct influence on pregnancy success and would be suitable candidates for genetic studies of RM.

One of the first proteins produced by conceptus is human chorionic gonadotropin (HCG). The synthesis of HCG begins shortly after fertilization; the β-subunit of the hormone has been detected in the two-cell stage embryo (Jurisicova et al. 1999). The main function of the hormone is to delay the apoptosis of the corpus luteum during the first trimester of the pregnancy, but HCG has also several paracrine effects in the process of implantation (Licht et al. 2001; Cameo et al. 2004), angiogenesis, placentation (Herr et al. 2007) and development of maternal immunotolerance (Kayisli et al. 2003). Low level of HCG in maternal serum during the first trimester of pregnancy is related to miscarriage, extrauterine pregnancy, and failure of *in vitro* fertilization procedure (Buyalos et al. 1992; Letterie and Hibbert 2000; Dumps et al. 2002; Tong et al. 2006).

HCG, like other gonadotropic glycoproteins, is composed of two subunits: common α- and hormone-specific β-subunit. HCG β-subunit is encoded by four highly homologous genes (*CGB, CGB5, CGB7, CGB8*) that reside in a common genome cluster together with an evolutionarily ancestral *LHB* gene at chromosome 19q13.32. Two other members of the *LHB/CGB* gene cluster, recently duplicated *CGB1* and *CGB2,* may give rise to a hypothetical protein that has no homology to any other known protein. The biological function of *CGB1* and *CGB2* is unknown.

No patients have been described with a phenotypic consequence from the polymorphisms in genes coding the subunits of HCG so far. Any mutation is supposed to be embryonic-lethal (Themmen and Huhtaniemi 2000). However, the natural variation within the genes is expected to occur with possible functional influences. The large resequencing study carried out in our laboratory revealed multiple polymorphisms in *LHB/CGB* genes (Hallast et al. 2005) but there is no data about their association with an individual's reproductive success. As HCG is coded by fetal genome, the design of an association study is complicated.

My study focuses on the detailed expression profile of six *CGB* genes both in total and individually during the normal and complicated pregnancies. Based on the results, the association of genetic variation of two most transcribed *HCGβ* genes and recurrent miscarriages will be addressed.

2. REVIEW OF THE LITERATURE

2.1. Recurrent miscarriage as a complex disease

2.1.1. The main causes of sporadic miscarriages

The most frequent condition causing a miscarriage of clinically diagnosed pregnancy is a chromosomal abnormality of the fetus/embryo encountered in >50% of gestations (Boue et al. 1975; Eiben et al. 1990). It has been estimated that 70% of pregnancies aborted before 6 gestational weeks are due to numeric cytogenetic errors (Sierra and Stephenson 2006). Pregnancy demise between 6 and 10 weeks of gestation occurs in approximately 15% of clinical pregnancies, of which 50% are due to numeric chromosomal anomalies (Jacobs 1987). After 10 gestational weeks the pregnancy loss is estimated at approximately 2 to 3%, of which only 5 to 6% are due to numeric chromosomal errors (Simpson 1990).

The most common chromosomal abnormalities are trisomies, arising *de novo* as a result of meiotic non-disjunction during gametogenesis in parents with a normal karyotype. The risk for meiotic non-disjunction increases both with maternal and paternal age (Hassold and Chiu 1985; Nybo Andersen et al. 2000; Slama et al. 2005). All trisomies have been observed except in chromosome 1 that is probably 100% lethal in preimplantation stage. The most frequent abnormality is a trisomy of chromosome 16 representing ~30% of all trisomies (Macklon et al. 2002). Only three of autosomal trisomies are observed at birth: trisomies of chromosome 21, 18, 13. They have estimated prenatal survival rate of 20%, 5% and 3%, respectively (Macklon et al. 2002). Sex chromosome aneuploidies mostly survive with an exception of 45X, which is prenatally lethal in 98% of cases (Hassold and Hunt 2001). There is no age difference between the mothers of liveborn trisomic children and mothers of spontaneously aborted trisomic embryos (Gardner 1996; Macklon et al. 2002). Numerical abnormalities are sporadic, and they do not usually recur in subsequent pregnancies. The recurrence risk is thought to be about 1% (Gardner and Sutherland 1996).

Structural chromosomal anomalies are defects in the structure of one or more chromosomes, mainly translocations (parts of chromosomes at wrong locations) and inversions (part of a chromosome is turned around). An individual carrying a balanced rearrangement would not usually have any phenotypic effect, except for the possibility of impaired fertility and reproduction. The chromosomes with structural anomaly have difficulty pairing up and dividing evenly during meiosis. As a result, gametes may possess an unbalanced amount of chromosomal material. These imbalances are usually lethal to a developing embryo or fetus, resulting in spontaneous abortion (Carp et al. 2001; Macklon et al. 2002).

2.1.2. Risk factors associated with recurrent miscarriage

Recurrent miscarriage or habitual abortion is defined as three or more consecutive pregnancy losses before 22 gestational weeks or expulsion of an embryo/fetus weighing less than 500g. Accepting a 15% pregnancy loss rate, it can be calculated that the incidence of RM by chance would be about 0.35% (0.15^3). Yet, the observed incidence of RM in several populations is three times higher being around 1–2% of couples planning a childbirth (Berry et al. 1995; Bricker and Farquharson 2002). Based on epidemiological studies, approximately two-thirds of the RM cases are thus associated with factors that increase the risk of miscarriage in these particular couples. The more pregnancy losses a couple has experienced previously, the higher is the probability to miscarry again during the next pregnancy. Concordantly, the frequency of normal karyotype of an aborted embryo/fetus increases significantly and that of an abnormal embryonic karyotype remains unchanged with the number of previous miscarriages (Fig. 1, Ogasawara et al. 2000). A couple suffering from RM has obviously more predisposing factors and clinical conditions that lead to the expulsion of chromosomally normal embryo/fetus from uterus than among couples with a sporadic miscarriage.

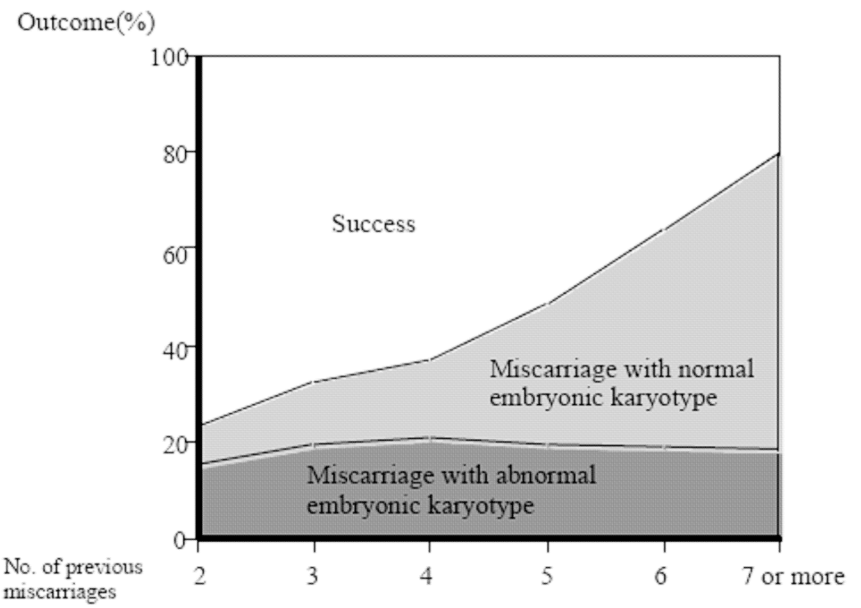

Figure 1. Estimated miscarriage rate with normal and abnormal embryonic karyotypes by number of previous miscarriages (adapted from Ogasawara et al. 2000).

Uterine abnormalities, various endocrine disturbancies, parental chromosome aberrations, the presence of antiphospholipid antibodies and trombophilic conditions are also found to be associated with RM. The prevalence of the factors among the patients with RM varies a lot in different studies (Table 1). The abnormalities may occur in the couples with normal fecundity pointing that RM is a complex disease and more than one single factor is involved in pathogenesis of RM.

Table 1. The list of risk factors and their prevalence in patients with RM.

Etiology	Prevalence in patient with RM	References
Chromosomal aberrations	2–7%	Coulam 1986; Hatasaka 1994; Stephenson 1996; Cramer and Wise 2000; Garcia-Enguidanos et al. 2002; Habayeb and Konje 2004; Stephenson and Sierra 2006
Anatomical abnormalities	1–16.7%	Coulam 1986; Hatasaka 1994; Propst and Hill 2000; Garcia-Enguidanos et al. 2002; Habayeb and Konje 2004; Devi Wold et al. 2006; Saravelos et al. 2008
Endocrine disorders	5–30%	Coulam 1986; Hatasaka 1994; Maione et al. 1995; Stephenson 1996; Cramer and Wise 2000; Habayeb and Konje 2004; Sierra and Stephenson 2006
Immunological factors	1–40%	Hatasaka 1994; Maione et al. 1995
Autoimmune	16–20%	Stephenson 1996; Habayeb and Konje 2004
Alloimmune	not stated	
Thrombophilic factors	14%	Habayeb and Konje 2004
Infections	0.5–2.4%	Maione et al. 1995; Stephenson 1996
Idiopathic	43–54%	Stephenson 1996; Li et al. 2002; Habayeb and Konje 2004

2.1.2.1. Anatomical risk factors

The incidence of uterine anomalies is suggested to be 1–6.7% in the general population and 3–16.7% in women with RM and poor reproductive outcome (Devi Wold et al. 2006; Saravelos et al. 2008). Many non-obstructing uterine abnormalities are asymptomatic and may be discovered only during the evaluation of RM or infertility. It should also be remembered that the patients with RM having an uterine abnormality may also have other factors that are more important in the pathogenesis of miscarriage, and the anatomical factor may be only a sheer coincidence.

However, if the abnormality is found, the risk for adverse pregnancy outcome increases (Table 2).

Table 2. Pregnancy outcome in patients with congenital and acquired uterine anomalies

	All pregnancies			Birth of live baby %
	Miscarriage %	Preterm birth %	Full-term birth %	
Septate uterus	65 (26–94)	21 (1–33)	14 (1–68)	32 (6–75)
Unicornuate uterus	51 (41–68)	15 (10–17)	34 (25–48)	39 (38–57)
Uterus *didelphus*	43 (32–52)	38 (20–45)	19 (12–44)	54 (41–64)
Bicornuate uterus	32 (28–35)	21 (14–23)	47 (42–56)	59 (57–63)
Arcuate uterus	13–45	13	x	x
Leiomyoma	40–60	x	x	60
Asherman syndrome	40–70	23	x	x

x – data not available

Compiled on data reported by Li et al. 1999; Bajekal and Li 2000; Propst and Hill 2000; Garcia-Enguidanos et al. 2002; Campo et al. 2003; Devi Wold et al. 2006.

2.1.2.2. Hormonal risk factors

Ovulation, implantation and the early stages of pregnancy are dependant on an integral maternal endocrine regulatory system. Undoubtedly, hormonal disorders are related to inability to conceive due to inadequate follicle maturation and/or failure to ovulate. Morphological and physiological changes of the endometrium caused by cyclic secretion of estrogens and progesterone ensure the suitable environment for embryo implantation. Progesterone deficiency could delay endometrial development, and thus could be a reason of unsuccessful implantation and formation of placenta. Several studies report lower than normal serum progesterone concentrations in RM patients with a delayed endometrium compared to those with normal endometrium whereas other studies have failed to detect differences between the two groups (Balasch et al.

1986; Babalioglu et al. 1996; Li et al. 2002). If plasma progesterone level is normal, the endometrium can still be progesterone deficient due to receptor abnormalities (Szekeres-Bartho and Balasch 2008). Overall, despite considerable medical use, there is currently insufficient information on the optimal dose, route and timing of progesterone supplementation during the first trimester to prevent miscarriage. However, progesterone treatment decreases the miscarriage rate in patients with RM (Oates-Whitehead 2003).

Other endocrinological disorders (LH hypersecretion, obesity, high androgen level, polycystic ovary syndrome and hyperprolactinemia) have also been associated with infertility and pregnancy complications, including RM (Jauniaux et al. 2006).

2.1.2.3. Immunological risk factors

Immunological factors in RM can be divided into autoimmune and alloimmune factors.

Autoimmunity is an immunological reaction against the individual's own tissue. An excessive amount of antiphospholipid antibodies, of which anticardiolipin antibodies are the most known and used in clinical conditions, is associated with RM and several other pregnancy complications: intrauterine growth retardation, pre-eclampsia and preterm delivery. The exact mechanism by which antiphospholipid antibodies cause RM is unknown but is proposed to be due to a thrombotic tendency resulting in decidual vasculopathy and placental infarction (Salmon and Girardi 2008). Antiphospholipid syndrome (APS) can be diagnosed if the patient with RM has anticardiolipin IgG or IgM antibodies at moderate or high level in blood on two or more occasions at least six weeks apart (Levine et al. 2002). Combination therapy with aspirin and heparin may reduce pregnancy loss in women with APS by 54% (Empson et al. 2005).

Alloimmunity is a condition in which the immune reactions are triggered by materials originated from another individual of the same species. An excessive maternal immune response against fetus has been postulated to be one of the causes of RM (Lim et al. 1996; Laird et al. 2003). Since half of the fetal genome derives from the father, the development of mutual state of immunotolerance between mother and fetus is critical for carrying pregnancy to full term. The trophoblastic cells use several unique strategies to protect the embryo from immunological attack (Gaunt and Ramin 2001):

1) Absence of MHC-I class molecules on the surface of trophoblastic cells.
2) Presence of unique HLA surface molecules (HLA-G) that make the invasive extravillous cytotrophoblast cells resistant to NK cell-mediated destruction and inflammatory signals.
3) Reduction of non-specific systemic immunoreactivity and increase of specific humoral arm (B-cells, antibodies) of immune system.

4) Expression of proteins that down-regulate the lytic reactions of the complement system.
5) Modification of immunoreactivity in feto-maternal interface.

Particular interest is focused on the association of RM with elevated number of NK cells either in peripheral blood or endometrium (Laird et al. 2003; Quenby and Farquharson 2006). However, due to limited data about the precise role of NK cells in implantation and placentation, the endometrial sampling and peripheral level measurements in patients with RM are not recommended in routine practice (Jauniaux et al. 2006). Consistently, number of studies have shown that the elevated concentration of proinflammatory or immunodystrophic cytokines (e.g. Th1 cytokines: IL-1, IL-2, TNF-α, IFN-γ) may harm the pregnancy. On the contrary, some maternally produced cytokines serve as placental growth factors and mediate tissue remodeling (IL-10, macrophage colony stimulating factor) to enhance the fetal growth and development (Aagaard-Tillery et al. 2006).

Several studies have focused on the theory that increased HLA compatibility between partners with RM causes inappropriate immune recognition of the trophoblast and subsequent miscarriage but the evidence remains still unclear (Beydoun and Saftlas 2005).

Nevertheless, the growing evidence that emphasize the essential role of alloimmune factors in pathogenesis of RM, their implementation in clinical management of RM is still delayed.

2.1.2.4. Thrombophilic risk factors

Pregnancy is a hypercoagulable state. Hormonal changes increase the concentration of procoagulants, and decrease anticoagulant and fibrinolytic activity of a pregnant woman. The evolutionary advantage of such a change in hemostasis is to counteract the inherent instability associated with villous hemochorial placentation, which is unique to humans (Rai and Regan 2006).

Several complications of pregnancy (miscarriage, placental abruption, preeclampsia, intrauterine growth restriction, deep vein thrombosis) may be caused by either acquired or hereditary defects in clotting and/or fibrinolytic pathways. The most frequently found mutations are G1691A in factor V (Leiden factor), and G20210A in factor II (prothrombin). Heterozygous and homozygous forms of the mutations increase the risk of RM several folds (Rey et al. 2003; Rai and Regan 2006). Hereditary deficiencies of anticoagulant proteins antithrombin, protein C, and protein S are heterogeneous in nature and caused by several different genetic mutations. Despite rare occurrence, they are strongly associated with clinical thrombosis (Rey et al. 2003). APC resistance either caused by factor V Leiden mutation or induced by several other factors

has also been identified as an independent risk factor for RM (Dawood et al. 2007).

Assessment of different markers of haemostatic function (APC resistance, free protein S and protein C, antithrombin III, etc) measured outside the pregnancy is recommended for the first step in the management of women with RM (Rai and Regan 2006). Deviation from the reference value of a functional test, the signs of thrombotic event and/or presence of any mutation among the patient's blood-related relatives may indicate to an underlying mutation.

The genetic defects causing thrombophilias are found 2-fold more likely among the patients with RM than in general population (ESHRE Capri Workshop, (2008). In addition to possible complications during the pregnancy, the prothrombotic state also confers a risk for development of cardiovascular diseases in later life (Smith et al. 2003).

2.1.2.5. Chromosomal risk factors

The incidence of structural chromosomal abnormalities, usually balanced translocations, is estimated to occur in 3–5% of either partner of couples with RM (Cramer and Wise 2000; Franssen et al. 2006). It has been demonstrated that these couples have a low risk of viable offspring with unbalanced chromosomal abnormalities. Their chance of having a healthy child is as high as a non-carrier couple, despite a higher risk of miscarriage (Franssen et al. 2006; Stephenson and Sierra 2006). A live birth rate among couples carring structural chromosomal anomaly is estimated to be 71–83% following evaluation and treatment of concomitant factors for RM (Franssen et al. 2006; Stephenson and Sierra 2006).

2.1.2.6. Microbiological risk factors

Any severe infection that leads to bacteraemia or viraemia can cause sporadic miscarriage. To cause RM, a microorganism should persist in the genital tract or the patient should suffer from persistent chronic systemic infections for a long time. Still, there is no direct proof that such organisms play a role in RM (Christiansen 1996; Romero et al. 2004). However, subclinical endometrial infection/inflammation is associated with several pregnancy complications, such as implantation failure, pregnancy loss and preterm delivery (Romero et al. 2004).

2.1.2.7. Extensive oxidative stress

Recently, the role of oxidative stress in pathogenesis of RM has been accentuated (Jauniaux et al. 2006). In normal pregnancies, the earliest stages of development take place in a low oxygen environment. This physiological hypoxia of the early gestational sac protects the developing fetus against deleterious and teratogenic effects of oxygen free radicals. In miscarriage, onset of the maternal blood flow to the developing placenta is precocious and disorganized leading to detrimental effects on the syncytiotrophoblasts. This mechanism is common to all miscarriages despite the time of occurrence (Jauniaux et al. 2006). In addition to miscarriage, oxidative stress-induced damage has been hypothesized to play a role in hydatiform mole, defective embryogenesis, drug-induced teratogenicity, preterm labor, intrauterine growth restriction and preeclampsia (Agarwal et al. 2005; Jauniaux et al. 2006). The expression of the markers of oxidative stress, e.g heat shock protein 70, lipid peroxidation, etc. was greater in tissues obtained from missed miscarriages compared with controls (Hempstock et al. 2003). On the contrary, the level of several antioxidants (gluthatione, gluthatione peroxidase, catalase, superoxide dismutase), were significantly lower in patients with RM than fertile controls (El-Far et al. 2007).

Reactive nitrogen species are other potential agents causing the oxidative stress and regulating the vascular tone. Nitric oxide, produced by the enzyme nitric oxide synthase (NOS) relaxes arterial and venous smooth muscles and inhibits platelet aggregation and adhesion. Lack of endothelial-derived NOS (eNOS) is associated with vasospasm and vascular infarction. It has been proposed that regular use of antioxidants should prevent and treat the diseases caused by excessive oxidative stress. Despite the hypothetical beneficial effect, the supplementation of vitamins with antioxidant properties, like vitamin C and E, prior to the pregnancy or at early pregnancy does not prevent miscarriage. However, taking the vitamin alone or in combination with other vitamins women may less likely develop preeclampsia and more likely have a multiple pregnancy (Rumbold et al. 2005).

2.1.3. Genetics of recurrent miscarriage

Only few studies have focused on the occurence of RM among relatives of women with RM. As summarized, the first-degree blood relatives of affected females have 2–7 fold increased risk of miscarriage compared to controls (Christiansen 1996).

The molecular genetic studies on RM have addressed both the aberrant expression of functional candidate genes at materno-fetal interface, and the poly-

morphisms of the genes in the physiological pathway involved in pathogenesis of pregnancy loss.

Thrombophilia-related genes

Mutations in several genes have been found to be related with adverse pregnancy outcomes: factor V (Leiden factor) G1691A, H1299R, Y1702C, factor II prothrombin G20210A, factor XIII V34L, β-fibrinogen (-455G>A), plasminogen activator inhibitor-I 4G/5G, human platelet antigen 1 a/b(L33P, MTHFR C677T, A1298C (Goodman et al. 2006). Leiden factor G1691A mutation and prothrombin G20210A increase the risk of RM before 13 gestational weeks at least by two fold (Rey et al. 2003; Rai and Regan 2006). Although elevated plasma homocysteine concentration has been suggested as a risk factor for RM, two meta-analyses focusing on the polymorphism C667T in *MTHFR* gene reducing the enzyme activity have given controversial results (Nelen et al. 2000; Ren and Wang 2006).

Immune response related genes

Since the conceptus produces the gene products originated from both parents, the immunological mechanisms responsible for the development of the tolerance to semiallogenic fetal "graft" by the maternal immune system is the most attractive target for genetic studies.

The expression of HLA-G, the most dominant HLA antigen in blastocysts and/or trophoblastic tissue, has been shown to be different in successful and failed pregnancies in some studies (Hviid 2006). 14 bp deletion/insertion polymorphism in exon 8 of the 3'UTR in HLA-G is related to decreased expression of the molecule. In contrary a "G" nucleotide at position -725 in promoter area of HLA-G increases the expression of HLA-G (Ober et al. 2003; Hviid et al. 2004). Both polymorphisms have been associated with the increased risk for RM (Hviid 2006), thus the expression level of the molecule has an ambiguous effect on reproductive success. The polymorphisms G*010103, G*0105N, G*010401 in HLA-G as well some variants of the other non-classical in HLA class Ib genes, HLA-E, C and F have been found to be related to RM with controversial effect (Hviid 2006; Tripathi et al. 2006; Kano et al. 2007; Hiby et al. 2008). In normal gestation, the HLA II class antigens located on maternal antigen-presenting cells should not recognize the trophoblastic tissue and present the trophoblast-derived peptides to maternal autoreactive T cells. Some HLA II class antigens, e.g. HLA-DRB1*03 tend to contribute to the pathogenesis of RM by presenting the fetal antigens to mother and/or enhancing the embryotoxic effect of several molecules (Kruse et al. 2004).

The aberrations in cytokine and chemokine levels have been shown both in maternal and fetal tissues and biological fluids during pathological pregnancies in multiple studies (Lim et al. 1996; Salamonsen et al. 2007; Whitcomb et al.

2007) but the underlying polymorphisms that lead to increased or decreased expression of a cytokine are still addressed in limited number of experiments.

Adhesion and angiogenesis related genes
Matrix metalloproteinase-2 (*MMP-2*), plasminogen activator inhibitor (*PAI*), integrin, *TGF-β, VEGF*, basic fibroblast growth factor (*bFGF)* and mucin 1 have been shown to have a decreased expression level in chorionic villi from RM patients compared to those from normal controls (Baek et al. 2002; Choi et al. 2003).

The polymorphisms in most of the angiogenesis-related genes that have been demonstrated to be associated with the implantation failure and poor pregnancy outcome are also important in several other pathologies: benign and malignant tumors, coronary artery disease, cerebro-vascular diseases, asthma, etc.

Apoptosis-related genes
The expression of several apoptosis-related genes: *caspase 3, 6, 7, 8, 9, 10, 12, BAD, BAX, BID, Fas*, and *FasL*, has increased in trophoblastic tissue from RM patients compared to normal controls (Choi et al. 2003). The most targeted apoptosis related genes are also involved in multiple physiological and pathological processes. For example, *p53* tumor suppressor gene polymorphism resulting in substitution of arginine with proline at codon 72 in exon 4 is related to poor prognosis of cancers (Boldrini et al. 2008), development of neurodegenerative and cardiovascular diseases, etc (Mercer et al. 2007; Zhang et al. 2007), but it is also been associated with increased longevity (Orsted et al. 2007).

Oxidative stress-related genes
The absence or null genotype of glutathione S-transferases M1 gene, one of the GST family (class mu) members that is a potent antioxidant, has been demonstrated to be a risk factor of several diseases. The patients with RM carry the null genotype of glutathione S-transferase M1 gene more frequently compared to fertile women (Sata et al. 2003). The absence of the gene increases the risk for RM more than 2-fold (Sata et al. 2003).

Candidate genes from animal models
One way to identify new genes associated with RM is to study genes required for the development of mice. A homozygous insertion mutation in *Amnionless* gene and the polymorphism in codon 511 of the aryl hydrocarbon receptor nuclear translocator gene cause the fetal loss in mice. In humans, the *Amnionless* gene is needed for absorption of cobalamin. Aryl hydrocarbon receptor-associated protein 9 acts as a chaperone involved in intracellular signal transmission. Despite the high similarity between the respective mouse and human genes, the association of studied polymorphisms with RM was not found (Kaare et al. 2006; Sullivan et al. 2006).

In summary, most of the studied candidate genes are involved in several physiological processes: inflammation, immunotolerance, apoptosis, tissue remodeling, regulation of vascular tone and metabolism. The polymorphisms in these genes are associated with different complex diseases and their contribution for pathogenesis of RM is not specific. The placental proteins coded by the fetal genome certainly have a direct influence on pregnancy success and would be suitable candidates for genetic studies of RM. However, the number of genetic studies focusing on the pregnancy-specific placentally expressed genes is still limited.

2.2. Human chorionic gonadotrophin

The history of HCG is closely related to its abundant production during the pregnancy. In 1928 Selmar Aschheim and Bernhard Zondek discovered a substance that induces rapid maturation of ovarian follicles and development of *corpus luteum*. Based on their observation, a pregnancy test, known as Zondek-Aschheim test, was developed. A woman's urine was injected into an immature rat or mouse. When the subject was not pregnant, there would be no reaction. In the case of pregnancy, the rat would show an estrous reaction despite its immaturity (Evans and Simpson 1930). The pregnancy "substance" was recognized as differing from pituitary gonadotropins and isolated from human placenta in 1970-ies (Bohn 1971). The crystal structure of HCG was described in 1994 (Lapthorn et al. 1994).

2.2.1. Molecular structure of HCG

Together with TSH, the gonadotropins HCG, LH and FSH form a family of glycoproteins. FSH, LH and TSH are produced in the anterior lobe of pituitary gland, while HCG originates from the placenta. The members of this family of relatively large proteins consist of a common α-subunit and a hormone-specific β-subunit that are associated through non-covalent interactions. The mature HCG is composed of 244 amino acids with a molecular mass of 36.7 kDa. Its total dimensions are 7.5×3.5×3 nanometers. The α-subunit comprises 92 amino acids with 10 cysteines, which are involved in intrasubunit disulfide linkages and two N-linked oligosaccharides. The premature polypeptide of β-subunit of HCG is 165 amino acids in length. The first 20 amino acids encode a signal peptid that is required for the transport of the protein across the membrane of endoplasmic reticulum (Moyle and Campbell 1996). Thereafter the signal peptid is cleaved. The mature polypeptide consists of 145 amino acids with 12 cysteine pairs that form six intrasubunit disulfide bridges, two N-linked oligosaccharides, and four O-linked oligosaccharides attached to the HCG-specific carboxy terminal peptide portion (Fig. 2). The additional 24 amino acids located

in the C-terminal extension give the longer circulating half-time and higher biopotency of HCG over LH (Themmen and Huhtaniemi 2000). Although the β-subunit of HCG confers functional differences, it has a considerable amino acid identity with other glycoproteins. The homology between β-subunit of LH and HCG (excluding the non-homologous C-terminal extension of HCG) is 83% (Themmen and Huhtaniemi 2000; Cole 2007). Compared to other glycoproteins, HCG is the most glycosylated; 25–40% of the molecular weight comes from oligosaccharides (Cole 2007).

The crystal structure of deglycosylated HCG has revealed that both subunits contain a cystine knot structure, similar to some remotely related signaling molecules such as transforming growth factor-beta (TGFβ), nerve growth factor, and platelet derived growth factor (Lapthorn et al. 1994). The similarity between these molecules explains why HCG has demonstrated in some extent growth stimulating effect.

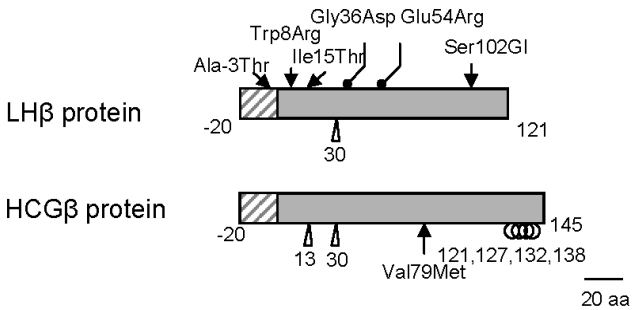

Figure 2. Schematic representation of β subunits of LH and HCG. The signal peptide at the beginning of protein is shown with diagonally striped box, the mature protein is depicted by the grey box. The numbers below the protein signify the start of the signal peptide and the lenght of the mature protein product. The positions of the N-linked glycosylation sites are indicated by triangles and the O-linked glycosylation sites by empty circles. Arrows show polymorphisms and a line with closed circles, an inactivating mutation (listed in Table 3).

2.2.2. Function of HCG

The main functions of HCG are to:

1) support *corpus luteum* function to guarantee the necessary level of progesterone during the first trimester;
2) prepare endometrium for the implantation of embryo and placentation;
3) improve the maternal blood supply;

4) modify the local immunosupression enabling the implantation of embryo, but preserving the immunological defense against pathogens;
5) ensure the quiescence of myometrium to prevent the premature expulsion of fetus from uterus.

Maternal recognition of pregnancy is initiated by the signals from developing embryo. HCG interacts with the LH/CG receptor that belongs to the superfamily of G protein coupled receptors. Its stimulatory signal is mediated via classical adenylyl cyclase-cAMP- protein kinase A pathway but besides the classical route, HCG also acts through immediate phosphorylation of ERK1/2 pathway (Themmen and Huhtaniemi 2000; Srisuparp et al. 2001). Upon hormonal stimulation LH/CG receptors desensitize and the signal could be relayed in a less efficient manner. The alternative signal transduction pathway helps to prevent the receptor desensitation-caused diminishing effect and leads to an increase of prostaglandin E2 and cyclooxygenase-2 in endometrium. Both molecules stimulate cell growth and differentitation being important in gamete maturation, fertilization, early embryonic development and implantation (Licht et al. 2001; Srisuparp et al. 2001).

The prevailing way of HCG action changes during the pregnancy (Licht et al. 2001). Initially, from 6–8-cell stage until the appearance in the serum, HCG acts preferable in a juxtracrine manner on the neighbouring tissues. In the endometrium, HCG modulates its differentation and function that facilitates the invasion of early embryo. After the appearance in the serum, HCG gains endocrine function, such as the rescue of the *corpus luteum*. The luteal rescue is associated with endothelial cell proliferation, angiogenesis, vascular stabilization and delayed apoptotic activity (Wulff et al. 2001; Stocco et al. 2007). Endocrine effect of HCG on the thyroid gland, the central nervous system, the immune system, and fetal testis, ovary and adrenal gland is also important. After the villous trophoblasts have gained responsiveness to HCG (>9th gestational weeks), HCG modulates trophoblastic differentiation, formation of placenta and self-regulation of its own biosynthesis in autocrine/paracrine way (Licht et al. 2001). HCG upregulates the secretion of, VEGF, the prime regulator of blood vessel growth, in a dose-regulated manner (Reisinger et al. 2007).

Due to its highly-negative charge, HCG may repel the immune cells of the mother, protecting the fetus during the first trimester. HCG-treated endometrial cells induce an increase in T cell apoptosis and facilitate the trophoblast invasion, which is known to expedite fetal development in the endometrium (Kayisli et al. 2003). 20–40% of all common epithelial carcinoma arising from mucosal epithelia such as bladder, cervix, lung and naso-pharynx have found to produce β-subunit of HCG (Iles 2007). In malignancies free β HCG acts as an autocrine growth factor by inhibiting apoptosis, enhancement of invasion and angiogenesis (Butler and Iles 2004; Reisinger et al. 2007). Structural homology and *in vitro* studies suggest that it may be achieved by the inhibition of TGFβ

receptor complex. HCG-H is also related to enhanced growth and invasion functioning through the same axis: HCG-H – TGFβ – apoptosis (Cole 2007).

HCG also helps to maintain pregnancy during the second and third trimester by inhibiting myometrial contractility. Rapid increase of intracellular calcium level mediated by gap junctions, induce the coordinated myometrial contractions in labor. The number of gap junctions (the intercellular communication channels), peaks at the time of delivery. HCG attenuates the stimulatory effect of oxytocin on gap junctions during the pregnancy acting as endogenous tocolytic agent (Kurtzman et al. 2001).

Receptors for LH/HCG have been identified in multiple mammalian brain structures. HCG may be involved in the behavioral regulation of pregnant women (Lei and Rao 2001). Adminstration of HCG promotes nerve regeneration *in vivo* and neurite outgrowth and survival of primary neurons *in vitro.* It induces neuronal differentiation through activation of stably expressed LH/HCG receptor (Meng et al. 2007).

HCG has been found in several fetal gonadal and non-gonadal tissues. HCG stimulates the production of dehydroepiandrosterone in fetal adrenal gland, which is later converted into estriol in the placenta. This conversion, as measured by maternal serum or urinary estriol levels, reflects fetal health and wellbeing. In addition, HCG stimulates testosterone production by Leydig cells, that is essential for masculinization of male fetus (Abdallah et al. 2004).

2.2.3. HCG in normal and pathological conditions

HCG is one of the first proteins synthesized by conceptus. β-subunit of HCG has been detected in the two-cell stage embryo (Jurisicova et al, 1999). After implantation, HCG is transported from syncytiocytotrophoblasts into maternal bloodstream where its concentration increases exponentially. At 3–4 weeks' gestation, the doubling time of dimeric HCG is 1.5–2.0 days and about 3.5 days at 9–10 weeks (Hay 1988). The maximum level of HCG is reached by 9–10 weeks of pregnancy. Concentration decreases from the 10^{th} to the 16^{th} week of gestation, being approximately 25% of peak concentration, thereafter the level of HCG falls to become 10% of peak trimester value (Fig. 3).

Although HCG is produced in some extent in several malignant tissues: bladder, renal, prostate, lung, gastrointestinal, neuroendocrine, breast and gynecological cancers (Stenman et al. 2004), its presence in serum or urine of a woman is highly specific to pregnancy. However, the extremely large interindividual variation of HCG level measured at the same moment of normal pregnancy, limits its usage for exact determination of gestational age. In cases of miscarriage, EP and failure of IVF procedure the concentration of HCG is lower than at the corresponding time of the normal pregnancy (Buyalos et al. 1992; Letterie and Hibbert 2000; Dumps et al. 2002; Tong et al. 2006). On the

opposite, the molar pregnancy is related to elevated production of HCG. Preeclampsia but not isolated pregnancy induced hypertension has also higher HCG levels than in normal gestation (Gurbuz et al. 2004). Several chromosomal aberrations, for example trisomy of chromosome 21 and 18, are associated with low hormone concentration (Brizot et al. 1995; 1996).

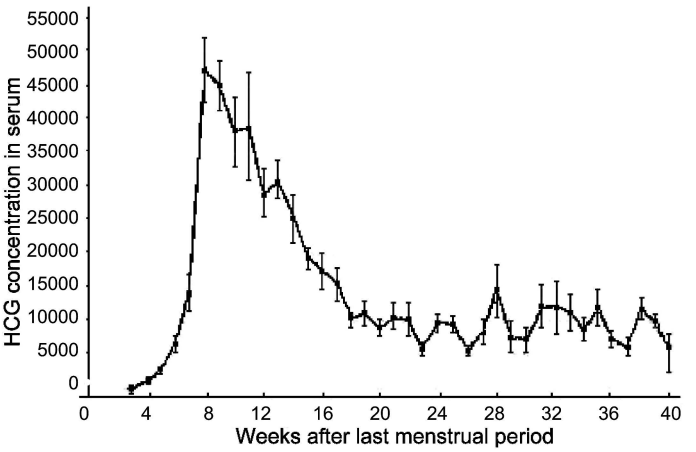

Figure 3. Mean (±SE) serum concentrations of human chorionic gonadotrophin throughout normal pregnancy (adapted from Braunstein et al 1976).

In addition to the intact dimeric HCG, various forms of HCG such as free α and β subunits, β core fragments, nicked and hyperglycosylated HCG (HCG-H) are detected in serum, urine and placental extracts. The bioactivity of the hormone and its subunits changes during the pregnancy being the highest during the first trimester. The bioactivity of HCG correlates with differentiation and invasiveness of trophoblasts (Srisuparp et al. 2001).

The HCG-H is a variant of the HCG that has additional sialic acid containing carbohydrates attached to serine residues in HCG specific C-terminal extension. The larger (>40000 versus 36,700 Da) and more negatively charged molecule is produced in cytotrophoblastic cells with $1/25^{th}$ the biological activity of regular HCG (Cole 2007). HCG-H has rather the autocrine and paracrine than the endocrine function promoting growth and invasion (Cole 2007). Up to 6^{th} gestational week HCG-H contributes more than 50% of total HCG, later its contribution decreases stabilizing at around 2% during the second and third trimester of normal pregnancy. Compared to regular HCG the hyperglycosylated HCG has less interindividual variation and overlapping values between pathological and normal conditions. Hence, a single test with cut-off value of 13 ng/ml HCG-H could be used between 4 to 7 weeks of gestation to differentiate a failure out-

come (<13 ng/ml) from term outcome (>13 ng/ml) (Sutton-Riley et al. 2006). Low maternal mid-trimester HCG-H levels predict the risk for developing subsequent preeclampsia, high and persisting concentration of HCG-H is an useful marker of invasive gestational trophoblastic disease (Cole 2007). The elevated level of HCG-H in second trimester may be indicative to trisomy 21. This is explained by an accumulation of cytotrophoblast, the major source of HCG-H, as a result of defective cytotrophoblastic differentiation into syncytiotrophoblasts in Down syndrome (Massin et al. 2001).

2.2.4. Therapeutic use of HCG

HCG is a widely used drug in reproductive medicine. It belongs to standard schemas in multiple assisted reproduction methods for triggering ovulation after stimulated growth and maturation of follicle. As the low hormone level and non-exponential increase of HCG during the first trimester of pregnancy is related to miscarriage, the HCG has been used for prevention of pregnancy loss in the cases of RM. A meta-analysis involving four trials with 180 female RM patients showed that HCG supplementation during the pregnancy was associated with a reduced risk of miscarriage (odds ratio 0.26, 95% CI 0.14–0.52, (Scott and Pattison 2000). However, the protective effect of HCG may be limited only to the subgroup of RM patients with oligomenorrhea and polycystic ovary syndrome (Pearce and Hamid 1994; Quenby and Farquharson 2006). The patients with thrombophilic or autoimmune disorders obviously do no benefit from the supplementation with HCG.

Some novel indications for treatment with HCG are proposed by small clinical trials. HCG may be used for tocolysis in cases of preterm labor with minimal adverse events (Kurtzman et al. 2001).

HCG is the first and only birth control vaccine that has successfully passed Phase II efficacy trials. The HCG vaccine is demonstrated to be highly effective and fully reversible, it is devoid of side-effects, as observed in >200 women during Phase I and Phase II trials. Vaccinated women have regular menstrual cycles and ovulate normally, they have no irregularity of bleeding in terms of spotting, and amenorrhoea or extra bleeding occurs (Naz et al. 2005). The shortcoming of the vaccine is that it generated above protective threshold titres in only 60–80% of women. That is highly satisfactory for vaccines against infectious diseases but a birth control vaccine has to be effective in >90–95% of recipients. Thus, it would require more potent adjuvants that could make the vaccine more immunogenic. Currently, the vaccinated individuals should be monitored for the presence/absence of sufficient antibody titres that can block conception (Naz et al. 2005).

In a pilot clinical trial, the application of a recombinant HCG in cases of primary breast cancer showed that it reduced significantly the proliferative

index and the expression of both the oestrogen receptor and progesterone receptor in cancer tissue (Janssens et al. 2007). The inhibitory effect of HCG on the progression of hormone-dependent breast cancer has previously been proposed in several observations, theoretical studies and animal experiments (Russo et al. 2005).

Inhibitory effects of HCG preparations on HIV infection of human placenta have been demonstrated by *in vitro* experiments (Polliotti et al. 2002) but its efficacy in treatment of HIV is currently under preclinical investigation (De Clercq 2005).

2.2.5. The genes coding for HCG subunits, their expression and variation

The α-subunit of HCG, common to all gonadotropic glycoproteins is encoded by a single gene located on chromosome 6q12-q21. α-subunit is highly conserved during the evolution, particularly in the region that interacts with the β-subunit. Most of the species differences are found in the amino-terminus and regions that form the first and the third loop. These nonconserved regions make relatively few contacts with the β-subunit (Moyle 1996). The only genetic alteration reported in the α-subunit gene is located in exon 3 causing amino acid change Glu56Ala. The mutated polypeptide failed to associate with the β-subunit and appeared to have higher molecular weight. This mutation is found only in carcinomas (Themmen and Huhtaniemi 2000). The lack of germ line mutation in the α-subunit gene could mean that such changes are lethal.

The β-subunit of HCG is encoded by a cluster of genes localized on chromosome 19q13.32 (Fig. 4). The whole cluster spanning approximately 52 kilobases consists of seven homologous genes: one luteinizing hormone beta (*LHB*) gene and six chorionic gonadotropin beta (*CGB*) genes. The genes that encode the β-subunit of HCG: *CGB, CGB5, CGB7* and *CGB8*, share 97–99% DNA sequence identity; similarity to functionally distinct *LHB* gene is 92–93% (Hallast et al. 2005). Despite high sequence similarity (85% identity) with the other genes in the cluster, *CGB1* and *CGB2* have been predicted to encode a novel hypothetical protein that differs from the functional HCGβ-subunit.

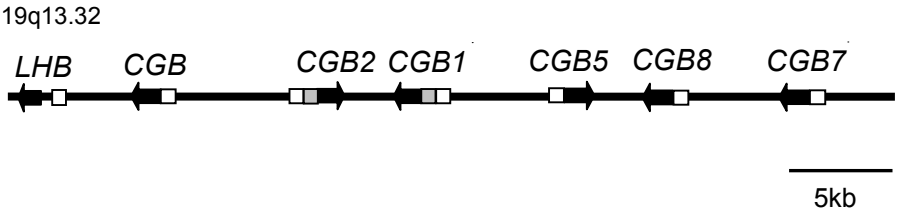

Figure 4. Schematic presentation of the *LHB/CGB* gene cluster. The genes are marked with black wide arrows in the direction of transcription on sense strand. Experimentally identified HCG beta promoter sequence (Otani et al. 1988; white bars) is also present, although more distally, upstream of *LHB, CGB1* and *CGB2* genes. *CGB1* and *CGB2* harbor an insertion of 736/724 bp, respectively (grey bars).

2.2.5.1. The comparative structure of the ancestral *LHB* and primate-specific *HCGβ* genes

During the primate evolution an ancestral *LHB* gene underwent a gene duplication, in which one copy was conserved as *LHB* while the other copy developed into *CGB* gene. The further duplications of *CGB* gene resulted in six gene copies in human (Talmadge et al. 1983; Maston and Ruvolo 2002; Henke and Gromoll 2008). In every primate species studied so far, the number of *LHB/CGB* genes seems to vary indicating an active genome dynamics of this region (Maston and Ruvolo 2002; Hallast et al. 2008; Henke and Gromoll 2008). The *LHB* and *HCGβ* transcripts encompass 1110 bp and 1467 bp, respectively (Henke and Gromoll 2008). *LHB* has a short 5'UTR (9 bp) and a consensus TATA box sequence that is located 38 bp upstream from ATG codon that is used for translation initiation. The *HCGβ* genes have, in contrast, a long 5'UTR, and transcription is initiated 365 bp upstream of the homologous promoter region in the *LHB* gene (Fig. 5). The TATA box in 5'UTR of *HCGβ* genes is retained in a non-functional state (Hollenberg et al. 1994).

CGB genes have a single-base deletion eight codons before the termination of *LHB* causing a frameshift that incorporates much of what is the 3'UTR in *LHB* into the third exon of *CGB* (Fig.5, Hollenberg et al. 1994).

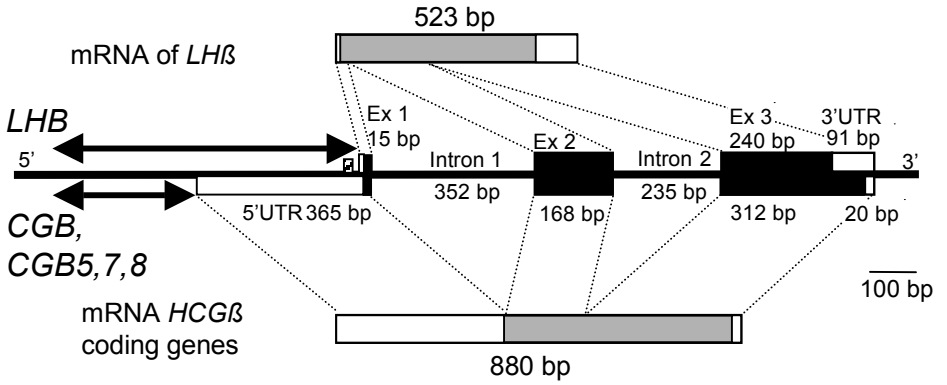

Figure 5. Structure of *LHB* and *HCGβ* genes (*CGB*, *CGB5*, *CGB7*, *CGB8*) and composition of their mRNA transcripts. The black boxes indicate the exons (Ex), white boxes the untranslated regions and solid horizontal lines introns. Two sided arrows mark the promoter region. Diagonally striped box before the 5'UTR and exon I in *LHB* is TATA box. Grey boxes in mRNA transcripts mark the translated regions for LH and HCG β-subunits.

2.5.2.2. The mutations and polymorphisms in *LHB* and *HCGβ* genes

As yet, only few mutations have been found in the gonadotropin β-subunit genes. All mutations are very rare and associated with loss of function (Fig. 2, Table 3). Three mutations Gly36Asp, Gln54Arg and a substitution G to C at position 536 (from translation start) in intron II have been described (Weiss et al. 1992; Valdes-Socin et al. 2004; Lofrano-Porto et al. 2007). The latter mutation disrupts the splicing of intron 2 of the *LHB* mRNA, resulting in the insertion of 236 nucleotides and frame shift in exon 3 as compared with the correctly spliced normal mRNA (Lofrano-Porto et al. 2007). As LH is not necessary for sexual differentation before birth, all patients had a normal phenotype at birth, including descended testes in males (Themmen 2005). The clinical signs caused by lack of bioactive LH appear after the pubertal age: delayed puberty, arrested spermatogenesis in males, and secondary amenorrhoa and infertility in a female individual (Table 3).

The best studied polymorphism in the *LHB* gene is a combination of two SNPs that are in complete LD and results in a combination of two amino acid changes: Thr8Arg/Ile15Thr. The first SNP is mainly responsible for the altered immunoreactivity and the second introduces an extra glycosylation site into the mutated LHβ peptide. The frequency of this v-LHβ allele differs widely between ethnic groups, being most common in aboriginal Australians (carrier frequency >50%; allelic frequency 28.3%) and totally lacking from Kotas of Southern India (Lamminen and Huhtaniemi 2001). V-LH possesses increased *in*

vitro bioactivity, whereas its half-life in circulation is shorter in comparison to wild-type LH. Association of v-LH with various clinical conditions suggests that it is biologically less active form of LH and may be related to suppression of gonadal function, including subfertility (Furui et al. 1994; Haavisto et al. 1995; Lamminen and Huhtaniemi 2001). Two other rare SNPs have been described in *LHB* gene. A SNP that causes an amino acid change Gly102Ser is related to subfertility in some populations (Ramanujam et al. 2000). A polymorphism causing an alanin to threonine change three amino acids before the signal peptid cleavage site leads to different *in vitro* signal transduction properties compared to wild type signal peptid (Jiang et al. 2002).

Only one possible polymorphism in the *HCGβ* genes has been reported, a SNP that causes a change from valine to methionine at position 79 (Fig. 2, Table 3, Miller-Lindholm et al. 1999). The peptide with substituted amino acid was unable to fold correctly and assemble with the hormone α-subunit in *in vitro* experiments. However, a subtle deficiency of bioactive HCG may be possible in individuals carrying this polymorphism. The methionine coding allele was found at a carrier frequency of 4.2% in 323 asymptomatic individuals from mid-west region of North America but none of 580 individuals from 5 European populations: Finnish, Danish, Greek, German and British (Miller-Lindholm et al. 1999; Jiang et al. 2004). None of 41 infertile patients carried the Val79Met polymorphism (Miller-Lindholm et al. 1999).

Table 3. List of currently known missense mutations and polymorphisms in the human *LHB* and *HCGβ* genes.

Location	Nucleotide/ amino acid change	Male phenotype	Female phenotype	Bioactivity	Reference
***LHB* mutations**					
Exon 3	A809G/ Gln54Arg	Absence of spontaneous puberty, no testosterone (one case)		Absent	Weiss et al. 1992
Exon 2	G519A/ Gly36Arg	↓ spermato-genesis, hypoplastic Leydig cells (one case)		Absent	Valdes-Socin et al. 2004
Intron 2	G536C	Hypo-gonadism, azoospermia (two cases)	Secondary amenorrhea, infertility (one case)	Absent	Lofrano-Porto et al. 2007

Location	Nucleotide/ amino acid change	Male phenotype	Female phenotype	Bioactivity	Reference
Polymorphisms in *LHB*					
Exon 2	T434C/ Trp8Arg & T456C/ Ile15Thr	Delayed tempo of pubertal progression	Slightly surpressed fertility	Increased *in vitro*, decreased half-life in circulation	Furui et al. 1994; Haavisto et al. 1995; Raivio et al. 1996
Exon 3	G952A Ser102Gly	Infertility	Menstrual disorders, subfertility	Decreased *in vitro*	Liao et al. 1998; Ramanujam et al. 2000
Exon 2	G404A Ala–3Thr	Normal	Normal	Alternative signal trans-duction pathway	Jiang et al. 2002
Polymorphism in *CGB5*					
Exon 3	G882A Val79Met	Normal	Normal	May be slightly decreased	Miller-Lindholm et al. 1999

The nucleotide number was counted according to the translation start site (including intronic sequences) and amino acid number according to mature protein.

2.2.5.3. Expression of *LHB* and *HCGβ* genes

Despite of their similarity, *LHB* and *CGB* have different expression patterns, transcriptional start sites and stop codons. *LHB* is expressed in anterior lobe of the pituitary gland in a pulsatile manner, whereas *CGB* genes are transcribed in normal placenta (Bo and Boime 1992) and in several non-trophoblastic normal and malignant tissues (Bellet et al. 1997; Reimer et al. 2000; Stenman et al. 2004). *HCGβ* genes have been reported to be expressed in minimal amount in normal (Bellet et al. 1997; Reimer et al. 2000) and in increased amount in malignant conditions of several non-trophoblastic tissues: testis, pituitary, adrenal and thyroid gland, breast, prostate, skeletal muscle, bladder, lung, oesophagus, uterus (Dirnhofer et al. 1996; Giovangrandi et al. 2001; Stenman et al. 2004; Hotakainen et al. 2007; Iles 2007).

CGB, CGB5 and *CGB8* give rise to identical proteins. *CGB7* product differs by three amino acids: Lys2Arg, Pro4Met and Asp117Ala (the nomenclature from the mature β-peptide). The gene variants encoding the protein with aspartic acid (*CGB*, *CGB5* and *CGB8*) in codon 117 are predominantly expressed in placenta, testis and malignant tumors. The normal non-trophoblastic cells express mostly the protein with alanine in the same codon (coded by *CGB7*) at very low level (Bellet et al. 1997; Stenman et al. 2004). Although the production of HCGβ-

subunit is associated with adverse prognosis, the correlation between stage of tumor and transcription level is still weak. However, the detection of HCGβ in complex with other tumor markers may be useful for stratification of patients with malignant disease for clinical trials and for identification the patients who might benefit from more aggressive therapy (Stenman et al. 2004).

2.2.6. HCGβ-subunit non-coding *CGB* genes

Despite high sequence similarity (85% identity) with the other genes in the cluster, *CGB1* and *CGB2* have been suggested to encode a novel hypothetical protein (132 amino acids in length) that is homological to neither the functional HCG β-subunit nor to any other known protein (Bo and Boime 1992). This change has been caused by an inserted DNA fragment (736 bp for *CGB1*, 724 bp for *CGB2*) that replaces 52 bp of the proximal end of promoter area and the whole 5'UTR of the HCGβ-subunit coding genes (Bo and Boime 1992; Hollenberg et al. 1994, Fig. 4, 6). *CGB1* and *CGB2* have recently arisen among Africal great apes (Hallast et al. 2007; Hallast et al. 2008). Differently from β-subunit coding genes, *CGB1* and *CGB2* have been shown to be transcribed with different splice variants (Bo and Boime 1992; Berger et al. 1994; Dirnhofer et al. 1996).

One of the alternative forms contains an extra 47 bp sequence involving a part of intron I of *CGB1* that covers 15 bp long sequence identical to exon I of *HCGβ* genes. This alternative form has been firstly described in two of 15 placentas (Bo and Boime 1992). The additional fragment arises when the splicing occurs at non-canonical splice site CAG/gaa that functions as a consensus splice site of *HCGβ* genes instead of *CGB1/2* specific canonical splice site AAG/gta located 32 bp upstream from initation codon of *HCGβ*.

The two other alternative forms contain an extra 166 bp or 176 bp sequence from the intronic part of the *CGB1/CGB2* genes and they have the same open reading frame as *CGB1/CGB2* gene. Both alternative forms of *CGB1/CGB2*: 166 bp and 176 bp have a predicted STOP codon shortly after transcription start producing a hypothetical polypeptide of 60 amino acids in length (Fig. 6).
Although the appropriate protein has not been isolated, mRNA of *CGB1* and *CGB2* has been detected in the placenta (Bo and Boime 1992) as well as in the testis (Berger et al. 1994), pituitary (Dirnhofer et al. 1996) and breast cancer tissue (Giovangrandi et al. 2001) predicting the functionality of these genes. Interestingly, in transgenic mice carrying a 36-kb cosmid insert containing all the six *CGB* genes, the *CGB1* and *CGB2* transcripts were also observed in brain at levels comparable to placenta, the expression site for all the *CGB* genes (Strauss et al. 1994).

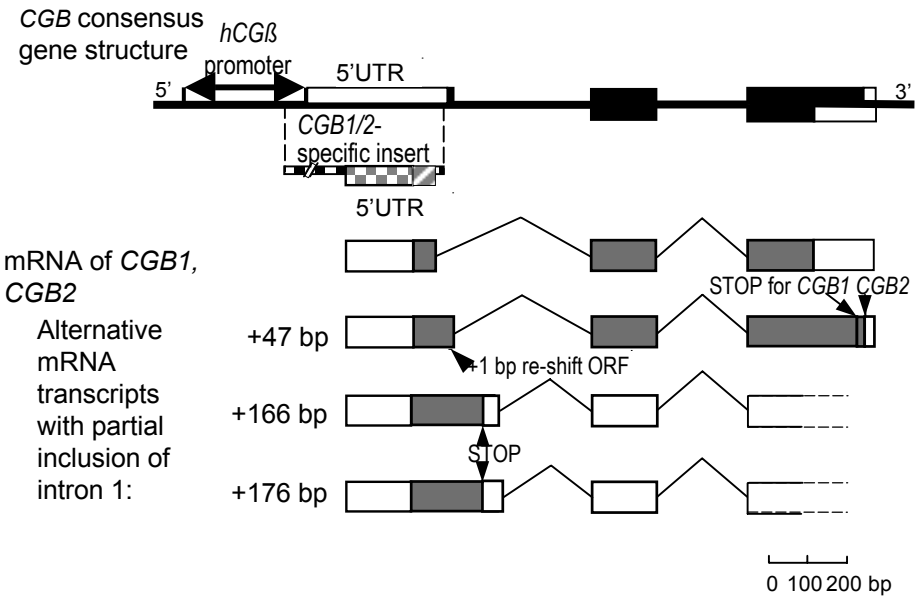

Figure 6. Schematic representation of the structure of the *CGB1* and *CGB2* and their alternatively spliced forms. The coding segments of all *CGB* genes are marked on the consensus gene structure with black boxes. Structures of *CGB1* and *CGB2* differ from a consensus *HCGβ* gene in the following aspects: (1) *HCGβ* 5'UTR has been replaced by a *CGB1/2*-specific insert coding for *CGB1/2* 5'UTR (wide chequered box, 174 bp), exon 1 (diagonally striped box, 58 bp) and part of intron 1 (22 bp) as well as provides a 481/469 bp upstream fragment, which could function as an additional promoter segment (narrow chequered box); (2) *HCGβ* exon 1 is a part of *CGB1* and *CGB2* intron 1; (3) open reading frames (ORF) of exon 2 and exon 3 of *CGB1 /2* (grey boxes) are shifted –1 bp compared to *HCGβ* genes; (4) the shifted ORF leads to earlier STOP codon and produces shorter exon 3. Alternative +47 bp *CGB1/CGB2* mRNA forms contain additional sequence from *CGB1/CGB2*-specific intron I (22 bp), the fragment corresponding to the *HCGβ* 5'UTR (10 bp) and exon I (15 bp), resulting in a re-shift the *CGB1/CGB2* ORF to the ORF of HCGβ-subunit coding transcripts. Due to sequence divergence in 3'UTR of *CGB1/CGB2*, the predicted STOP codons for +47 bp *CGB1* and +47 bp *CGB2* differ by 7 amino acids. Alternative transcripts +166 bp and + 176 bp contain additional 119 bp/129 bp sequence that is identical to the intronic part of all *CGB* genes. The predicted STOP codon for +166 bp /+176 bp forms is located at position 355 from transcription start.

3. AIMS OF THE STUDY

The general aim of the present work was to investigate the role of the genetic variation and expression of the genes encoding the β-subunit of human chorionic gonadotropin in pathogenesis of recurrent miscarriages.

Based on this, the present study was aimed:

1) to determine the contribution of an individual *HCGβ* gene into β-subunit production in trophoblastic tissue;
2) to determine the expressional profile of all six *CGB* genes in trophoblastic tissue during the normal pregnancy and in different first trimester pathologies: recurrent miscarriage, ectopic and molar pregnancy;
3) to compare the transcription activity of the HCG β-subunit coding genes and hormone concentration in normal and pathological conditions;
4) to detect the expression of all *CGB* genes in normal non-trophoblastic tissues;
5) to identify the variants of the most actively transcribed *HCGβ* genes that are associated with recurrent miscarriages.

4. SUBJECTS AND METHODS

4.1. Ethical consideration

The study was approved by the Ethics Committee of the University of Tartu, Estonia (protocols no 117/9, 16.06.03, 126/14, 26.04.2004) and the Ethics Committee of the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital out-patient clinic for women for RM (protocol no 298/E2/ 2000).

4.2. Study subjects and collected material

4.2.1. Material for mRNA expression studies (Paper I and II)

The female individuals (n=30) who had experienced the normal pregnancy until the tissue sampling were included the study to determine the expression of *CGB* genes in normal trophoblastic tissue. Additionally, the patients with recurrent miscarriage (n=11), ectopic pregnancy (n=8) and molar pregnancy (n=2) were included to assess the transcription of *CGB* genes in different pathological conditions affecting the pregnancy during the first trimester.

Chorionic villi/placental samples were obtained during or shortly after the following procedures:

1) elective therapeutic abortion during first trimester of pregnancy (4–12 weeks of gestational age, n=10);
2) therapeutic abortion during second trimester due to medical indications of pregnancy, no fetal anomalies were detected (17–21 weeks of gestational age, n=8);
3) normal delivery at term resulting from uncomplicated pregnancy (38–42 weeks of gestational age, n=12);
4) surgical removal of ectopic pregnancy (6–14 weeks of gestational age, n=8);
5) uterine curettage because of recurrent (a patient should have had ≥2 spontaneous abortions before the case) incomplete or missed abortion (6–17 weeks of gestational age, n=11);
6) uterine curettage because of molar pregnancy (9–10 weeks of gestational age, n=2).

Collecting the clinical samples was carried out during several years. Therefore, the reduced number of trophoblastic samples extracted from EP (n=6) and RM (n=7) were used for Gene Scan Fragment analysis in Paper I. The samples representing the molar pregnancies were available only for experimental part of Paper II.

All the procedures were performed in Tartu University Hospital Women's Clinic in 2003–2006 and the material was collected and handled by the author. Trophoblastic samples were snap-frozen in liquid nitrogen and stored at –80°C or placed immediately into RNAlater solution (Ambion Inc, Austin TX) and kept at –20°C until RNA isolation.

The blood samples from the individuals were taken on the day of abortion, delivery or surgery and frozen at –20°C. A total HCG and free β-HCG in the serum were measured by chemiluminescent enzyme immunometric assay IMMULITE at United Laboratories, Tartu University Hospitals.

Blastocysts were kindly donated by six couples undergoing IVF procedure (Dr. Peeter Karits). The samples of selected tissues were obtained from Research Tissue Bank of Tartu University Hospital (cerebral cortex, colon, liver, skeletal muscle, kidney, pancreas, spleen, small intestine); Dr. Tõnu Vooder and Kristjan Välk, University of Tartu, Chair of Biotechnology, Lung Clinic of Tartu University Hospital (lung, thymus) and Dr. Margus Punab, University of Tartu, Unit of Andrology (testis).

Blastocysts and tissue material were handled in similar way as samples from trophoblast. For all biopsy/autopsy samples the histological examination was carried out to confirm that the non-malignancy of the tissues. In addition to clinical samples the cDNA from Human Multiple Tissue cDNA Panel I and II (Clontech Laboratories Inc, Mountain View, CA) was used to broaden the range of studied tissues.

4.2.2. Patients and controls for association study (Paper III)

The couples suffering from RM and fertile women were recruited and blood samples for the DNA extraction were collected at the Women's Clinic of Tartu University Hospital and Nova Vita Clinic, Centre for Infertility Treatment and Medical Genetics, Tallinn, Estonia in 2003–2007 by author; and in the Department of Gynaecology and Obstetrics of the Helsinki University Hospital in Finland during 2001–2004 by Dr. V.-M. Ulander, Dr. K. Aittomäki (Table 4). Written informed consent was obtained from every study participant.

In both participating centers the recruited patients fulfilled the same inclusion criteria: they had at least ≥3 abortions during the first trimester of pregnancy at the age of 18–40 years. As both maternal and paternal gene variants contribute equally to the function of a fetal genome, the patient group included both parents who had experienced RM. In Estonian sample cohort the patient group consisted of 32 couples and 29 females with RM, and additional three couples with ≥3 unsuccessful IVF procedures. The Finnish sample set consisted of 40 couples and 5 female patients with RM. In 43 women, all miscarriages had taken place during the first trimester (<13 weeks). In addition to first trimester losses, two women had experienced second trimester (from 13

to 23 + 6 weeks) miscarriages and one woman had a third trimester (≥24 weeks) intrauterine fetal death (Kaare et al. 2006; Ulander 2007).

Uterine anomalies were assessed by ultrasonography or hysterosonogram. Maternal and paternal karyotypes were tested from peripheral blood lymphocyte cultures and shown to be normal in all patients.

The female patients with no successful pregnancies before miscarriages were defined as having primary RM (n= 69), the patients with secondary RM had at least one delivery before miscarriages (n=40).

The control group (n=195) consisted of fertile women with no history of miscarriage and either at least one normal pregnancy (the Finnish subjects, n=100) or more stringently, ≥3 successful deliveries (the Estonian subjects, n= 95). Their male partners were not recruited into control group as detailed information about their past reproductive history was difficult to get.

The blood samples were obtained from all individuals and kept at –20°C until DNA extraction.

Table 4. Individuals included to the association study.

	Estonian cohort	Finnish cohort	Total
Individuals suffering from RM:	99:	85:	184:
Females	64	45	109
Male partners	35	40	75
Fertile women	95	100	195
Total	194	185	379

4.3. DNA/RNA extractions and cDNA synthesis

DNA was extracted from peripheral blood in-house procedure or Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, USA), both based on the salting method.

Total RNA from 30–1000 mg of tissue was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA) or NucleoSpin® II Isolation Kit (Macherey-Nagel, GmbH&Co KG, Düren, Germany). The integrity of RNA was checked by 2100 Agilent Bioanalyzer using RNA 600 Nano LabChips (Agilent Technologies, Santa Clara, CA) and quantified by measuring absorption ratios at 260/280 nm (Helios Alpha&Beta Spectrophotometer, Thermo Spectronic). RNA was reverse transcribed to cDNA using random hexamers and SuperScript™ III Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA) or oligo(dT)18 primers and M-MuLV Reverse Transcriptase (First Strand cDNA Synthesis Kit, Fermentas Life Sciences) according to manufacturers' protocols.

The minus reverse transcription controls were treated in similar way except that the reactions lacked reverse transcriptase.

4.4. Gene expression analysis (Paper I and II)

Two approaches were used to carry out gene expression analysis. A semi-quantitative RT-PCR in combination with Gene Scan Fragment analysis was based on the detection of fragments derived from the digestion of a fluorescent-labeled PCR product. The method enabled to discriminate the expression of each individual *CGB* gene but lacked the sensitivity to detect weak signals emitted by the gene transcripts with low expression level.

On the contrary, a real-time RT-PCR method as the most sensitive method, enabled to determine the transcription of the genes with low expression: *CGB1* and *CGB2* with their transcripts, as well as *HCGβ* genes in trophoblastic and non-trophoblastic tissues. The discrimination of individual *CGB* gene was not possible with real-time RT-PCR method because of high homology of the genes.

4.4.1. Design and implementation of Gene Scan Fragment analysis

The results of re-sequencing study (95 worldwide individuals) in our laboratory (Hallast et al. 2005) that described the polymorphic positions in all six *CGB* genes were taken into account to design of primers for amplification of *HCGβ, CGB1/CGB2* and *LHB* genes. The primers were positioned to the different exons (exon I and III for co-amplification of *HCGβ* genes and *LHB* gene) or on the border of exons (for co-amplication of *CGB1* and *CGB2*) to prevent the amplification the possible contaminated DNA (Fig. 7). The 5'-end of reverse primers were labeled with an ABI compatible fluorescent dye (6-FAM, TET, HEX; Metabion International AG, Deutschland). The RT-PCR of *LHB* was attempted with three primer pairs. For normalization two reference genes were selected: a widely used *GAPDH* and a largest subunit of *RPII* as the most stable gene on a methodological report comparing reference genes for expression studies (Radonic et al. 2004). The localization and sequences of the primers are listed in Table 5 and depicted in Figure 7.

The RT-PCR mixture in total volume of 25 µl consisted of 1 µl first-strand reaction product, 1 U Smart-Taq Hot DNA Polymerase (AppliChem GmbH), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP (Fermentas Life Sciences), 2.5 µl 10x PCR reaction buffer containing $(NH_4)_2$ SO_4 and 400 nmol of forward and reverse primers. Each cDNA sample was also the amplification target for the two reference genes *GAPDH* and *RPII* under the same conditions. Amplification was attained by GeneAmp PCR System 2700 thermal cycling (Applied

Biosystems, Foster City, CA).

In order to discriminate of each individual *CGB* gene, a PCR product was treated with *HhaI* and *DraI* for *HCGβ* genes and *BcnI* for *CGB1/CGB2* that resulted with fragments of different lenght in basepairs (Fig. 7; Lazar et al. 1995; Miller-Lindholm et al. 1997).

A mixture of gene-specific fragments and reference gene products in equal volumes was combined with fluorescently labeled GeneScan-500 TAMRA internal size standard (Applied Biosystems-Perkin Elmer), and electrophoresed in an Applied Biosystems™ 373 DNA. All samples were amplified in triplicate, and each PCR product combination (reference and target genes, internal markers) was electrophoresed at least twice.

The gel was analyzed by GeneScan 2.1 software that allowed determining the length, height and area of the peaks (in relative fluorescent units) corresponding to the gene-specific fragment. The relative expressional level of each gene was calculated as followed: the value of the peak height of each target gene was divided with the value of peak height of the reference gene from the same lane of the gel.

For statistical analysis a Pearson correlation test and a random effects linear regression model, allowing a patient-level random intercept were used. All statistics were performed using R 2.0.1, a free software environment for statistical computing and graphics (http://www.r-project.org/).

Table 5. Primers and probes used for gene expression (A) and association study (B).

Primer or probe	Fig.7 Label	Sequence with Fluorescent Dye	Product size
A (i). GeneScan Fragment analysis			
HCGβ genesF	1	GACCCCACCATAGGCAGAG	541 bp
HCGβ genesR	2	GGTAGTTGCACACCACCTGA-FAM	
CGB1/CGB2F	3	GGAGGGAGGAAGGGGAACT	207 bp/ 206 bp
CGB1R^{G}	4	GCAACAGCAGCAGCCTCTTT-FAM	
CGB2R^{G}	5	CAACAGCAGCAGCCCCTTT-FAM	
GAPDH_F^{ML}		CCATGGAGAAGGCTGGGG	196 bp
GAPDH_R^{ML}		CCAAGTTGTCATGGATGACC-HEX	
*RPII*_F		CTTCACGGTGCTGGGCATT	239 bp
*RPII*_R^{R}		GTGCGGCTGCTTCCATAA-TET	

Primer or probe	Fig.7 Label	Sequence with Fluorescent Dye	Product size
A (ii). Real-time RT-PCR experiment			
*CGB1/2*F	6	AACACCCCTCACTCCCTGTCT	139 bp
*CGB1/2*R	7	ATGCTCAGCAGCAGCAACA	
*CGB1*A	A	FAM-ACATGTCAAAGAGGCTG-MGB	
*CGB1*G	B	VIC-CATGTCAAAGGGGCTG-MGB	
alt47C1majR	8	GCAGCAGCCTCTGGAACATCT-3'	167/ 165 bp
alt47C1min/C2R	9	AGCAGCCCCTGGAACATCTC-3'	
CGB1†	C	FAM-CATGTCCACATTCCCAGTG-MGB	
CGB2†	D	FAM-CATGTCCACATCCCCAGT-MGB	
alt176C1/C2intF	10	GCCATCACTGGCATGAGAAG	96 bp
alt176C1majR	11	AGCAGCAGCCTTGAAGCTTACT	
alt176C1min/C2R	12	AGCAGCAGCCCTGAAGCTTAC	
alt176C1/2	E	FAM-CTCTTTCTGGAGGAGCGT-MGB	
*CGB*allF	13	TCACCGTCAACACCACCATCT	121 bp
*CGB*totalR	14	ATGGACTCGAAGCGCACATC	
*CGB*all	F	FAM-CCACCATGACCCGCGT-MGB	
plasmid construction for positive control			
*CGB8*F		AGCACCTTTCTCGGGTCAC	791 bp
*CGB8*R		GGCCTTTGAGGAAGAGGAGT	
*CGB1*F		GAGGAAGGGGAACTGCATCT	686/ 689 bp
*CGB2*F		AGGGAGGAAGGGGAACTGTA	
*CGB1, CGB2*R		TGCGGATTGAGAAGCCTTTA	
*CGB1*Fnested		GAAGGGGAACTGCATCTGAG	406 bp*
*CGB1*Rnested		GGTAGTTGCACACCACCTGA	
B. Case – control association study			
CGB5 promoter			
CGB5pr_F	15	TTTAGTAGAGACAGGGATTCACCA	2243 bp
CGB5pr_R	16	AGACCACGGTGAAGTGATCTCAG	
CGB5 gene			
CGB5_F	17	CAGGAAAGCCTCAAGTAGAGGAG	1757 bp
CGB5_R	18	CGCTCGACGATGTTTTCTATTTT	

Primer or probe	Fig.7 Label	Sequence with Fluorescent Dye	Product size
CGB8 Long range PCR			
CGB8_F	19	CACGCCTGTAATTGTCGGAGGCTGT	8384 bp
CGB8_R	20	GAAAAGAGAGTGAAGATGGGGGACGAC	
CGB8 nested PCR			
CGB8n_F	21	CCCGGATAACTTTTCGTATTTTTA	2544 bp
CGB8n_R	22	TCCTCAGATCAACTCTCATGGAT	

F, forward primer; R, reverse primer; a primer originally published by [G]Giovangrandi et al., 2001; [ML]Miller-Lindholm et al.,1997; [R]Radonic et al. 2004;

† the probe was used to detect *CGB1/CGB2 + 47 bp* alternative mRNA form;

* the primers amplified *CGB1* + 176 bp mRNA form, 581 bp and *CGB1* + 166 bp mRNA alternative form, 571 bp.

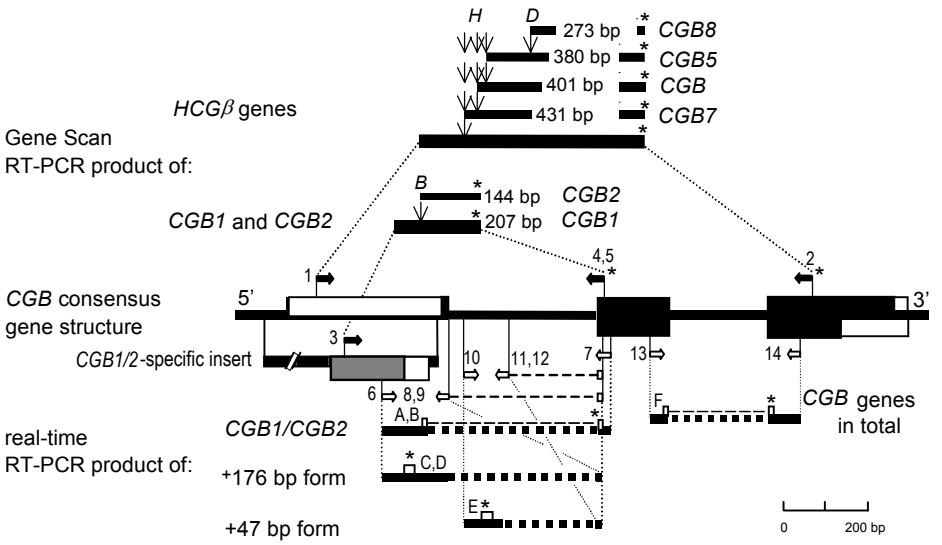

Figure 7. The design of gene expression study: (i) Gene Scan Fragment analysis above; (ii) real-time RT-PCT method below. The coding segments of all *CGB* genes are marked with black boxes, non-coding segments with white boxes. *CGB1/CGB2*-specific insert has a specific first exon (incl. 5'UTR). The PCR products are marked with black bold lines; the connecting dotted lines show the intronic part that is not included to transcripts. The black horizontal arrows indicate the binding sites for the forward and the fluorescent-labeled (*) reverse primers used for Gene Scan Fragment analysis. The vertical arrows indicate the restriction enzyme sites for *HhaI (H)*, *DraI (D)* and *BcnI (B)* that enable to discriminate each individual *CGB* gene. Primers and fluorescent-labeled (*) probes used for real-time RT-PCR experiments are shown with white arrows or boxes. The oligonucleotide sequences of the all primers (marked with numbers) and probes (marked with letters) are listed in Table 5.

4.4.2. Real-time RT-PCR amplification and data analysis

4.4.2.1. Design and experimental procedures

Primers and probes for real-time RT-PCR were designed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA). The primers for co-amplification of all *CGB* genes were located on exon II and exon III and the probe was placed on their boundary. The setup for the specific amplification of *hCGβ* genes was not reliable because of high variability in the region that discriminates *CGB, CGB5, CGB7* and *CGB8* from β-subunit non-coding *CGB1* and *CGB2* (Hallast et al. 2005).

For amplification of different splice forms of *CGB1/CGB2,* a probe (for *CGB1/CGB2*) or the reverse primers (for *CGB1/CGB2* alternative form + 49 bp and +176 bp) were positioned on the boundary of form-specific splice site of exon I and II. The quantification of mRNA transcript *CGB1/CGB2*+166 bp was omitted because of an unfavorable sequence (ACCCCCA) on the form-specific exon I/exon II boundary for positioning either primers or a probe. As the *CGB1* harbours a polymorphism (G/A) at the beginning of exon II, the mixture of allele-specific primers or probes was used (Fig. 7, Table 5). In subsequent calculations the results obtained from two differentially labeled probes for *CGB1/CGB2* were averaged.

The primer-probe mix of the reference genes (*GAPDH*, Hs99999905_m1, amplicon length 122 bp; *HPRT1*, Hs99999909_m1, 100 bp) were purchased from Applied Biosystems (Foster City, CA). *GAPDH* is expressed at approximately the same level with *HCGβ*-subunit coding genes and *HPRT* is transcribed in the similar activity as *CGB1/CGB2* genes.

The real-time RT-PCRs were performed using Applied Biosystems 7900HT Fast Real-time PCR system in 384 micro-well plates. The 10 µl PCR reactions consisted of 1 µl cDNA product, 5 µl TaqMan® Universal PCR Master Mix (2X) containing AmpliTaq Gold® DNA Polymerase and AmpErase® UNG (Applied Biosystems, Foster City, CA), and the primers and a probe in following concentration: 500 nM forward and reverse primer, 100 nM probe for target genes; or 0.5 µl ready-to-use endogenous control gene primer-probe mix (20X). Each sample was run in triplicate. Statistical analysis included the data only from the reactions where minus cDNA gave the negative result.

4.4.2.2. Construction of transcripts-specific and negative control plasmids

Three transcripts were purified from normal first trimester placenta: i) *CGB8* (representing four *HCGβ* genes), ii) *CGB1* (=*CGB2*), iii) *CGB1*+176 bp splice-form in order to optimize the reaction conditions and prepare the standard curves for absolute quantification Primers are listed in Table 5. All inserts were cloned into pTZ57R/T vector (InsTAclone™ PCR Cloning Kit, Fermentas Life Sciences, Burlington, Canada) and plasmid DNA was extracted with Nucleo Bond® PC 500 Plasmid Purification Kit (Macherey-Nagel GmbH&Co KG, Düren, Germany). A cloned *CGB1* genomic fragment, which contained the regions targeted by Taqman primers and probes, was used as an additional negative control for the oligo specificity to mRNA, and to exclude genomic contamination.

4.4.2.3. Data analysis

For quantification of the target genes two methods were used: comparative Ct (cycle threshold, cycle number at which the PCR amplification crosses the background fluorescence) method and standard curve method. The calculations by Ct method was carried out by two formulas differing in taking into account PCR efficiency (Livak and Schmittgen 2001; Pfaffl 2001). The calculated relative expression level of *CGB* genes by two methods was strongly correlated (r=0.95–0.99). For applying comparative ΔΔCt method a sample of trophoblastic tissue from the first trimester of normal pregnancy with the highest expression level of target gene (*CGB, CGB1/CGB2,* and their alternative forms) was selected as a calibrator.

Standard curve of serial dilutions of plasmid cDNA was run over eight logs covering 10^7–10^1 copies of target transcript per reaction. Copy number of *CGB* transcripts was quantified according to guidelines by Applied Biosystems (http://www.appliedbiosystems.com/support/apptech/#rt_pcr) and as described by Reimer *et al.* (2000).

Statistical analysis was performed by R 2.4.1, a free software environment for statistical computing and graphics (http://www.r-project.org/). A linear regression model and Pearson correlation test was used.

4.5. Case-control association study (Paper III)

4.5.1. Design of the study

Two the most transcribed *CGB* genes: *CGB5* and *CGB8* were selected to carry out the association study. The genes in *LHB/CGB* gene cluster share 85–99% DNA sequence identity, thus the one-step amplification of the genomic region of a target gene was possible only for *CGB5* (~ 1.7 kb fragment). The specific amplification of *CGB8* was achieved by using two steps: long-range PCR ~ 8.3 kb; nested PCR ~2.5 kb fragment. The oligonucleotide primers and PCR conditions that had been applied in previous re-sequencing study in our laboratory (Hallast et al. 2005) were used. Additional primers were designed for the analysis of the 5'upstream region of the *CGB5* gene (450 bp) using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All primers are listed in Table 5. The schematic representation of the genomic region harboring *CGB5* and *CGB8* and the positions of all primers are depicted in Figure 8.

The resequenced region involving *CGB8*covered 2050 bp, including the entire *CGB8* (1474 bp), 400 bp of 5' upstream region. The resequenced region for *CGB5* (1468 bp) covered the full genic region and part of 3'downstream region (Fig. 8).

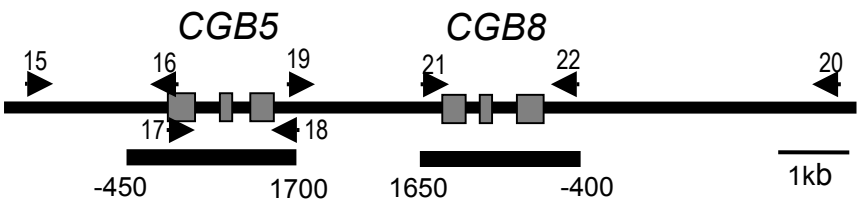

Figure 8. The region consisting of *CGB5* and *CGB8.* The exons are depicted with grey boxes. Primers used for ampification of genic part and 5'upsteam region of *CGB5* and *CGB8* are shown with black arrows along the gene, and marked with numbers. The resequenced region with range (relative to transcription start site) for *CGB5* and *CGB8* is shown with black bold line (4.2 kb in total). The oligonucleotide sequences are listed in Table 5.

4.5.2. Resequencing and data analysis

Amplifications of 100 ng genomic DNA (Long PCR Enzyme Mix; Fermentas International Inc.) were performed in a GeneAmp® PCR System 2700 thermal cycler (Applied Biosystems). PCR amplifications with minor modifications were carried out as reported previously (Hallast et al 2005). Purification of PCR products and resequencing reactions were performed as recommended by manufacturer (Big Dye Terminator v 3.1 Cycle Sequencing Kit, Part No. 4336919, Applied Biosystems, Warrington, UK). The sequences were resolved with either ABI 3730 X1 or ABI 3730 XL DNA Analyzer (Applied Biosystems).

Publicly available software was used to analyze the sequence data; bases were called with Phred, sequences were aligned with Phrap, and data were visualized by using Consed (Ewing and Green 1998; Ewing et al. 1998; Gordon et al. 1998). Polymorphisms were identified using the PolyPhred program (Version 6.02; http://www.phrap.org/phredphrapconsed.html; Bhangale et al. 2006) and confirmed by manual checking. A genetic variant was verified only in case it was observed in both forward and reverse orientations. In case of indel heterozygosity two independent forward and two reverse primers were used to confirm the genotype of the subject. The nomenclature of the identified polymorphisms was based on the following GenBank reference sequences: NM_033043.1 GI:15451747 for *CGB5*, NM_033183.2 GI:146229337 for *CGB8*. SNP code includes gene and sample set name (e.g.c5=*CGB5*; E=Estonians, F=Finns), and location relative to transcription start site.

The descriptive statistics of linkage disequilibrium (LD), r^2 was calculated for pairs of markers and summarized by Haploview software (Barrett et al. 2005).

Allele frequencies obtained by re-sequencing case-control study (Study III) were estimated and conformance to Hardy-Weinberg Equilibrium (HWE) was calculated (α= 0.05). In total 8 rare SNPs in 5'upstream region of *CGB5* were deviating from HWE, as one individual was homozygous for minor allele of all these SNPs. The significance of the association between the identified SNPs in *CGB5* and *CGB8* genes and occurrence of RM was tested using Cochran-Armitage test for trend implemented in statistical analysis package JMP® 6.0.3 with Genomics module 2.0.6 (http://www.jmp.com/software/genomics/). The same test was applied to address the interpopulation (Estonians, Finns) differentiation.

Odds ratio (OR) with 95% confidence intervals (CI) were calculated to show the strength and direction of the association. In all tests, $p<0.05$ was considered statistically significant.

5. RESULTS

5.1. Expression of *CGB* genes in trophoblastic tissue

5.1.1. The contribution of *HCGβ* genes to hormone β-subunit production (Paper I)

The contribution of an individual *HCGβ* gene (*CGB, CGB5, CGB7, CGB8*) into β-subunit production was determined by RT-PCR combined with restriction that enabled to distinguish mRNA products specific to each *CGB* gene (Fig. 9). The relative quantization of the fluorecent-labeled gene-specific fragments was performed by Gene Scan Fragment analysis.

The most prevalent contribution pattern of individual genes to HCGβ-subunit production was *CGB8* > *CGB5* ≈ *CGB* >> *CGB7* (Table 6, Fig. 9,10). For the third trimester placentas, the pattern was slightly altered, *CGB8*≈ *CGB5*>*CGB*>>*CGB7* (Table 6). Compared to the first trimester, the expression level of *CGB5* was higher ($p<0.05$) and of *CGB* lower ($p<0.005$).

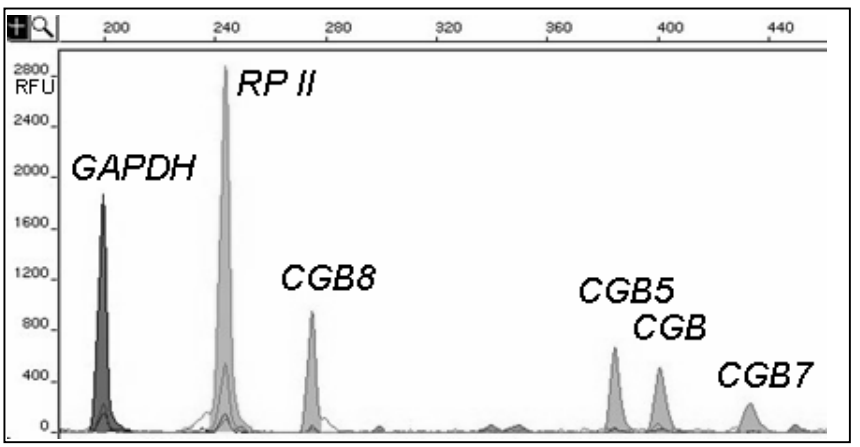

Figure 9. GeneScan electrophoretogram showing the amplified fluorescent-labeled (6-FAM, HEX or TET) products of reference genes and *HCGβ* genes after treatment by restriction enzymes. The x-axis shows the size of the detected fragments in base pairs, and the y-axis represents the relative intensity of fluorescence (RFU). The peaks indicate the DNA fragments: *GAPDH* at 196 bp, *RPII* at 239 bp, *CGB8* at 273 bp, *CGB5* at 380 bp, *CGB* at 401 bp, *CGB7* at 433 bp. The trophoblastic tissue from ectopic pregnancy (6 weeks) expresses the genes: *CGB8*>*CGB5*>*CGB*>*CGB7*.

The major β-subunit contributing gene was *CGB8*, giving almost a half of total mRNA transcripts, during the second trimester of normal pregnancy, and in cases of EP and RM. During the first and third trimester of normal pregnancy, its contribution was 39% (Table 6).

Two the most transcribed genes *CGB8* and *CGB5* provided 70±7.8% (range 56–84.7%) of total HCG β-subunit mRNA transcripts.

In concordance with high variation of intergenic mRNA transcription level, the interindividual variation of *HCGβ* genes was also high. The expression of *CGB5* among individuals within the same groups had the widest variation range (9.6–55.5%, Fig. 10).

Table 6. Individual contribution (percentage and standard deviation) of *CGB, CGB5, CGB7* and *CGB8* into summarized HCG β–subunit determining placental mRNA during the normal pregnancy, in cases of ectopic pregnancy and recurrent miscarriage.

	CGB8	*CGB5*	*CGB*	*CGB7*
I trimester, (4–12 wks, n=10)	39.3±1.8	25.5±2.7	27.1±1.5	8.1±1.2
II trimester, (17–20 wks, n=8)	48.1±2.8**	25.7±4.2	20.0±2.3**	6.2±1.8
III trimester, (38–42 wks, n=12)	39.2±2.5	36.0±3.8**	20.1±2.1**	4.7±1.6*
Ectopic pregnancy, (6–14 wks, n=6)	48.0±3.2*	18.7±4.7	25.3±2.6	8.0±2.1
Recurrent miscarriage (6–9 wks, n=7)	47.1±3.7*	23.3±4.9	22.0±2.8	7.6±2.2

**p<0.005; *p<0.05: Significant difference of the expression level of each gene during II and III trimester placentas as well as in cases of EP and RM compared to I trimester of normal pregnancy as a reference level.

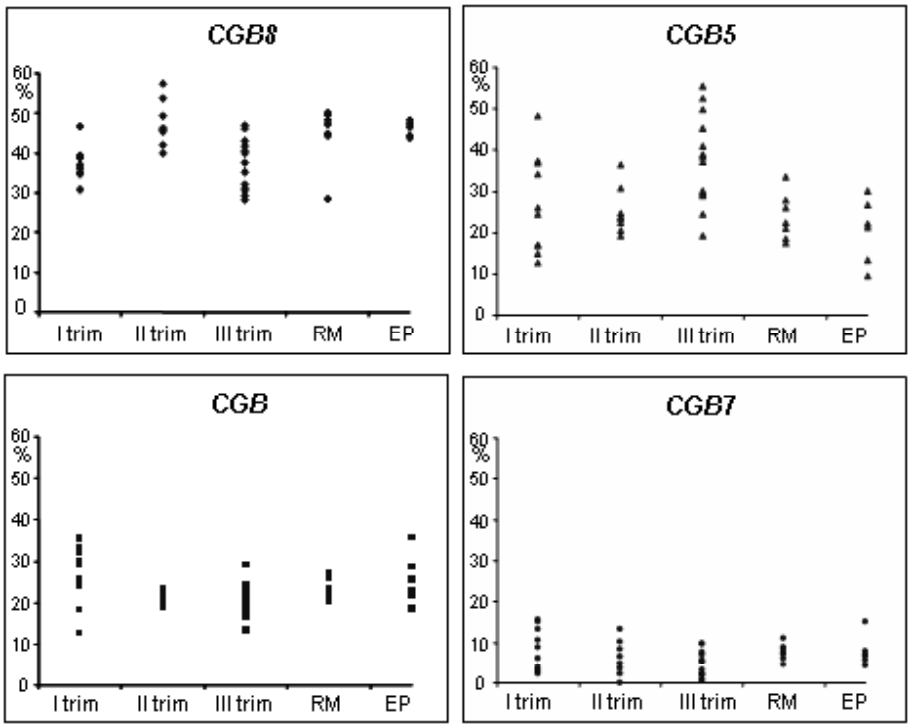

Figure 10. The percentage from total of HCG β-subunit mRNA transcription of *CGB8*, *CGB5, CGB* and *CGB7* determined in placentas representing the first, second and third trimester of normal pregnancy; recurrent miscarriage (RM) and extrauterine pregnancy (EP). Each dot represents the averaged value of an individual's experiments (three independent PCR amplifications, each amplicon was digested and electrophoresed at least twice).

5.1.2. Transcription of *HCGβ* genes during the normal and complicated pregnancy

The summarized expression of HCGβ-subunit coding genes in trophoblastic tissue in the normal and complicated pregnancy was assessed by two methods:
(i) semi-quantitative RT-PCR Gene Scan Fragment analysis (Paper I);
(ii) real-time RT-PCR method (Paper II).

The highest transcription level of *HCGβ* genes during normal pregnancy was detected in the first trimester (Fig. 11A,B). Data from both experiments confirmed the significant decrease of expression level of the genes during the second trimester ($p \leq 0.001$). In the third trimester of pregnancy the mRNA transcription increased but did not reach the level detected in the first trimester (Fig. 11A, B, Fig.12).

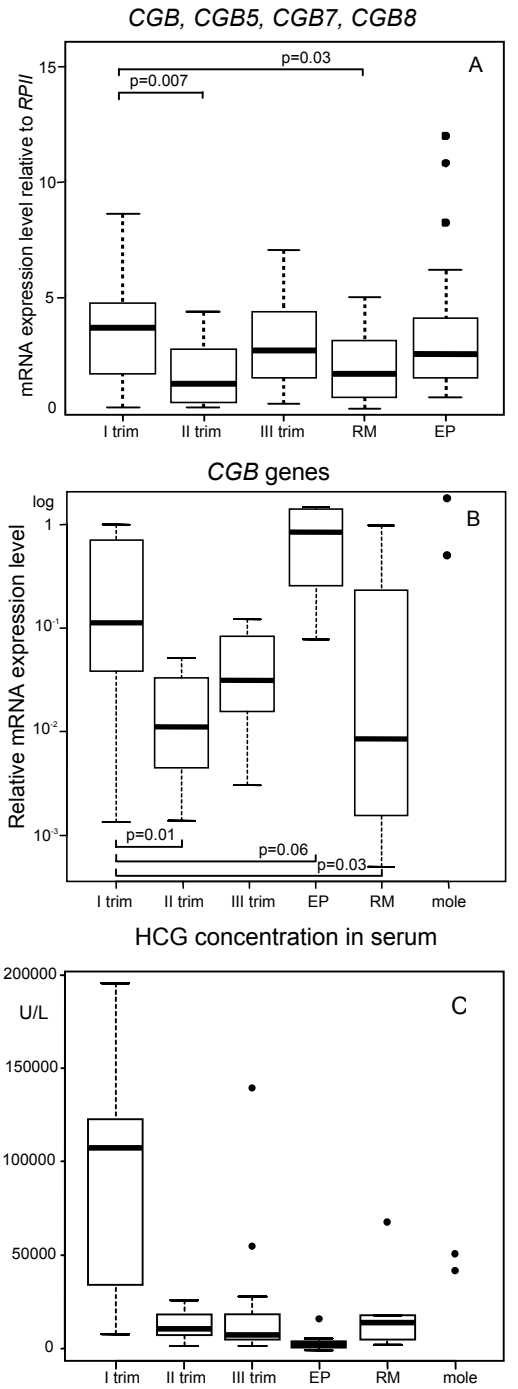

Figure 11. The relative expression level of (A) HCG β-subunit coding genes / (B) all *CGB* genes in trophoblastic tissue; and (C) HCG concentration in the serum measured on the day of tissue sampling during the first, second and third trimester of normal pregnancy, in case of recurrent miscarriage, ectopic and molar pregnancy.

The transcription was determined by (A) semi-quantitive RT-PCR combined with Gene Scan Fragment analysis; (B) real-time RT-PCR method. A sample from first trimester of normal pregnancy with highest expression was selected for a calibrator.

The boxes represent the 25th and 75th percentiles; the median is denoted as the line that bisects the boxes.

The significant reduction of the summarized transcriptional activity of *CGB* genes was detected in the cases of RM compared to normal pregnancy at the same gestational age (p=0.03; Fig. 11A,B, Fig.12). Again, the results obtained from two methods were concordant with each other. Although the β-subunit non-coding *CGB1* and *CGB2* were also included into summarized transcript pool in experiments using real-time RT-PCR method, their presence did not change the result.

In cases of EP, the total *CGB* mRNA level tended to be higher than the distribution described for normal intrauterine gestation, however the difference was not statistically significant (Fig. 11A,B, Fig.12).

The transcription level of all *CGB* genes in molar pregnancy (n=2) determined by real-time RT-PCR method was at the upper range of the distribution obtained from normal first trimester pregnancy (Fig. 11B, Fig.12).

In mRNA extracted from pooled blastocysts material (n=6), the summarized level of *CGB* transcripts was as high as detected in trophoblastic tissue from first trimester of normal pregnancy. This supports the suggestions that the activation of *CGB* genes occurs in early preimplantation stage and is essential for normal implantation (Bonduelle et al. 1988; Jurisicova et al. 1999).

The high interindividual variation of mRNA transcription level of *CGB* genes in trophoblastic tissue was observed among the samples representing the same gestational age and clinical condition (Fig. 12). This was consistent with previous reports on expression of *CGB* genes (Miller-Lindholm et al. 1997).

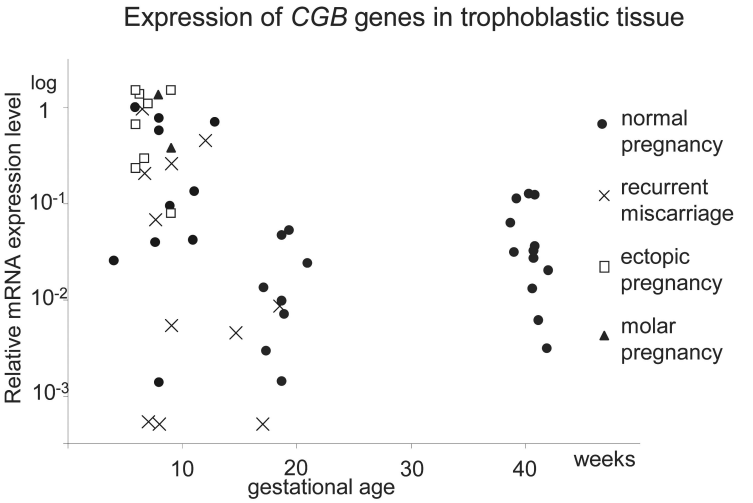

Figure 12. Transcription pattern of *CGB* genes in trophoblastic tissue during the normal and complicated pregnancies (recurrent miscarriage, ectopic and molar pregnancy) by gestational age. Relative expression level was determined by real-time RT-PCR comparative Ct method. A sample from first trimester of normal pregnancy with the highest expresson was selected for a calibrator (expression level = 1).

5.1.3. Comparison of HCG concentration in maternal serum with expression level of *CGB* genes

HCG concentration in maternal serum was the substantially higher during the first trimester of normal pregnancy as compared to the second and the third trimester (Fig. 11C). Hormone level was significantly lower both in cases of EP (p=0.0001) and RM (p=0.002) compared to the gestational age adjusted normal pregnancy.

The expression level of *CGB* genes in total in placenta showed a moderate but significant correlation to HCG concentration in the serum among all samples except EP (Fig.13). In RM, low HCG in serum is associated with low transcriptional activitity of the *CGB* genes. Differently, in cases of EP, the expression of *CGB* genes was higher than in trophoblastic tissue from normal pregnancy adjusted to gestational age and HCG concentration in maternal serum (p=0.01).

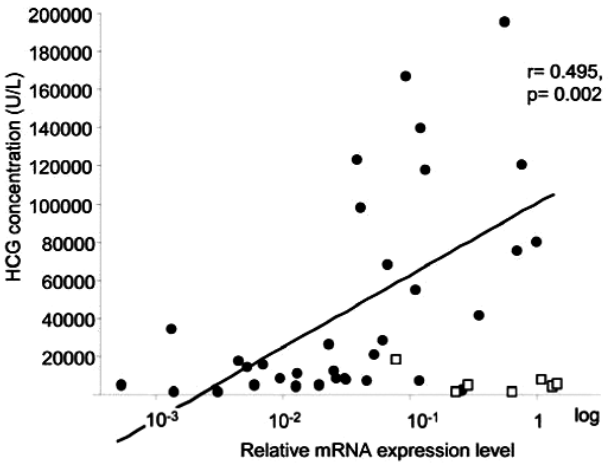

Figure 13. Correlation between HCG concentration in maternal serum and relative mRNA expression level of *CGB* genes in trophoblastic tissue. Transcription was determined by real-time RT-PCR comparative Ct method. A sample from first trimester of normal pregnancy with the highest expresson was selected for a calibrator sample. The trophoblastic samples representing intrauterine pregnancy: normal pregnancy I, II and III trimester, recurrent miscarriage and molar pregnancy, are shown with filled circles (●); ectopic pregnancy are marked with empty squares (□). The regression line and correlation coefficient are based on the data of intrauterine pregnancies.

5.1.4. The transcription of HCG β-subunit non-coding *CGB1* and *CGB2* genes

The expression of *CGB1* and *CGB2* was also determined by two methods: semi-quantitative RT-PCR Gene Scan Fragment analysis and real-time RT-PCR.

The study setup for *CGB1* and *CGB2* combining RT-PCR with restriction and Gene Scan Fragment analysis enabled to discriminate the genes (Fig. 7) and demonstrate the comparatively more abundant transcription of *CGB1* compared to *CGB2* (Paper I). The peaks representing *CGB1* on the Gene Scan electropheretograms were detected in 80% and *CGB2* in 50% of analyzed specimens. An alternative form +47 bp was detected only for *CGB1*.

The real-time PCR method enabled to detect the mRNA of the genes with very low expression and revealed the differences of the transcription level during the normal and complicated pregnancy (Paper II).

The expression of the three alternative *CGB1/CGB2* transcripts followed the same pattern that was seen in summarized *CGB* genes. The strongest expression was during the first trimester of normal pregnancy compared to the second and third trimester (Fig. 14). A reduced transcriptional activity was observed in cases of RM, and more abundant *CGB1/CGB2* mRNA transcripts was detected in trophoblastic tissue from EP compared to normal pregnancy at the same level as seen in normal first trimester pregnancy (Fig. 14).

The expression level of alternative splice form *CGB1/CGB2* +176 bp mRNA introducing a premature stop-codon was at approximately the same level than it was detected for originally described *CGB1/CGB2*. The other reported alternative splice-form (+47 bp) was substantially less transcribed remaining at the borderline of detection limit of real-time RT-PCR.

The transcription of different splice forms of *CGB1/CGB2* correlated within a sample (r=0.82–0.89).

The transcript-specific plasmids were used to create standard curves for absolute quantification of co-amplified *CGB1/CGB2* and all *CGB*. This enabled to estimate the contribution of *CGB1/CGB2* into summarized expression of *CGB* genes. Despite *CGB1* and *CGB2* genes form one third of the total six *CGB* gene copies, they provided only ~1/2000 of the *CGB* mRNA transcripts in the first trimester of normal pregnancy. During the second and third trimester the contribution was even lower (~1/20 000). In comparison to normal implantation process, the trophoblastic tissue from EP was characterized by higher transcriptional activity of *CGB1/CGB2* and/or lower expression level of HCG β-subunit coding genes: *CGB1/CGB2* contributed 1/400–1/2500 of summarized *CGB* transcripts.

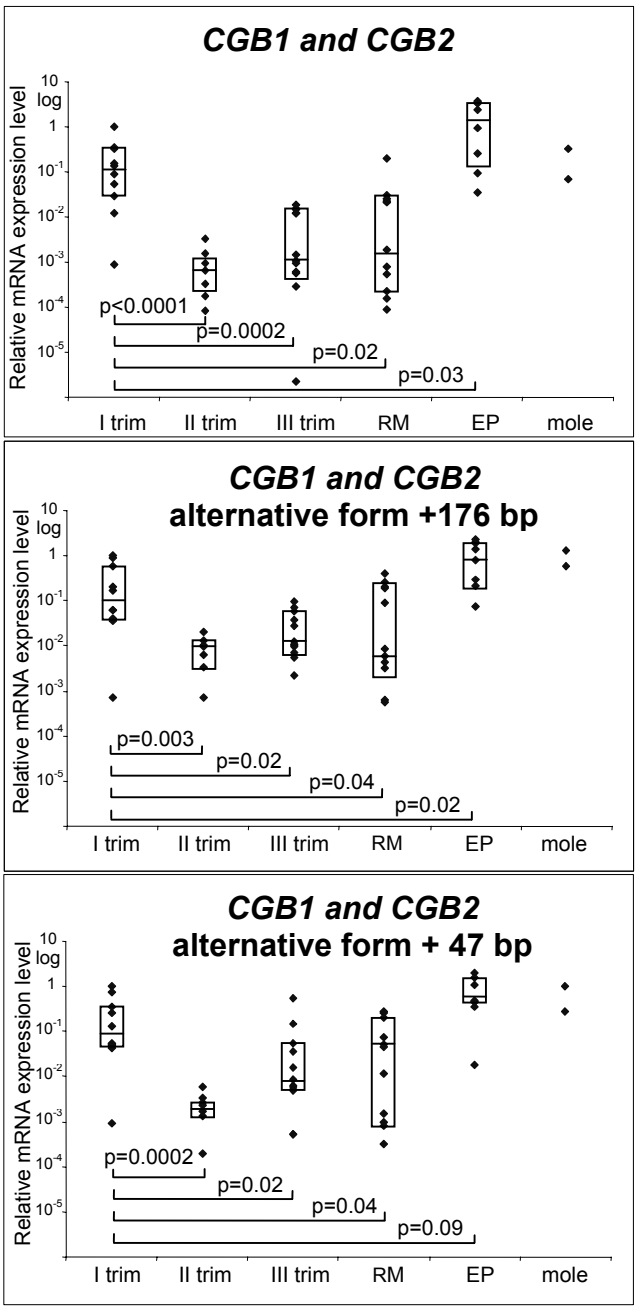

Figure 14. The relative mRNA expression level of *CGB1* and *CGB2* transcript, alternative form +176 bp and +47 bp in placenta during the normal pregnancy, in cases of recurrent miscarriage (RM), extrauterine pregnancy (EP) and molar pregnancy determined with real-time RT-PCR comparative Ct method. A sample from first trimester of normal pregnancy with highest expression was selected for a calibrator. The boxes represent the 25th and 75th percentiles, the median is denoted as the line that bisects the boxes.

Despite the low general expression level *CGB1/CGB2* were also detected in blastocyst material both by real-time RT-PCR as well even a semi-quantitative Gene Scan Fragment analysis (Fig. 15).

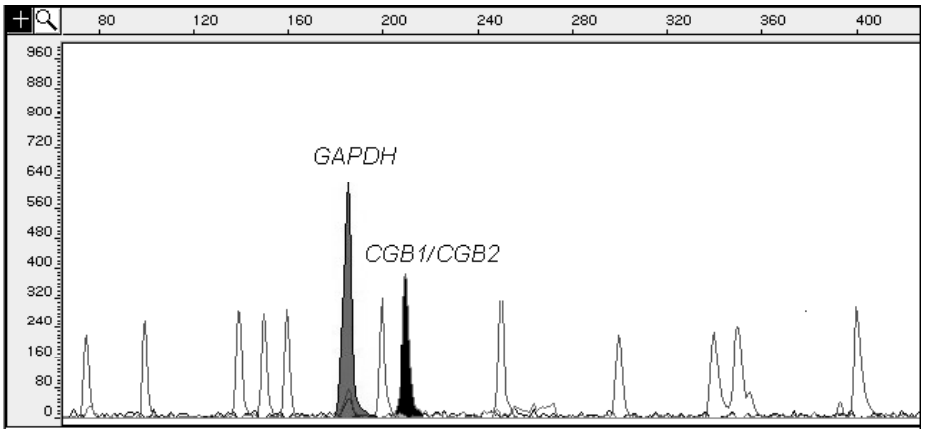

Figure 15. Gene Scan Fragment analysis electrophoretogram showing the amplified fluorescent-labeled products of *CGB1* and *CGB2* (6-FAM; 207 bp) gene and *GAPDH* (HEX; 196 bp) from cDNA of a single blastocyst material. The x-axis shows the size of the detected fragments, and the y-axis represents the relative intensity of fluorescence. The empty peaks mark the GeneScan-500 TAMRA internal size standard.

5.1.5. The expression of *LHB* gene (Paper I)

The expression of *LHB* gene in trophoblastic tissue representing the normal and complicated pregnancy was determined with three different primer pairs. As the transcription *LHB* was not detected in any samples by semi-quantitative RT-PCR Gene Scan Fragment analysis, the further experiments were not done.

It can be concluded that the expression of *LHB* genein placenta is fully down regulated during pregnancy.

5.2. The transcription of *CGB* genes in normal non-trophoblastic tissues (Paper II)

The expression of *CGB* genes in non-trophoblastic non-malignant tissues was determined with semi-quantitative RT-PCR combined with Gene Scan Fragment analysis and real-time RT-PCR TaqMan method.

Initially, cDNAs from Human Multiple Tissue cDNA Panels I and II were screened for presence of transcripts of HCG β-subunit coding genes and *CGB1/CGB2* by RT-PCR and GeneScan analysis of amplified fragments. When a detectable peak on electrophoretogram was seen at least in one run of three, the additional samples were prepared for real-time PCR experiment. The results

obtained from tissue panel cDNA and "in-house" synthesized cDNA were coincident with small non-significant discrepancies. All *CGB* genes in total are transcribed in greater extent (100–1000 copies per reaction) in testis, prostate, thymus, skeletal muscle and lung being approximately 4 orders of magnitude less expressed than in averaged samples from trophoblastic tissue of first trimester of normal pregnancy. In several tissues: ovary, small intestine, kidney, spleen, liver, heart, brain and colon the *CGB* genes in total were detected in very low level (9–100 copies per reaction) (Table 7).

Table 7. Expression of *CGB* genes in human tissues determined by Gene Scan Fragment analysis and Taqman real-time RT-PCR methods

	Summarized *CGB*		*CGB1/ CGB2*	
	Gene Scan[b]	TaqMan[c]	Gene Scan[b]	TaqMan[c]
Trophoblastic tissue, I trim:				
Calibrator sample[a]	+	11873350	+	15000
Normal pregnancy (n=0/10)	+	2308383	+	879
EP (n=0/8)	+	13163945	+	15067
RM (n=0/11)	+	194371	+	134
Placenta, II trim (n=0/8)	+	147295	+/-	15
Placenta, III trim (n=8/12)	+	161600	+/-	28
Testis (n=45/5)	+	722	+	453
Prostate (n=32/0)	+/-	491	+/-	17
Thymus (n=18/5)	+/-	280	+	4
Skeletal muscle (n=8/3)	nd	254	nd	nd
Lung (n=4/5)	+/-	160	+	nd
Small intestine (n=32/3)	nd	86	nd	nd
Ovary (n=14/0)	nd	50	nd	3
Kidney (n=5/3)	nd	28	nd	5
Spleen (n=5/3)	nd	23	+/-	nd
Heart (n=3/0)	nd	20	nd	nd
Brain (n=2/3)	nd	20	nd	nd
Liver (n=1/3)	nd	28	nd	nd
Colon (n=20/3)	nd	9	nd	6
Pancreas (n=15/3)	nd	nd	+/-	nd
Peripheral blood leucocytes	nd	nd	nd	nd

trim – trimester, nd – not detected, n – number of samples from pooled cDNA panel / from biopisy or autopsy

[a] the calibrator sample (first trimester normal pregnancy) selected based on the highest expression of the target gene and used in relative quantification;

[b] detection of the target gene in at least in two runs of three (+); one run of three (+/-);

[c] mRNA molecules of the target gene/50 ng of total RNA used for cDNA synthesis

The expression of *CGB1/CGB2* was detected only in limited non-trophoblastic tissues: testis, prostate, thymus, ovary, kidney, colon and peripheral blood leucocytes. In testis *CGB1/CGB2* are considerably more expressed than in other tissues exceeding the transcription rate even in the placenta during the second and third trimester of normal pregnancy (Table 7).

5.3. Variation in *CGB8* and *CGB5* is associated with RM (Paper III)

5.3.1. Detailed variation of *CGB8* and *CGB5*

The entire genic and 5'upstream regions of *CGB5* and *CGB8* genes were fully resequenced in a sample collection consisting of Finnish and Estonian RM cases (n=184; n=85 Finns, n=99 Estonians) and fertile controls (n=195; n=100 Finns, n=95 Estonians). For every subject, the entire resequenced region covered 4.2 kb (Fig. 8).

In total of 71 polymorphisms were identified: 18 and 3 SNPs in the 5' upstream regions; 29 and 19 SNPs in the genic part of *CGB5* and *CGB8*, respectively; and 2 SNPs 3'downstream of *CGB5* (Fig.16). 48 SNPs (68% of total of 71 SNPs) were novel variants, previously not described in dbSNP database (http://www.ncbi.nih.gov/SNP) and literature. Neither SNP in *CGB5* nor *CGB8* has been covered by the most recent version of HAPMAP (http://www.hapmap.org/; release March 2008). All identified polymorphisms were submitted to dbSNP database and are listed in Appendix I and II.

The density of the polymorphic sites in intronic, exonic and adjoining untranslated regions was 26.4, 9.1 and 14.1 SNPs per 1000 bp, respectively. This exceeded 2.5–6 fold the average SNP density in the respective regions of resequenced 1630 genes in 82 unrelated individuals (Schneider et al. 2003). High genetic variation of genes in *LH/CGB* gene cluster has also observed in the large resequencing study in our laboratory (Hallast et al.2005).

Two thirds of the identified polymorphic sites (n=41; 58%) were shared by Estonian and Finnish sample collections. In both sample sets, there were 15 population-specific SNPs represented as singletons or low-frequency variants (<2%). Majority of the shared SNPs had no differences in Estonian and Finnish sample sets (Appendix I). Significant difference in allele frequencies was detected for in case of 8 SNPs out of 71, most of these are rare variants. None of these SNPs had different distribution of minor allele between the RM patients and fertile women. Linkage disequilibrium between the identified SNPs in the resequenced region was nearly absent in both population samples. However, some SNPs at neighbouring loci had strong LD forming a gene conversion motif, for example four SNPs in 5-upstream region in *CGB5*: c5EF-155, c5EF-147, c5EF-144, c5EF-142.

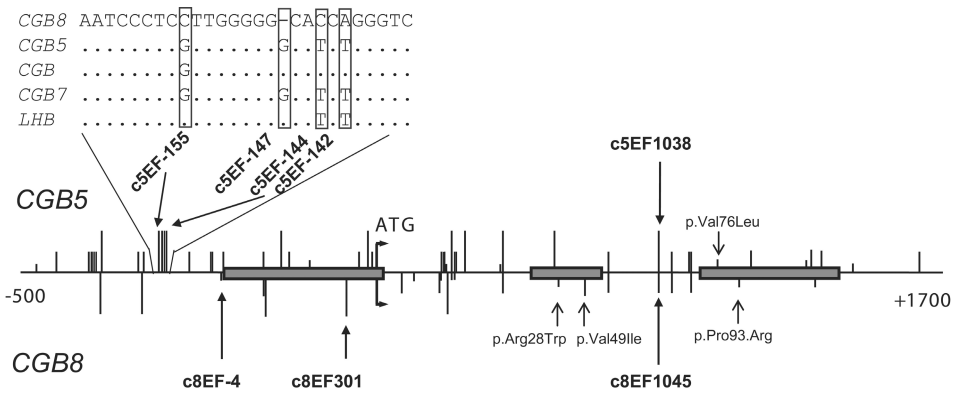

Figure 16. The localization of SNPs in *CGB5* and *CGB8*. The exons are depicted with grey boxes, translation initation site ATG is shown. The longest vertical lines mark SNPs with minor allele frequency >5%, the shortest lines represent singleton SNPs in pooled Finnish and Estonian sample set (n=384). SNPs with significantly different prevalence among RM patients (n=184) and fertile controls (n=195) are pointed by capped arrows and marked with bold letters. SNP code includes gene and population name (c5= *CGB5,* E= Estonians, F= Finns), location relative to mRNA start site. The SNPs causing non-synonymous amino acid changes are pointed with arrows and marked. The aligned consensus sequences of the 5'upstream element of *LHB/HCGβ* genes are shown with highlighted positions that are distinctive for each gene. The minor alleles in positions c5EF-155, c5EF-147, c5EF-144, cEF5-142 of *CGB5* are identical with the nucleotides in *CGB8* corresponding sites (G/C, G/del, T/C, T/A).

5.3.2. *CGB8* and *CGB5* variants reducing the risk for RM

The occurrence of all identified SNPs in *CGB5* and *CGB8* was compared among the patients with RM and fertile women separately for the Estonian (RM cases n=99; fertile women defined as controls n=95) and the Finnish subjects (cases n=85; controls n=100). The low population stratification enabled the joint analysis in order to increase the statistical power of the study.

In the sample set that included all individuals (cases n= 184, fertile controls n=195), a significant association with RM was detected for *CGB5* 5'upstream polymorphisms (c5EF-155, c5EF-147, c5EF-144 and c5EF -142, Table 8). Analysis of the Estonian and the Finnish subsamples supported trend for association in both study populations independently. However, the p-values reached did not reach statistical significance due to reduced samples sizes.

Table 8. Variants in *CGB5* and *CGB8* genes significantly associated with RM. Association p-values and odds ratio with 95% CI was calculated by Cochran-Armitage test for trend.

	Estonians (n=194)			Finns (n=185)			All individuals (n=379)			
	MAF (%)			MAF (%)			MAF (%)			
SNP	Fertile women n=95	RM patients n=99	p-value	Fertile women n=100	RM patients n=85	p-value	Fertile women n=195	RM patients n=184	p-value	OR (95%CI)
CGB5										
c5EF-155	13.16	8.08	0.083	11.50	6.55	0.129	12.31	7.38	0.024	0.57 (0.35–0.93)
c5EF-147	13.16	8.08	0.083	11.00	5.95	0.094	12.05	7.10	0.018	0.54 (0.32–0.91)
c5EF-144	13.16	8.08	0.083	13.00	7.74	0.131	13.08	7.92	0.023	0.58 (0.36–0.93)
c5EF-142	13.16	8.08	0.083	13.00	7.74	0.131	13.08	7.92	0.023	0.58 (0.36–0.93)
c5EF1038	14.47	9.09	0.079	14.00	7.06	0.036	14.36	8.15	0.007	0.53 (0.32–0.85)
CGB8										
c8EF-4	0	1.01	0.163	0	0.64	0.265	0	0.85	0.071	na*
c8EF301	5.79	5.05	0.740	8.85	3.21	0.034	7.33	4.24	0.072	0.55 (0.29–1.06)
c8EF1045	0.53	0.51	0.977	3.13	0	0.025	1.83	0.28	0.042	0.15 (0.02–1.03)

*na =not applicable as monomorphic among patients

Table 9. Gender-specific analysis of the variants in *CGB5* and *CGB8* genes significantly associated with RM in full sample set. Association p-values and odds ratio with 95% CI was calculated by Cochran-Armitage test for trend.

	Fertile women n=195	Female RM patients n=109			Male RM patients n=75		
SNP	MAF %	MAF %	p-value	OR (95%CI)	MAF %	p-value	OR (95%CI)
CGB5							
c5EF-155	12.31	7.34	0.058	0.59 (0.32–1.03)	7.43	0.105	0.57 (0.29–1.13)
c5EF-147	12.05	6.88	0.039	0.53 (0.28–0.98)	7.43	0.115	0.57 (0.28–1.15)
c5EF-144	13.08	7.34	0.031	0.53 (0.29–0.95)	8.78	0.171	0.64 (0.34–1.22)
c5EF-142	13.08	7.34	0.031	0.53 (0.29–0.95)	8.78	0.171	0.64 (0.34–1.22)
c5EF1038	14.36	6.88	0.006	0.45 (0.25–0.81)	10	0.182	0.66 (0.36–1.22)
CGB8							
c8EF-4	0	0.91	0.058	na*	0.67	0.098	na*
c8EF301	7.33	5.14	0.296	0.68 (0.33–1.4)	2.86	0.059	0.37 (0.13–1.02)
c8EF1045	1.83	0.47	0.162	0.25 (0.03–2.04)	0	0.104	na*

*na =not applicable as monomorphic among patients/fertile women

The significant association with all four *CGB5* promoter polymorphisms results from higher MAF in fertile women (12.05%–13.08%) compared to RM group (7.10%–7.92%). This difference between the control group and RM cases was consistent in both study populations (Table 8).

Among the *CGB5* genic SNPs a strong protective effect was detected for the minor allele of intron 2 c5EF1038, represented with the frequency 14.36% in fertile women compared to 8.15% in the RM group (Table 8). In the separate analysis of Finnish subgroup the polymorphism had also statistically significant association with RM (p=0.036; OR=0.48 [95% CI 0.24–0.97]) but among Estonians the association remained close to limit of statistical significance (p=0.079; OR=0.57 [95% CI 0.30–1.08]). No increase in protection toward RM was detected for the combination of the minor alleles of the *CGB5* 5'upstream and the intronic SNPs.

Population-specific associations we detected in the Finnish sample collection with two rare SNPs (MAF <10%) in *CGB8*: c8EF301 (p=0.034) and c8EF1045 (p=0.025) (Table 8). The protective variant in the intron 2 of *CGB8* (c8EF1045) is located at the same position within the gene as the *CGB5* intronic variant (c5EF1038).

Notably, for all the five *CGB5* SNPs the protective effects remained significant even when only the female RM patients (n=109) were included as cases into the statistical analysis (Table 9). Some differences between the female and their male partners were observed, the excess of minor allele of c5EF1038 was more significant in female patients and c8EF301 in males (Table 8). However, this may be incidental effect arisen from the substantially different sample size between the genders.

Interestingly, a significant deficit of an intronic (c5EF1038) and four 5'upstream SNPs (c5EF-155, c5EF-147, c5EF-144, c5EF-142) compared to fertile women was detected only among patients with primary RM (MAF 5.79–6.52%, p=0.009–0.064) but not with secondary RM (MAF 8.75%, p=0.185–0.391, Fig. 17). Again, the reduced number of patients in both groups may cause biased conclusions.

Despite there is neither experimentally proved (Hollenberg et al. 1994; Johnson and Jameson 2000; Ghosh et al. 2003) nor *in silico* predicted (MatInspector 2.2 http://www.genomatix.de/products/MatInspector/; (Cartharius et al. 2005) transcription factor binding sites in the motif c5EF-155…c5EF-142 that would explain the differential expression of *CGB5* and *CGB8* genes, the involvement of this sequence fragment in regulation of transcription cannot be ruled out.

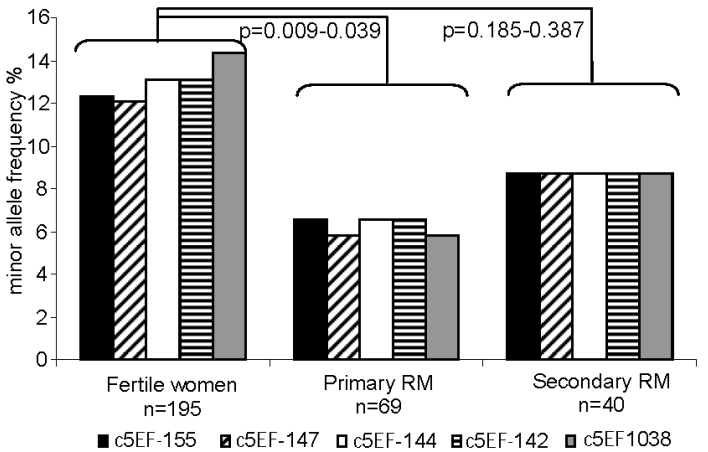

Figure 17. Minor allele frequencies of four 5'upstream and an intronic SNP among fertile women and female patients with primary and secondary RM.

5.3.3. Rare gene variants increase the susceptibility to RM

5.3.3.1. Non-synonymous amino acid substitutions

Three polymorphisms causing a non-synonymous amino acid substitution were found only among the patients with RM:
CGB5 p.Val76Leu (G/C, c5F1178) in a single Finnish RM patient;
CGB8 p.Arg28Trp (G/A, c8E806) in a single Estonian patient;
CGB8 p.Pro93Arg (C/G, c8E1237) in a single Estonian patient.
In addition, one non-synoymous amino acid and *CGB8* p.Val49Ile (G/A, c8EF869) was detected in one Finnish and two Estonian patients, and also seven Estonian fertile women (Fig. 16).

5.3.3.2. The polymorphisms in promoter region of *CGB8*

The resequenced *CGB8* 5'upstream region harbours only 3 SNPs (two common and one rare) compared to the respective region for *CGB5* with 18 polymorphic sites (Fig. 16).

The rare allele A of SNP at the position 4 bp from transcription start site (T/A, c8EF-4) was solely represented in patients, one from Finland and two from Estonia (Cochran-Armitage trend test, p=0.071; Table 8, 9). This polymorphism is located within the AP1-like sequence that overlaps the *HCGβ* transcription initiator element critical for basal transcription and downstream of

the Ets-2 binding site acting as a major enhancer of *HCGβ* gene expression (Fig. 18, Ghosh et al. 2003).

Two other polymorphisms in *CGB8* promoter region: T/C, c8EF-287 and G/T, c8EF-186 are located within the Sp1/AP-2 binding sites detected by functional assays exploring *CGB5* promoter region (from *CGB5* mRNA start site -318 to -279 and -220 to -188; Johnson et al. 1997; Johnson and Jameson 1999; 2000). These transcription factor binding sites reside in the critical region for the trophoblast-specific expression (-305 to -279) and cAMP-responsiveness (-311 to -202) of the *HCGβ* gene transcription (Albanese et al. 1991; Hollenberg et al. 1994).

Interestingly, no individuals carrying both minor alleles of c8EF-287 (C; MAF=25.2%) and c8EF-186 (T; MAF=39.7%) were detected. The carriers' frequency was expected to be 10%. The nucleotides in these positions may possess a combinatory effect affecting the binding affinity of Sp1 and AP-2 transcription factors.

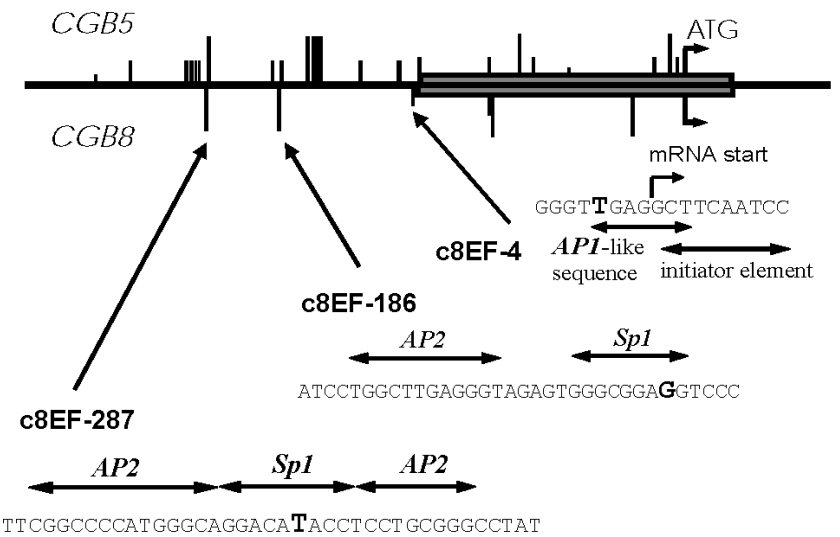

Figure 18. The detailed representation of 5'upstream region and exon I (grey boxes) in *CGB5* and *CGB8*. Translation start is shown with ATG. The longest vertical lines mark SNPs with minor allele frequency >5%, the shortest lines represent singleton SNPs in pooled Finnish and Estonian sample set (n=384). All three detected SNPs (c8EF-287, c8EF-186, c8EF-4) in 5'upstream region of *CGB8* are highlighted (bold letters) and their adjacent sequence harbouring experimentally detected transcription factor binding sites are shown (Albanese et al. 1991; Johnson and Jameson 1999; Ghosh et al. 2003).

6. DISCUSSION

6.1. Expression of *CGB* genes in normal and complicated pregnancies

6.1.1. Comparison of two methods for assessment of transcription

Application of two alternative methods to determine the expression of *CGB* genes enabled to benefit from the advantages and overcome limitations of either approach. RT-PCR combined with restriction and Gene Scan Fragment analysis allowed to determine the expression of each individual *HCGβ* gene. That was not applicable with real-time RT-PCR method due to high homology (up to 99%) between the genes in *LHB/CGB* genome cluster.

The results on the expression level of HCGβ-subunit coding genes determined by two methods were highly concordant. Thus, it can be concluded that the RT-PCR in combination with Gene Scan Fragment analysis proved to be a reliable method for quantification the genes with abundant expression level.

Real-time RT-PCR method demonstrated its advantage for quantification of the mRNA with low transcription level as in case of *CGB1/CGB2*. The comparative analysis of the results obtained by two methods revealed that the RT-PCR in combination with Gene Scan Fragment analysis allowed reliable detection of the transcripts in amount of >100 copies per reaction.

6.1.2. High interindividual and intergenic variation

The high interindividual variation of mRNA transcription level of *CGB* genes observed in this study (Fig. 12) is also demonstrated by a previous report addressing the expression of *HCGβ* genes in 27 first trimester placentas from 6–16 gestational weeks (Miller-Lindholm et al. 1997). *CGB5* accounted for 40–82%, *CGB8* 12–32%, *CGB* 1–42%, and *CGB7* <3% of the total β-subunit mRNA transcription (Miller-Lindholm et al. 1997). Consistant with these findings we also observed the widest interindividual variation range for *CGB5* (10–56%). However, the most prevalent pattern detected in this study: *CGB8* > *CGB5* ≈ *CGB* >> *CGB7* differs from that reported previously: *CGB5* > *CGB8* ≈ *CGB* >> *CGB7* (Bo and Boime 1992; Miller-Lindholm et al. 1997).

The expression varies most of all within the samples representing the first trimester of pregnancy (Fig. 11, 12). During this period the production of HCG increases rapidly (Hay 1988), so does the expression of hormone producing genes. Inclusion of the samples from 4 to 12 gestational weeks can partly explain the wide variation. However, the overlap of hormone concentration

characterizing the different gestational age and pathological conditions is widely known in medical practice.

6.1.3. Gene expression and hormone level may not be concordant in pathological pregnancies

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In most clinical conditions (normal pregnancy, miscarriage, molar pregnancy), the transcriptional activity of HCG β-subunit coding genes correlates with HCG level in maternal serum but in some pathologies (EP, trisomy 21) a discrepancy between the gene expression and hormone concentration can be observed (Brizot et al. 1995; Jauniaux et al. 2000; Feng et al. 2006).

Ectopic pregnancy

In contrast to significantly reduced hormone concentration compared to gestational age matched normal pregnancies; the expression of the *CGB* genes in EP is not down-regulated and has a narrower expressional window (Fig. 11, 12).

Low level of HCG in EP might refer to deficient hormone assembly in fallopian tube. This process is obviously the most effective in endometrium that also expresses both hormone subunits and produces HCG during the secretory phase of the menstrual cycle and the early pregnancy (Wolkersdorfer et al. 1998; Zimmermann et al. 2003). Despite the early placentation in the fallopian tube that occurs in the same manner as it does in the uterus, tubal placentation is membranaceous in nature and gets inadequate blood supply (Randall et al. 1987). Thus, tubal epithelium is not able to function as effectively as endometrium. Low hormone level may activate the transcription of *CGB* genes via a negative autoregulatory pathway.

In addition, the transcription of *CGB* in EP may be maintained in unaltered state to cope with extensive oxidative stress triggered by abnormal placentation in the fallopian tube. It has been shown that the oxidative stress stimulates the synthesis of placental proteins, such as HCG, to repair the damage and rescue the pregnancy (Jauniaux et al. 2006).

Trisomies 13, 18 and 21

The discrepancy between expression of *CGB* genes and HCG level is observed in the cases of trisomy 21 (Brizot et al. 1995). Trisomy 21 or Down syndrome is characterized by elevated, not reduced maternal serum concentrations of HCG and free HCGβ-subunit in spite of low transcription of *CGB* genes. In this case, the defective differentiation leads to the accumulation of extravillous cytotrophoblasts producing the structurally and functionally distinct hyperglycosylated hormone variant (HCG-H) instead of the regular HCG (Cole 2007).

HCG-H has been shown to increase invasiveness of the trophoblastic cells via autocrine/ paracrine route.

Very low level of HCG in trisomies 18 and 13 are direct consequences of poor differentation of the cytotrophoblast (Jauniaux et al. 2000). The transcription of *CGB* genes in both trisomies is significally reduced (Brizot et al. 1996).

Molar pregnancy

The elevated expression level of summarized *CGB* genes demonstrated in two tissue samples from molar pregnancy in this study (Fig. 11, 12) is concordant with the findings reported in 7 regressive and 7 persistent complete hydatidiform mole tissues (Feng et al. 2006). The high transcription of both subunits of HCG may be the consequence of larger mass of the placenta compared to the normal pregnancy. The admixture of cyto- and syncytiotrophoblasts in hydatidiform mole do not decrease the expression *CGB* genes (Hoshina et al. 1983). The up-regulation of the *CGB* genes may also be caused by alterations in the signal transduction machinery within the molar trophoblast (Petit et al. 1996).

Another essential hormone for conception, implantation and placentation, glycodelin or placental protein 14, a potent immunomodulatory protein, exhibits the similar expression pattern with *CGB* in both protein and mRNA level: down-regulated in first trimester miscarriage and up-regulated in mole pregnancy (Toth et al. 2008).

6.1.4. Low expression of *HCGβ* genes and RM

Miscarriage is the final event that may be initiated by several factors. Depending on the severity and exposition time of a detrimental factor the explusio of fetus/embryo from maternal environment may happen at different gestational ages and with different clinical scenarios ranging from empty gestational sac to late pregnancy loss.

The low expression of *CGB* genes, observed in miscarriages, may be either a primary or secondary event leading to pregnancy loss. It has been shown that at the beginning of the process, in threatened miscarriage, when the damage of the syncytiotrophoblast is limited, the *CGB* gene expression is not affected. The production of HCG even increases to protect the embryo/fetus from damage by excessive oxidative stress (Jauniaux etal. 2006; Johns et al. 2007). This is gained by activated differentiation of cytotrophoblasts into hormone producing syncytiotrophoblasts (Hay 1988; Johns et al. 2007). If the signal from the embryo/fetus is still lost for longer time, the trophoblastic tissue degrades and HCG level decreases (Greenwold et al. 2003) due to the low transcription of *CGB* genes. In cases, where the trophoblastic tissue is not able to increase the production of HCG to cope with detrimental factors, the pregnancy stops much

earlier. In some cases, low HCG concentration is sufficient for the normal course of the pregnancy, but if other risk factors are also present, the low hormone level and/or inability to increase the production turns to be fatal.

6.2. Association of variation in *CGB8* and *CGB5* with RM

6.2.1. Special aspects addressed in design of the study

The design of the case-control study to find the RM-associated variants of *HCGβ* genes was complicated by several aspects.

Firstly, HCG is coded by fetal genome, thus the most appropriate target for a case-control study could be trophoblastic tissue samples from miscarriaged versus normal pregnancies. Miscarriages due to a chromosomal abnormality should be excluded by cytogenetic analysis. As it is technically and ethically (cervical dilatation and uterine curettage should be avoided if less invasive options are available) complicated to get sufficient number of tissue samples, the couples suffering from RM were included into the study. Both parents, the equally contributing donors to fetal genotype were involved to find out the possible variants associated with pregnancy failure. The inclusion of the couples suffering from RM and fertile women as control individuals have been applied in several previous studies addressing other placentally expressed genes, HLA-G, -C and -E (Hviid 2006, Aldrich et al. 2001, Pfeiffer et al. 2001).

However, the inclusion of the parents of affected individuals (=fetuses/ embryos) necessitated the larger sample size for the study. Inclusion of two independent sample set representing different populations (Estonian and Finnish) allowed to elucidate the possible population-specific associations and find the risk or protective variants that are relevant in both studied sample sets, thus reflecting the population-independent effects.

Secondly, the *CGB* genes are clustered together on chromosome 19 and have arisen by several duplication events. High sequence similarity (>92%) between these genes and high diversity, large number of population-specific variants and low LD (Hallast et al. 2005) hindered the selection of reliable tag-SNPs and limited the use of any genotyping method. Many of the variants found in the database were not detected in our study. These could be the representatives of a multisite variation or may just reflect differences in the nucleotide content of identical positions in duplicated genes. In case, no previous detailed re-sequencing has been done to recover true SNPs, SNP tagging was not applicable for association analysis based solely on the information of SNP databases and direct resequencing of duplicated genes proved to be the most informative strategy.

And thirdly, the β-subunit of HCG is coded by four *CGB* genes that are not expressed equally. Gene Scan Fragment analysis revealed that *CGB8* and *CGB5*

gave the major contribution (~2/3) in total hormone β-subunit transcripts. In the case-control study, these two genes were addressed as a genetic variance in these genes has obviously the greatest effect on pregnancy outcome.

6.2.2. Variants in *CGB5* and *CGB8* may elucidate their functional role in hormone production

The promoter areas of *LHB* and *CGB* genes harbour several binding sites for transcription factors, and the divergent nucleotides between the genes have been shown to influence the transcription activity (Hollenberg et al. 1994; Ghosh et al. 2003). For example, a deletion of the AGA sequence at position -20 to -18 or a substitution of dinucleotide GG with TT at position -54 from transcription start of *HCGβ* genes reduces the *CGB5* promoter activity 5-fold. The combination of both changes causes > 20-fold reduction in promoter strength (Hollenberg et al. 1994). Comparison of promoters of *LHB* and *HCGβ* genes showed that the change of expression site from pituitary to placenta was caused by the accumulation of multiple, combinatorial regulatory elements and not by acquisition of single distinct tissue-specific enhancer (Hollenberg et al. 1994). The latter has been shown to regulate the tissue-specific expression in growth hormone/chorionic somatomammotropin gene cluster. Like the *LHB/CGB* gene cluster it has also a pituitary expressed ancestral growth hormone gene and placentally expressed duplicated gene copies (Nachtigal et al. 1993). Thus, as multiple divergent nucleotides and their combinations regulate the promoter activity and transcription level of a *CGB* gene, the high genetic variation could be one possible reason for wide interindividual differences in expression pattern of *CGB* genes.

However, the β-subunit of HCG is a combination of the products coded by four genes (*CGB8, CGB5, CGB, CGB7*). For maintenance of early pregnancy, the total level of hormone is more important than the expression of the individual gene (Miller-Lindholm et al. 1997). Low expression of one or two *CGB* genes may be compensated by others. In some cases, low HCG concentration is sufficient for normal course of the pregnancy, but still the lower values of HCG in serum are related to miscarriage. In the cases of threatened miscarriage with successful completion of pregnancy, the concentration of HCG in maternal serum is higher compared to the cases that end with abortion (Johns et al. 2007). This demonstrates the ability of trophoblast to increase the hormone production in response to unfavorable conditions for survival. In critical situations, some variants of *CGB* genes may turn to be advantageous. In *CGB5*, five polymorphisms were associated with protective effect reducing the risk of RM up to 1.8 fold. Four SNPs are located in 5'upstream area of *CGB5* (c5EF-155, c5EF-147, c5EF-144, c5EF-142). The motif consisting of all minor alleles of these SNPs is identical to the

corresponding area in *CGB8* gene. As *CGB8* probably harbors the most effectively functioning promoter, the replacement of the major alleles in the promoter motif in *CGB5* with *CGB8*-like promoter motif may facilitate the production of HCG in critical situations and reduce the risk of reproductive failure.

The resequencing of both genes showed that *CGB8,* and especially its promoter region, is under the stronger functional constraint compared to *CGB5* in spite of high DNA sequence similarity (98-99%) between these genes. There were > 2 times less polymorphisms in *CGB8* genomic region (n=22) compared to *CGB5* (n=49*)*. Two common polymorphisms (c8EF-287 MAF 25.2%, and c8EF-186 MAF 39.7%) were located within the binding sites of transcription factors Sp1/AP-2 that have been shown to be critical for transcription of *HCG* genes (Albanese et al. 1991; Johnson and Jameson 1999). The combination of minor allele variants of both these SNPs seems to be strictly avoided as there was no individual who was homozygous for minor allele of either SNP and carried one or both minor alleles of the another SNP. Additionally, three rare variants in *CGB8* that may exhibit an effect on hormone action (p.Arg18Trp, p.Pro93Arg, and c8EF-4 within proximal promoter) were present exclusively in RM patients. Thus, the *CGB8* as the most expressed HCG β-subunit coding gene has probably the most critical role in regulation of the hormone production and pregnancy success.

6.2.3. RM-associated gene variants by population, gender and type of RM

The deficit of minor alleles of five polymorphisms in *CGB5* (c5EF-155, c5EF-147, c5EF-144, c5EF-142, c5EF1038) among RM patients compared to fertile controls was at approximately equal in both Estonian and Finnish sample sets. Two other protective gene variants (minor alleles of c8EF301 and c8EF1045) had significant association with RM only among Finns. Discrepant results between the populations may be caused by the specific demographic history of the Finnish population (Norio et al. 1973) or too small sample size of this study to detect association of SNPs with low allele frequency (MAF <10%).

The differences between populations regarding the low-frequency polymorphisms have also been found in other studies. An amino acid substitution Val79Met (nomenclature based on mature protein; from ATG p.Val99Met) in *CGB5* exon 3 has been reported at carrier frequency 4.2% in 323 asymptomatic individuals from the Midwest of the United States (Miller-Lindholm et al. 1999) but it was absent in a 580 individuals originating from five European populations (Jiang et al. 2004) as well as in this study.

Gender-specific analysis showed that the association of four *CGB5* SNPs was significant when only the female RM patients (n=109) were considered as "cases" (Table 8). Despite the male RM patients carried also less minor alleles

of five *CGB5* polymorphisms compared to fertile controls, the difference (4.3–4.9%) did not reach significant p-value due to reduced sample size (n=75). The weaker effect may also be caused by greater heterogeneity within the group representing male RM patients because the correct information about their reproductive history is often unavailable.

Some observations indicate that in spite of complexity of pathogenesis of RM, the primary and secondary RM may be considered as two different entities having various prevailing mechanisms leading to abortion. The immunological factors may play a greater role in women with a series of miscarriages after a birth (secondary RM) than in women with RM who had never had a successful pregnancy (primary RM) (Christiansen et al. 2004). For example, the HLA-DR3 allele found to be associated with RM displays a much stronger association to secondary than to primary RM (Kruse et al. 2004). Non-immunological risk factors for RM, the factor V Leiden mutation, seem to be associated only with primary RM (Foka et al. 2000; Wramsby et al. 2000). In the light of these findings, the association of polymorphisms in *CGB5* detected in this study may be different among women with primary and secondary RM. However, substantially larger number of individuals with both primary and secondary RM should be examined to confirm the observation that the variants in *CGB5* are associated mostly with primary RM.

6.3. The expression of *CGB* genes in non-trophoblastic tissues – link to malignancy?

In concordance with two previous studies addressing the transcriptional profile of *CGB* genes in normal non-malignant non-trophoblastic tissues (Bellet et al, 1997: semi-quantitative detection; Reimer et al. 2000: real time quantification with TaqMan) and gene expression microarray data (http://expression.gnf.org, 1153_f_at; Affymetrix Annotation) the strongest non-trophoblastic *CGB* transcriptional activity was detected in testis, prostate and skeletal muscle.

In lesser extent the expression of *CGB* genes has been shown in thymus, colon, lung and kidney by real-time experiments (this study, Reimer et al. 2000). Additionally, according to other tissues studied in literature, *CGB* expression has been detected also in uterus, mammary, thyroid, adrenal and pituitary glands (Dirnhofer et al. 1996; Bellet et al. 1997; Reimer et al. 2000). The role of HCG may be related to autoregulatory mechanisms that regulate the growth of tissue. HCG acts probably in an auto-/paracrine way; a transcrine route has been suggested for the testis, prostate and uterus (Berger et al. 2007). However, the further experiments should be done to elucidate the functional significance of HCG in non-trophoblastic tissues.

Several non-trophoblastic tumors: bladder, renal, prostate, lung, gastro-intestinal, neuroendocrine, breast and gynecological cancers, have been shown

to produce HCG (Stenman et al. 2004). The role of HCG in the carcinogenesis might be associated with the enhancement of invasion, angiogenesis, the inhibition of apoptosis and escape from immune surveillance (Butler and Iles 2004; Herr et al. 2007; Reisinger et al. 2007) – the same means are used by the trophoblasts to ensure successful implantation and placentation. At the genomic level, the activation of the transcription of *CGB, CGB5* and *CGB8* has been associated with malignant transformation of non-trophoblastic cells (breast, bladder, prostate, thyroid, testis and renal cancer; Bellet et al. 1997; Giovangrandi et al. 2001; Hotakainen et al. 2007). Interestingly, in comparison to the normal tissues, the HCG producing tumor samples express proportionally lower amount of *CGB7* differing by one non-synonymous change from other *HCGβ* genes (Ala117Asp in the mature protein; from ATG Ala137Asp) and more preferentially placentally expressed *CGB, CGB5* and *CGB8* (Bellet et al. 1997). Based on this study, some variants in *CGB5* are associated with pregnancy success probably because of the ability to increase the hormone production in response to critical conditions during the imminent abortion. If the variation in HCGβ-subunit coding genes is also related to malignant transformation of normal tissue facilitating the switch on the transcription of the placentally expressed *CGB, CGB5* and *CGB8* genes remains to be addressed in furthers studies.

6.4. The expression of *CGB1* and *CGB2*, the genes with unknown biological function

The expressional activity of *CGB1* and *CGB2* was correlated with the total level of *CGB* transcripts suggesting the *in consort* transcriptional activation of the gene cluster. The consorted manner of the expression might be explained by the transcriptional activation of the genome cluster through simultaneous chromatin modifications and/or by highly homologous DNA sequence (>95%) in the promoter regions of all *CGB* genes. Compared to *HCGβ* genes the transcription of *CGB1* and *CGB2* was significantly lower. The reduced expression results from *CGB1/CGB2* specific-insertion that replaces the proximal promoter region and the 5'UTR of *HCGβ* genes (Fig. 4, 6). This region harbors two functionally important binding sites for Ets-2, a potent transcription enhancer of of *HCGβ* genes (Ghosh et al. 2003).

In consistence with expression of *HCGβ* genes during the normal pregnancy the highest transcription level of *CGB1* and *CGB2* in trophoblastic tissue was detected during the first trimester (Fig. 14). In cases of EP, the expression of *CGB1* and *CGB2* was significantly higher and in RM lower than during the normal pregnancy at the same gestational age. The contribution of *CGB1* and *CGB2* into total transcript pool of all *CGB* genes differed slightly from what

was calculated for the normal first trimester pregnancy. It has been demonstrated that the invasion of the placental bed occurs in the same manner as in the uterus and fallopian tube, indicating that the ability to implant and initiate the placentation is determined by trophoblastic tissue of fetal origin rather than by maternal tissues (Randall et al. 1987). Miscarriaged pregnancies are related to poor trophoblast invasion (Meegdes et al. 1988; Lyall 2002). During the second and third trimester the process of implantation has been replaced by the growth and maturation of the placenta, thus the genes regulating implantation and early function of placenta can be down-regulated. It is also noteworthy that the expression of *CGB1* and *CGB2* was detected already at blastocyst stage.

The relative abundant expression of *CGB1* and *CGB2* in testis may also be related to the reproductive success. Testis serves as a highly specialized "gymnasium" where sperm cells are adequately equipped, trained and selected for their ability to survive as foreigners in the female genital tract and contribute to embryonic development (Filippini et al. 2001).

The resequencing of *CGB1* and *CGB2* in total of 95 individuals representing three population (Estonians – Europe, Mandenkas – Africa; Chinese Han – Asia) revealed polymorphism pattern that suggests a strong evolutionary selection on these genes (Hallast et al. 2007).

Although the variation and expression of *CGB1* and *CGB2* indicate their possible involvement in early stage of the pregnancy, the protein product of *CGB1* and *CGB2* is still not characterized and the question whether the genes are pseudogenes remains open. The pseudogene status is supported by extremely low transcription level and no structural similarity of the predicted hypothetical protein to the published structures. There is growing evidence that transcribed pseudogenes are not entirely without purpose, they might act as potential regulators of gene expression and effectors of the function of known genes (Hirotsune et al. 2003). This type of regulatory transcripts tend to be expressed at low levels, sometimes at or below detection limit; the transcripts are located close to annotated genes or even within genes, in introns or on the opposite DNA strand (Khaitovich et al. 2006). The transcripts produced by the pseudogenes located between protein coding genes show conserved tissue-specific expression patterns concordant with the protein-coding transcripts of known genes. All alternative splice forms *CGB1/CGB2* (+47 bp, 166 bp or 176 bp; Fig. 6, 14) may also serve as pseudogenes with regulatory function.

7. CONCLUSIONS

1. The transcriptional activity of the human *CGB* genes revealed high intergenic and interindividual variability. The most prevalent contribution profile of the individual *CGB* genes to the *HCGβ* mRNA was *CGB8*>*CGB5*≈ *CGB*>> *CGB7*. *CGB8* and *CGB5* are the most actively transcribed genes and contribute ~70% to the total production of HCG β-subunit. Thus, the variation in these genes may have the greatest functional significance.
2. The full transcription of all *CGB* genes occurred already in blastocyst stage and was high throughout the pregnancy. The expression of *CGB* genes was significantly reduced in cases of recurrent miscarriages but was not altered in ectopic and molar pregnancies.
3. The summarized expression of HCG β-subunit coding genes was moderately, but significantly correlated with HCG concentration in serum. In cases of RM, the reduced hormone level was consistent with low mRNA level suggesting that low transcriptional activity of *CGB* genes contributes in pathogenesis of RM. In EP, the low hormone but high expression level of *CGB* genes indicates the failure of auto-regulatory feedback or aberrations in posttranslational mechanisms.
4. Despite the specialization to the expression in placenta, the *CGB* genes are also transcribed in several non-malignant non-trophoblastic tissues: testis, prostate, thymus, skeletal muscle, lung, at minimum level. The relative abundance of *CGB1* and *CGB2* in testis may indicate their functional role in the male reproductive tract.
5. Four promoter and two intronic variants of *CGB5* and *CGB8* carrying the protective polymorphisms reduce the risk of RM up to 1.8 fold. All these SNPs occur more frequently in fertile women compared to the RM patients. Additionally, three non-synonymous amino acid substitutions in *CGB5* and *CGB8* and a rare promoter polymorphism located within transcription initiator element of *CGB8* were identified only in RM cases as possible risk variants for RM. The high genetic variation in *HCGβ* genes may also explain the large interindividual differences in HCG level during the pregnancy.

Implications and further development of the study:
The identified gene variants associated with decreased or increased risk to RM promote further experiments to uncover their functional effect on gene expression and HCG hormone activity. I hope that the results of this study can be transformed to the diagnostic application and could contribute in the improvement of early and preventive treatment of recurrent miscarriage.

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9. SUMMARY IN ESTONIAN

Inimese kooriongonadotropiini beeta alaühikut kodeerivate geenide polümorfismide ja ekspressioonimustrite seos korduva raseduse katkemisega

Sissejuhatus

Raseduse katkemine on sagedaseim rasedustüsistus. Ligikaudu 70% viljastunud munarakkudest ei arene normaalseks raseduseks, mis lõpeb elusa lapse sünniga. Enamikel juhtudel toimub katkemine nii varases järgus, et rasedust ei jõutagi veel tuvastada (Macklon et al. 2002). Kliiniliselt diagnoositud raseduse korral on iseenesliku katkemise risk ~10–15% (Wilcox et al. 1988; Cramer ja Wise 2000, Zinaman et al. 1996)

Korduv raseduse katkemine (KRK) on vähemalt kolme järjestikuse kliiniliselt diagnoositud raseduse katkemine enne 22. rasedusnädalat või kui loode kaalus katkemise ajal alla 500g. KRK teoreetiline esinemissagedus pelgalt juhuse tõttu on hinnanguliselt 0,35%, kuid tegelikult esineb KRK ~1% last soovivatest paaridest (Bricker ja Farquharson 2002; Berry et al. 1995). Kõige enam on seostatud KRK-ga ema hormonaalseid ja vere hüübimissüsteemi häireid, immunoloogilisi ja anatoomilisi tegureid ning vanemate kromosoomianomaaliaid. Ligikaudu 50% juhtudel ei leidu kõrvalekaldeid üheski seni kasutusel olevas diagnostilises testis (Bricker ja Farquharson 2002).

KRK-ga patsientide esimese ringi sugulastel on raseduse katkemise risk 2–7 korda suurem viidates pärilike tegurite osalusele patogeneesis (Christiansen 1996). Enamikes töödes on uuritud KRK seotust geenidega, mis osalevad immuuntolerantsi välja kujunemisel, apoptoosis, angiogeneesis ja vere hüübimissüsteemis. Kõik need geenid on seotud paljude erinevate funktsioonidega ema organismis, mistõttu on seos KRK-ga tihti mittespetsiifiline. Platsentas toodetud valgud ning neid kodeerivad geenid on otseselt seotud raseduse kulu ning loote arenguga olles sobilikeks kanditaatgeenideks ning markeriteks KRK päriliku eelsoodumuse leidmiseks.

Inimese kooriongonadotropiin (HCG) on platsenta süntsüütsiotrofoblasti rakkudes toodetud hormoon. Hormooni β-alaühikut on leitud juba 2-rakulises embrüos, seega on HCG üks esimesi loote poolt toodetud valke (Jurisicova et al. 1999). Pärast embrüo pesastumist emaka limaskesta jõuab HCG ema vereringesse ning uriini, kust hormooni määramine võimaldab HCG-d kasutada raseduse kliiniliseks diagnostikaks. HCG on oluline rasedusaegse kollaskeha funktsiooni säilitamiseks, et tagada vajalik progesterooni hulk. Lisaks soodustab HCG embrüo implantatsiooni, platsenta verevarustust ning immuuntolerantsi kujunemist ema ja loote vahel. Mitmete rasedustüsistuste (raseduse katkemine, peetunud rasedus, emakaväline rasedus) korral on HCG tase tavapärasest ma-

dalam (Buyalos et al. 1992; Letterie ja Hibbert 2000; Dumps et al. 2002; Tong et al. 2006).

HCG koosneb kahest alaühikust: α-ühikust, mis on sarnane FSH, LH ja TSH α-alaühikuga ning hormoonspetsiifilisest β-alaühikust. HCG β-alaühikut kodeerib 4 *CGB* geeni: *CGB, CGB5, CGB7* ja *CGB8*, mis on teineteisega 97–99% ulatuses identse DNA järjestusega. *CGB* geenid on tekkinud samasse geeniklastrisse (19q13.32) kuuluva *LHB* geeni duplitseerumise tulemusena. Lisaks neljale β-alaühikut kodeerivale *CGB* geenile kuulub samasse geeniklastrisse kaks geeni, *CGB*1 ja *CGB2*, mille bioloogiline roll on seni veel teadmata.

Kui *FSHB* ja *LHB* geenides on kirjeldatud üksikuid üliharuldasi mutatsioone, mis kõik seonduvad olulise viljakuse languse ja/või soolise arengu häirumisega, siis α-alaühiku geenis ega ka *CGB* geenides pole selliseid mutatsioone seni kirjeldatud. Tõenäoliselt on kõrvalekalded neis geenides seotud embrüo hukkumisega raseduse väga varases staadiumis (Themmen ja Huhtaniemi 2000).

Töö eesmärk ja ülesanded

Doktoritöö eesmärgiks oli hinnata inimese kooriongonadotropiini β-alaühikut kodeerivate geenide varieeruvuse ja ekspressiooni rolli korduvate raseduse katkemiste patogeneesis.

Lähtudes eesmärgist püstitati järgnevad töö ülesanded:

1) määrata iga *HCGβ* geeni panus hormooni β-alaühiku sünteesis trofoblasti koes;
2) määrata kõigi kuue *CGB* geeni ekspressiooni tase trofoblasti koes normaalse raseduse ajal ning esimese trimestri patoloogiate: korduv raseduse katkemine, emakaväline rasedus ja moolraseduse, korral;
3) võrrelda *HCGβ* geenide transkriptsiooni taset trofoblasti koes ning HCG kontsentratsiooni ema vereseerumis normaalse ja tüsistunud raseduse korral;
4) määrata *CGB* geenide ekspressioonitase teistes inimese normaalsetes kudedes (v.a trofoblast);
5) kirjeldada enim ekspresseeritud *HCGβ* geenide varieeruvus ning leida korduva raseduse katkemisega seonduvad geeni variandid.

Kasutatud uurimismetoodikad ja tulemused

CGB geenide ekspressioon

CGB geenide ekspressiooni taseme hindamiseks koguti bioloogiline materjal järgnevalt:

A. Trofoblasti kude ja ema seerum:
   I Normaalne rasedus:
      1. trimester (4–12 näd., n=10);
      2. trimester (17–21 näd., n=8);
      3. trimester (38–42 näd., n=12)
   II Tüsistunud rasedus:
      korduv raseduse katkemine (≥2 eelnenud katkemist; 6–17 näd., n=11);
      emakaväline rasedus (6–14 näd., n=8);
      moolrasedus (9–10 näd., n=2)

B. Muud koed:
   Blastotsüst (n=6, Nova Vita kliinik); ajukoor, maks, skeletilihas, neer, pankreas, põrn, peensool (n=3, TÜ Kliinikumi koepank:); munand (n=5, TÜ Androloogiakeskus, dr. M. Punab); kops, tüümus (n=5, TÜ Kopsukliinik, dr. T.Vooder)

CGB geenide ekspressiooni hindamiseks kasutasime kahte meetodit:

1) semikvantitatiivset RT-PCR meetodit kombineeritult restriktsiooni ja elektroforeesil põhineva Gene Scan fragmentanalüüsiga. Meetod võimaldas määrata spetsiifiliselt iga 4 *HCGβ* alaühikut kodeeriva geeni transkriptoorset aktiivsust.

Kõige levinum ekspressioonimuster raseduse esimesel trimestril oli *CGB8* > *CGB5* ≈ *CGB* >> *CGB7,* kolmandal trimestril oli *CGB8* ja *CGB5* transkriptsiooni tase ligikaudu võrdne. Kaks enim ekspresseeritud geeni, *CGB8* ja *CGB5* moodustasid ~70±7,8% (vahemik 56–85%) kogu hormooni β-alaühiku transkriptidest.

2) reaal-aja RT-PCR meetodit, mis võimaldas oluliselt täpsemalt määrata kõikide *CGB* geenide summaarse ja eraldi *CGB1/CGB2* ekspressiooni taseme nii trofoblasti koes kui ka teistes kudedes.

Kõikide *CGB* geenide (sh. *CGB1/CGB2*) transkriptsioon oli tuvastatav mõlema meetodi abil juba väga varases raseduse järgus, blastotsüsti staadiumis. Emakasisese raseduse korral korreleerus *CGB* geenide ekspressiooni tase trofoblasti/platsenta koes HCG kontsentratsiooniga ema seerumis ($r=0,495$, $p<0,002$) olles kõrgeim normaalse raseduse I trimestril. Raseduse II ja III trimestril *CGB* geenide ekspressioon vähenes. Võrreldes normaalse rasedusega oli sama gestatsiooniajaga KRK-st pärit trofoblasti koes *CGB* geenide transkriptsioon oluliselt madalam ($p=0,03$) viidates asjaolule, et madal hormooni tase on tingitud *HCGβ* geenide mRNA sünteesi vähesusest. Nii moolraseduse kui ka ER korral jäi *CGB* geenide ekspressiooni tase normaalse rasedusega samale tasemele või ületas seda pisut. ER korral esinev lahknevus: madal seerumi

HCG, kuid kõrge *HCGβ* geenide transkriptsiooni tase trofoblasti koes võib olla tingitud häiretest tagasiside- ja/või transkriptsioonijärgsetes hormooni sünteesi mehhanismides.

CGB geenid on vähesel määral ekspresseeritud mitmes ekstraplatsentaarses koes. Suhteliselt rohkem, kuid siiski ~10000 korda vähem kui raseduse esimesel trimestril transkribeeriti *CGB* geene munandis, eesnäärmes, tüümuses, skeletilihases ja kopsus. Munasarjas, peen- ja jämesooles, neerus, põrnas, maksas, südames, ajus oli *CGB* ekspressioonitase veelgi madalam.

Kuigi *CGB1* ja *CGB2* DNA järjestus on 85% ulatuses identne HCG β-alaühikut kodeerivate geenidega, on nende geenide 5'upstream-piirkonda integreerunud lisalõik (736 nukleotiidi *CGB1-s*, 724 nukleotiidi *CGB2-s*). See võimaldas spetsiifiliselt amplifitseerida ja analüüsida nende kahe β–alaühikut mittekodeeriva geeni ekspressiooni. *CGB1* and *CGB2* geenide transkriptsiooni tase korreleerus summaarse *CGB* ekspressiooniga, kuid oli oluliselt väiksem kui neljal *HCGβ* geenil. Normaalse raseduse I trimestril moodustasid *CGB1/CGB2* transkriptid ~1/2000 ning II ja III trimestril vaid ~1/20000 kõikidest *CGB* transkriptidest. Munandis leidus *CGB1/CGB2* transkripte ligikaudu samaväärsel tasemel kui normaalse raseduse I trimestri trofoblasti koes andes üle poole kõikidest munandi koe *CGB* transkriptidest.

HCGβ geenide varieeruvuse seos korduva raseduse katkemisega

Kahe kõige enam ekspresseeritud *HCGβ* geeni, *CGB8* ja *CGB5* varieeruvuse seotuse hindamiseks KRK-ga resekveneerisime mõlemad geenid 109 KRK-ga naisel ning 75 mehel, kelle naispartneril oli olnud KRK (kokku 184 patsienti) ning 195 viljakal naisel. Uuritud indiviidid pärinesid Eestist (n=194) ja Soomest (n=185) ning olid kogutud järgnevate kriteeriumite alusel: patsientidel oli anamneesis vähemalt 3 raseduse katkemist ning neil polnud leitud tsütogeneetilisel uuringul kromosoomianomaaliaid; kontrollidel oli vähemalt 1 (Soome valimis) või 3 (Eesti valimis) normaalset rasedust ja ei ühtki raseduse katkemist. Mõlema partneri kaasamine KRK-ga patsientide gruppi oli vajalik, sest HCG tootmist kodeerivad loote geenid, mis on pärit mõlemalt vanemalt. Andmete statistiliseks analüüsiks kasutasime Cochran-Armitage trend-testi.

Vaid 23 polümorfismi kõikidest leitud 71-st (32%) olid varasemalt kirjeldatud avalikes andmebaasides. 41(58%) polümorfismi esines mõlemas populatsioonis, 15 harva polümorfismi (üksikleid või minoorse alleeli sagedus <2%) esines vaid ühes valimis.

CGB5 viie polümorfismi (c5EF-155, c5EF-147, c5EF-144, c5EF-142, c5EF1038) osas ilmnes minoorse alleeli kandjatel KRK-e suhtes kaitsev efekt (šansside suhe kuni 0,53–0,58, p=0,007–0,024). Minoorset alleeli esines oluliselt sagedamini viljakatel naistel (12,05%–14,36%) kui KRK-ga patsientidel (7,10%–8,15%). Sama tulemuse andis ka ainult naispatsientide võrdlemine kontrollidega (šansside suhe kuni 0,45–0,59, p=0,006–0,059).

CGB8 geeni II intronis leidus üks kaitsva efektiga polümorfism (c5EF1045), mis paiknes samas positsioonis *CGB5* introni polümorfismiga. *CGB8* polümorfism esines vaid 1 KRK-ga patsiendil ja 7 kontrollisikul (p=0,042). Lisaks leidsime patsientide hulgas 2 aminohappe muutust põhjustavat polümorfismi *CGB8*-s (p.Arg28Trp, p.Pro93Arg) ning ühe *CGB5*-s (p.Val76Leu).

Võrreldes *CGB5*-iga, oli *CGB8*-s enam kui poole vähem polümorfisme (vastavalt 49 ja 22 polümorfismi), milles viimase promootorpiirkonnas paiknes vaid kolm SNP-d. Üks polümorfismidest (c8EF-4) asus transkriptsiooni initsiaatorelemendi seondumiskoha vahetus läheduses ning esines vaid kolmel KRK-ga patsiendil. On tähelepanuväärne, et kõigi uuritavate hulgas ei leidunud ühtki indiviidi, kes kandsid korraga kahe ülejäänud *CGB8* promootorpiirkonna polümorfismi minoorse alleeli. Arvestades polümorfismide sagedusi (c8EF-287, MAF 25,2 % ja c8EF-186 MAF 39,7%) oleks selliseid indiviide pidanud olema ~10%. Mõlemad polümorfismid paiknevad transkriptsioonifaktorite Sp1/AP2 seondumispiirkonnas. Seega, *CGB8* ning eriti selle promootorpiirkonna vähene varieeruvus rõhutab tema olulisust HCG β-alaühiku transkriptsioonil.

Järeldused

1. *CGB* geenide ekspressiooni tase on indiviiditi ja geeniti laiades piirides varieeruv. HCG β-alaühikut kodeerivad geenid ei ole võrdselt aktiivsed, sagedaseim ekspressioonimuster on *CGB8*>*CGB5*≈*CGB*>>*CGB7*.
   ~70% kõikidest hormooni β-alaühiku transkriptidest on kodeeritud *CGB8* ja *CGB5* poolt. Seega varieeruvus neis kahes geenis mõjutab raseduse kulgu kõige enam.
2. *CGB* geenide ekspressioon oli tuvastatav juba blastotsüsti staadiumis ning püsis kõrgel tasemel kogu normaalse raseduse jooksul. Võrreldes normaalse rasedusega oli korduva raseduse katkemise (KRK) korral *CGB* geenide transkriptsiooni tase madalam ning emakavälise raseduse ja moolraseduse korral see ei erinenud olulisel määral.
3. HCG tase normaalse raseduse ajal korreleerus β-alaühikut kodeerivate geenide ekspressiooniga trofoblasti koes. KRK korral oli madal nii *HCGβ* geenide ekspressiooni tase koes kui ka hormooni tase ema seerumis. Emakavälise raseduse korral esinev madal hormooni hulk ema veres vaatamata geenide kõrgele ekspressioonitasemele on ilmselt tingitud posttranskriptoorsete mehhanismide häirumisest.
4. Lisaks trofoblasti koele on *CGB* geenid vähesel määral ekspresseeritud mitmes normaalses koes: munandis, eesnäärmes, tüümuses, skeletilihases ja kopsus. *CGB1 ja CGB2* suhteliselt aktiivne transkriptsioon munandis viitab nende geenide võimalikule rollile mehe reproduktiivtraktis.

5. Kuue polümorfismi minoorse alleeli kandjatel (neli *CGB5* promootorpiirkonnas, kaks *CGB5* ja *CGB8* II introni identses positsioonis) on risk korduva raseduse katkemisele kuni 1,8 korda väiksem kui mažoorse alleeli kandjatel. Kõik need kuus polümorfismi esinesid viljakatel naistel sagedamini kui KRK-ga patsientidel. Lisaks esines KRK-ga patsientide hulgas kolm aminohappe muutust põhjustavat polümorfismi (kaks *CGB5*-s ja üks *CGB8*-s) ning üks polümorfism *CGB8* transkriptsiooni initsiaatorelemendi seondumiskoha vahetus läheduses. Seega mitmed harvad variandid *CGB5* ja *CGB8* geenides võivad suurendada KRK-e riski. *HCGβ* geenide suur geneetiline varieeruvus võib põhjustada HCG taseme erinevusi sama raseduse kestuse ja kliinilise seisundiga indiviidide vahel.

Edasised töö suunad ja praktiline väljund

Tuvastatud geenivariandid, mis seonduvad suurenenud või alanenud riskiga korduvaks raseduse katkemiseks võimaldavad jätkata uuringut, et selgitada funktsionaalne seos geeni variandi ning hormooni taseme vahel.

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APPENDIX I

List of SNPs identified in *CGB5* and *CGB8* in Estonian and Finnish sample sets.

SNP code[a]	Posit. from ATG	Allele[b] major/minor Aminoacid change[c]	Minor allele frequency (%) Estonians (n=194)	Finns (n = 185)	population difference. p-value[d]	rs number
c5F-447	-812	T/G	0	S (Co)	0.304	
c5F-399	-764	T/C	0	0.82	0.167	
c5EF-322	-687	T/C	0.52	0.82	0.667	
c5EF-315	-680	T/G	S (Pa)	1.9	0.071	
c5EF-314	-679	C/A	S (Pa)	1.09	0.234	
c5EF-309	-674	C/T	S (Pa)	1.09	0.234	
c5EF-306	-671	T/G,C	S (Pa)	2.45	0.037	
c5EF-291	-656	C/T	21.65	17.66	0.162	rs4801789
c5F-204	-569	A/G	0	1.36	0.051	
c5F-191	-557	T/C	0	1.36	0.051	
c5EF-155	-520	G/C	10.57	9.24	0.543	
c5EF-147	-512	G/del	10.57	8.7	0.373	
c5EF-144	-509	T/C	10.57	10.6	0.989	
c5EF-142	-507	T/A	10.57	10.6	0.989	
c5EF-82	-447	G/A	1.8	1.63	0.853	
c5EF-30	-395	G/C	0.77	1.36	0.429	
c5E-28	-393	C/T	1.55	0	0.016	
c5EF-1	-366	A/G	S (Co)	S (Co)	0.973	
c5E101	-265	C/T	0.52	0	0.166	
c5EF138	-228	A/G	12.11	6.76	0.011	rs710899
c5E157	-209	C/T	0.52(Pa)	0	0.166	
c5F206	-160	C/T	0	S (Co)	0.305	
c5F324	-42	G/A	0	0.54(Co)	0.146	
c5EF345	-21	G/C	22.16	16.76	0.055	rs12610392
c5F354	-12	G/del	0	1.08	0.040	
c5E519	154	G/T	0.52	0	0.166	
c5E525	160	A/G	0.52	0	0.166	rs35621293
c5E527	162	G/A	0.52	0	0.166	
c5E529	164	G/A	S (Pa)	0	0.328	
c5EF544	179	T/G	33.25	25.68	0.024	rs3956245
c5E551	186	C/T	0.77	0	0.089	rs4002422
c5F553	188	T/C	0	0.54(Co)	0.146	
c5EF580	215	G/A	21.65	17.03	0.104	rs34524624
c5F660	295	A/C	0	S (Pa)	0.305	
c5EF666	301	C/T	32.73	25.41	0.027	rs35871536
c5EF789	424	G/A p.Pro24Pro	21.13	17.03	0.148	rs35133942
c5E912	547	G/C	0.77	0	0.089	
c5E918	553	C/G	0.52	0	0.166	rs33933429
c5EF1038	673	C/T	11.86	10.81	0.651	rs34335161
c5EF1069	704	A/G	1.55	2.43	0.376	rs33976607
c5F1111	746	A/T	0	0.54(Co)	0.146	
c5EF1115	750	C/T	3.35	0.54	0.005	rs34935416

SNP code[a]	Posit. from ATG	Allele[b] major/minor Aminoacid change[c]	Minor allele frequency (%) Estonians (n=194)	Finns (n = 185)	population difference. p-value[d]	rs number
c5F1178	813	G/C p.Val76Leu	0	S (Pa)	0.305	
c5EF1258	893	C/T p.Tyr102Tyr	4.64	0.81(Co)	0.001	rs35756580
c5E1390	1025	C/A p.Pro146Pro	S (Co)	0	0.328	
c5EF1402	1037	T/C p.Ser150Ser	S (Co)	S (Pa)	0.973	
c5EF1426	1061	G/A p.Ser158Ser	S (Co)	S (Pa)	0.973	
c5E1501	1136	T/C	S (Pa)	0	0.328	
c5EF1660	1295	A/T	1.29	1.08	0.502	
c8EF-287	-659	T/C	26.49	23.85	0.417	rs4801790
c8EF-186	-558	G/T	40.21	39.08	0.754	rs8102901
c8EF-4	-376	T/A	0.52 (Pa)	S (Pa)	0.627	
c8EF105	-268	G/C	4.12	0.57(Co)	0.003	rs34212754
c8EF108	-265	C/T	39.95	39.08	0.808	rs13345685
c8EF301	-72	T/A	5.41	6.32	0.597	rs35930240
c8EF432	60	A/C	0.52	0.57(Co)	0.913	
c8F461	89	T/C	0	S (Pa)	0.290	
c8EF523	151	G/T	S (Co)	0.86(Pa)	0.264	
c8F526	154	T/G	0	S (Co)	0.290	rs2387591
c8EF541	169	G/C	39.69	39.37	0.928	rs13345575
c8F551	179	G/T	0	S (Co)	0.290	
c8F558	186	T/C	0	S (Co)	0.290	
c8EF673	301	T/C	S (Co)	S (Co)	0.938	
c8E806	434	C/T p.Arg28Trp	S (Pa)	0	0.343	
c8EF869	497	G/A p.Val49Ile	2.32	S (Pa)	0.017	
c8EF925	553	G/C	2.06	3.16	0.341	rs2303050
c8EF1045	673	C/T	0.52	1.72(Co)	0.112	rs33943298
c8EF1076	704	G/A	1.8	2.01	0.836	
c8EF1122	750	T/C	1.8	2.01	0.836	
c8E1237	865	C/G p.Pro93Arg	S (Pa)	0	0.343	
c8E1418	1046	A/T pArg153Arg	S (Pa)	0	0.343	

[a] SNP code includes gene and sample name (e.g.c5=*CGB5*; E=Estonians, F=Finns), location relative to mRNA start site; GenBank references: NM_033043.1 GI:15451747 for *CGB5*, NM_033183.2 GI:146229337 for *CGB8*; non-synonymous changes detected only in patients are underlined;

[b] alleles at the coding strand; [c]coding from ATG including signal protein;

[d] Cochran-Armitage test for trend;

S – singleton SNP; Co – only among fertile women with no miscarriages; Pa – only among RM patients

APPENDIX II

List of the SNPs in *CGB5* and *CGB8* submitted to dbSNP (http://www.ncbi.nlm.nih.gov/SNP/)

c5F-447	105106983
c5F-399	105106984
c5EF-322	105106985
c5EF-315	105106986
c5EF-314	105106987
c5EF-309	105106988
c5EF-306	105106989
c5EF-291	105106990
c5F-204	105106991
c5F-191	105106992
c5EF-155	105106993
c5EF-147	105106994
c5EF-144	105106995
c5EF-142	105106996
c5EF-82	105106997
c5EF-30	105106998
c5E-28	105106999
c5EF-1	105107000
c5E101	105107001
c5EF138	105107002
c5E157	105107003
c5F206	105107004
c5F324	105107005
c5EF345	105107006
c5F354	105107007
c5E519	105107008
c5E525	105107009
c5E527	105107010
c5E529	105107011
c5EF544	105107012
c5E551	105107013
c5F553	105107014
c5EF580	105107015
c5F660	105107016
c5EF666	105107017
c5EF789	105107018
c5E912	105107019
c5E918	105107020
c5EF1038	105107021
c5EF1069	105107022
c5F1111	105107023
c5EF1115	105107024

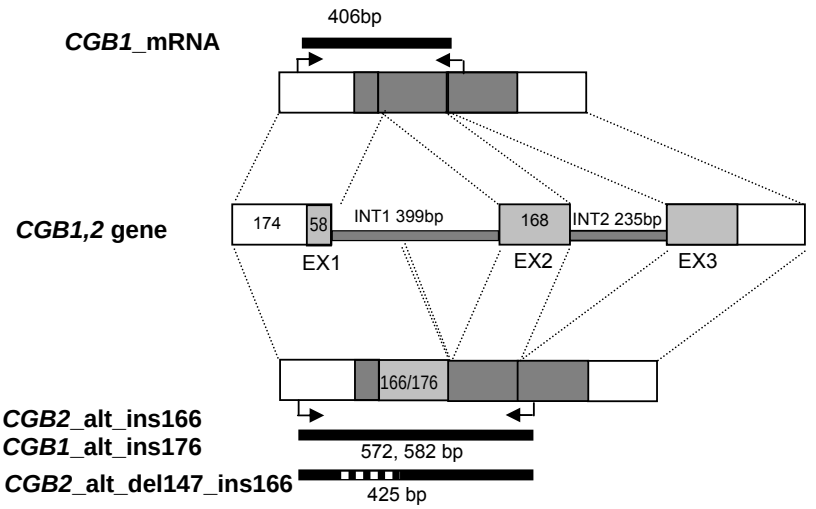
c5F1178	105107025
c5EF1258	105107026
c5E1390	105107027
c5EF1402	105107028
c5EF1426	105107029
c5E1501	105107030
c5EF1660	105107031
c8EF-287	105107032
c8EF-186	105107033
c8EF-4	105107034
c8EF105	105107035
c8EF108	105107036
c8EF301	105107037
c8EF432	105107038
c8F461	105107039
c8EF523	105107040
c8F526	105107041
c8EF541	105107042
c8F551	105107043
c8F558	105107044
c8EF673	105107045
c8E806	105107046
c8EF869	105107047
c8EF925	105107048
c8EF1045	105107049
c8EF1076	105107050
c8EF1122	105107051
c8E1237	105107052
c8E1418	105107053

APPENDIX III

List and sequences of the transcripts of *CGB1* and *CGB2* submitted to GenBank:

CGB1_mRNA 406bp
dbEST Id: 62657544
GenBank Acc: GE745564

GAAGGGGAACTGCATCTGAGAGAGAGCAGCCAATTGGGTCCGCTGACTCGGGCCAGGTT
CCCGTGCCGCGTCCAACACCCCTCACTCCCTGTCTCACTCCCCCACGGAGACTCAATTT
ACTTTCCATGTCCACATTCCCAGTGCTTGCGGAAGATATCCCGCTAAGAGAGAGACATG
TCAAAGGGGCTGCTGCTGTTGCTGCTGCTGAGCATGGGCGGGACATGGGCATCCAAGGA
GCCGCTTCGGCCACGGTGCCGCCCCATCAATGCCACCCTGGCTGTGGAGAAGGAGGGCT
GCCCCGTGTGCATCACCGTCAACACCACCATCTGTGCCGGCTACTGCCCCACCATGACC
CGCGTGCTGCAGGGGGTCCTGCCGGCCCTGCCTCAGGTGGTGTGCAACTACC



CGB2_alt_ins166 572bp
dbEST Id: 2657545
GenBank Acc: E745565

GAAGGGGAACTGCATCTGAGAGAGAGCAGCCAATTGGGTCCGCTGACTCGGGCCAGGTT
CCCGTGCCGCGTCCAACACCCCTCACTCCCTGTCTCACTCCCCCACGGAGACTCAATTT
ACTTTCCATGTCCACATCCCCAGTGCTTGCGGAAGATATCCCGCTAAGAGAGAGACATG
TCAAAGGTAGGGTAGATCGACATTTCCGGGCACCAAAGATGGAGATGTTCCAGGAAAGA
CTGCAGGGCCCCTGGGCACCTTCCACCTGCTTCCAGGCCATCACTGGCATGAGAAGGGG
CAGACCAGTGTGAGCTGTGGAAGGAGGCCTCTTTCTGGAGGAGCGTGACCCCCAGGGCT
GCTGCTGTTGCTGCTGCTGAGCATGGGCGGGACATGGGCATCCAAGGAGCCGCTTCGGC
CACGGTGCCGCCCCATCAATGCCACCCTGGCTGTGGAGAAGGAGGGCTGCCCCGTGTGC
ATCACCGTCAACACCACCATCTGTGCCGGCTACTGCCCCACCATGACCCGCGTGCTGCA
GGGGGTCCTGCCGGCCCTGCCTCAGGTGGTGTGCAACTACC

CGB2_alt_del147_ins166 425 bp
dbEST Id: 62657547
GenBank Acc: GE745567

GAAGGGGAACTGCATCTGAGAGAGAGACATGTCAAAGGTAGGGTAGATCGACATTTCCA
GGCACCAAAGATGGAGATGTTCCAGGAAAGACTGCAGGGCCCCTGGGCACCTTCCACCT
GCTTCCAGGCCATCACTGGCATGAGAAGGGGCAGACCAGTGTGAGCTGTGGAAGGAGGC
CTCTTTCTGGAGGAGCGTGACCCCCAGGGCTGCTGCTGTTGCTGCTGCTGAGCATGGGC
GGGACATGGGCATCCAAGGAGCCGCTTCGGCCACGGTGCCGCCCCATCAATGCCACCCT
GGCTGTGGAGAAGGAGGGCTGCCCCGTGTGCATCACCGTCAACACCACCATCTGTGCCG
GCTACTGCCCCACCATGACCCGCGTGCTGCAGGGGTCCTGCCGGCCCTGCCTCAGGTGG
TGTGCAACTACC

CGB1_alt_ins176 582 bp
dbEST Id: 62657546
GenBank Acc: GE745566

GAAGGGGAACTGCATCTGAGAGAGAGCAGCCAATTGGGTCCGCTGACTCGGGCCGGGTT
CCCGTGCCGCGTCCAACACCCCTCACTCCCTGTCTCACTCCCCCACGGAGACTCAATTT
ACTTTCCATGTCCACATCCCCAGTGCTTGCGGAAGATATCCCGCTAAGAGAGAGACATG
TCAAAGGTAGGGTAGATCGACATTTCCGGGCACCAAAGATGGAGATGTTCCAGGAAAGA
CTGCAGGGCCCCTGGGCACCTTCCACCTCCTTCCAGGCCATCACTGGCATGAGAAGGGG
CAGACCCGTGTGAGCTGTGGAAGGAGGCCTCTTTCTGGAGGAGCGTGACCCCCAGTAAG
CTTCAAGGCTGCTGCTGTTGCTGCTGCTGAGCATGGGCGGGACATGGGCATCCAAGGAG
CCGCTTCGGCCACGGTGCCGCCCCATCAATGCCACCCTGGCTGTGGAGAAGGAGGGCTG
CCCCGTGTGCATCACCGTCAACACCACCATCTGTGCCGGCTACTGCCCCACCATGACCC
GCGTGCTGCAGGGGGTCCTGCCGGCCCTGCCTCAGGTGGTGTGCAACTACC

PUBLICATIONS

CURRICULUM VITAE

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Education

1979–1990	Tartu High School No.5, *cum laude*
1990–1996	University of Tartu, Faculty of Medicine, *cum laude*
1996–1998	Internship in General Medicine, University of Tartu
1998–2003	Residency in Obstetric and Gynecology, Women's Clinic of Tartu University Hospitals
2003–2009	PhD studies, University of Tartu

Professional employment

1991–1996	Nurse-anaesthetist, Tartu Lung Clinic
1997–2002	Pharmaceutical Information Assistant, Magnum Medical AS
2003–2004	Gynecologist-obstetrician, Rakvere County Hospital
2004–2006	Consulting gynecologist, Nova Vita Centre of Infertility Treatment and Medical Genetics
2004–2007	Researcher, Department of Obstetrics and Gynecology, University of Tartu
2003–	Gynecologist-obstetrician; Women's Clinic of Tartu University Hospitals
2007–	Research assistant, Institute of Molecular and Cell Biology, University of Tartu

Academic interests

The scientific work has been focused on the following areas:

1. Recurrent miscarriage: genetic factors, clinical management.
2. Role of the genetic variation and expression patterns of the genes encoding placental and pituitary hormones in human reproductive success.
3. Insulin resistance and hyperinsulinemia in reproductive failure, possible autoimmune mechanisms related to insulin resistance.

List of publications

1. Rull K, Nagirnaja L, Ulander VM, Kelgo P, Margus T, Kaare M, Aittomäki K, Laan M. Chorionic Gonadotropin Beta gene variants are associated with recurrent miscarriage in two European populations. *J Clin Endocrinol Metab*. 2008; 93(12):4697–4706.

2. Rull K, Hallast P, Uusküla L, Jackson J, Punab M, Salumets A, Campbell R, Laan M. Fine scale quantification of HCG beta gene transcription in human trophoblastic and non-malignant non-trophoblastic tissues. *Mol Hum Reprod*. 2008; 14(1):23–31.

3. Lazarus JV, Rull K, Wyn Huws D, Rasch V, Liljestrand J. A survey of midwives' views on providing aspects of antenatal care in Estonia. *Midwifery*. 2008; 24(4):399–404.

4. Hallast P, Rull K, Laan M. The evolution and genomic landscape of CGB1 and CGB2 genes. The evolution and genomic landscape of CGB1 and CGB2 genes. *Mol Cell Endocrinol*. 2007; 260–262:2–11.

5. Grigorova M, Rull K, Laan M. Haplotype structure of FSHB, the beta-subunit gene for fertility-associated follicle-stimulating hormone: possible influence of balancing selection. *Ann Hum Genet.* 2007; 71(Pt 1):18–28.

6. Rull K, Laan M Expression of beta-subunit of HCG genes during normal and failed pregnancy. *Hum Reprod*. 2005; 20(12):3360–3368.

7. Haller K, Mathieu C, Rull K, Matt K, Béné MC, Uibo R. IgG, IgA and IgM antibodies against FSH: serological markers of pathogenic autoimmunity or of normal immunoregulation? *Am J Reprod Immunol.* 2005; 54:262–269.

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Haridus

1979–1990	Tartu 5. Keskkool, kuldmedal
1990–1996	Tartu Ülikool, arstiteaduskond, ravi eriala *cum laude*
1996–1998	Tartu Ülikool, üldarstlik internatuur
1998–2003	Tartu Ülikool, residentuur sünnitusabi ja günekoloogia erialal
2003–2009	Tartu Ülikool, doktorantuur

Teenistuskäik

1991–1996	õde-anestesist, Tartu Kopsukliinik
1997–2002	raviminfo töötleja, Magnum Medical AS
2003–2004	valvegünekoloog, AS Rakvere Haigla
2004–2006	günekoloog-konsultant, Nova Vita lastetuse ja meditsiinigeneetika uuringute keskus
2004–2007	sünnitusabi ja günekoloogia teadur, Tartu Ülikool
2003–	arst-õppejõud sünnitusabi ja günekoloogia erialal SA Tartu Ülikooli Kliinikumi Naistekliinik
2007–	spetsialist, TÜ Molekulaar- ja Rakubioloogia Instituut

Teadustöö

Teadustöö on olnud seotud järgmiste valdkondadega:

1. Korduv raseduse iseeneslik katkemine: pärilikud tegurid, kliiniline diagnostika ja ravi.
2. Platsenta ja ajuripatsi hormoonide taset määravate geenide varieeruvuse ning ekspressiooni seos inimese reproduktiivse edukusega.
3. Insuliinresistentsuse ja hüperinsulineemia roll polütsüstiliste munasarjade sündroomi patogeneesis, võimalikud seosed autoimmuunhäiretega.

Publikatsioonid

1. Rull K, Nagirnaja L, Ulander VM, Kelgo P, Margus T, Kaare M, Aittomäki K, Laan M.Chorionic Gonadotropin Beta gene variants are associated with recurrent miscarriage in two European populations. *J Clin Endocrinol Metab*. 2008; 93(12):4697–4706.

2. Rull K, Hallast P, Uusküla L, Jackson J, Punab M, Salumets A, Campbell R, Laan M. Fine scale quantification of HCG beta gene transcription in human trophoblastic and non-malignant non-trophoblastic tissues. *Mol Hum Reprod*. 2008; 14(1):23–31.

3. Lazarus JV, Rull K, Wyn Huws D, Rasch V, Liljestrand J. A survey of midwives' views on providing aspects of antenatal care in Estonia. *Midwifery*. 2008; 24(4):399–404.

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5. Grigorova M, Rull K, Laan M.. Haplotype structure of FSHB, the beta-subunit gene for fertility-associated follicle-stimulating hormone: possible influence of balancing selection. *Ann Hum Genet*. 2007; 71(Pt 1):18–28.

6. Rull K, Laan M Expression of beta-subunit of HCG genes during normal and failed pregnancy. *Hum Reprod*. 2005; 20(12):3360–3368.

7. Haller K, Mathieu C, Rull K, Matt K, Béné MC, Uibo R. IgG, IgA and IgM antibodies against FSH: serological markers of pathogenic autoimmunity or of normal immunoregulation? *Am J Reprod Immunol.* 2005; 54:262–269.

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