A modest but significant effect of CGB5 gene promoter polymorphisms in modulating the risk of recurrent miscarriage

Kristiina Rull, M.D., Ph.D., a,b Ole Bjarne Christiansen, M.D., Ph.D., c,d Liina Nagirnaja, M.Sc., a
Rudi Steffensen, Ph.D., a Tõnu Margus, M.Sc., f,g and Maris Laan, Ph.D. a

a Human Molecular Genetics Research Group, Department of Biotechnology, Institute of Molecular and Cell Biology, and b Department of Obstetrics and Gynecology, University of Tartu, Tartu, Estonia; c The Fertility Clinic, University Hospital Copenhagen, Rigshospitalet, Copenhagen, Denmark; d Department of Obstetrics and Gynecology, and e Department of Clinical Immunology, Aalborg Hospital, Aalborg, Denmark; f Department of Bioinformatics, Institute of Molecular and Cell Biology, University of Tartu; and g Estonian Biocenter, Tartu, Estonia

Objective: To confirm the effect of single nucleotide polymorphisms (SNPs) in chorionic gonadotropin beta (CGB) genes in modulating the susceptibility to recurrent miscarriage (RM) in Danes and in a meta-analysis across Danes and the discovery samples from Estonia and Finland.

Design: Case-control association study, restriction fragment length polymorphism genotyping, resequencing.

Setting: Fertility clinics at the Rigshospitalet, Copenhagen, and Aalborg Hospital, Aalborg, Denmark.

Patient(s): Four hundred fifty Danish women and men from couples with RM and 119 women with children and no miscarriages in a new study. A total of 634 women and RM couples and 314 female controls in a combined study of Estonians, Finns, and Danes.

Intervention(s): None.

Main Outcome Measure(s): Distribution of CGB5 and CGB8 allele and haplotype frequencies in patients and controls.

Result(s): For the majority of studied SNPs, the allelic and haplotypic distribution differed statistically between the Danish and the previous Estonian-Finnish sample. In Danes, two CGB5 promoter SNPs (c5–155; c5–142) exhibited a nonsignificant trend for higher allele frequency in fertile women compared with RM patients. The meta-analysis of results from three populations confirmed a modest but significant effect on carriage of c5–155C (odds ratio = 0.64; 95% CI 0.44–0.94) and c5–142A (odds ratio = 0.66; 95% CI 0.45–0.94) variants in reducing the risk of RM. None of the investigated genetic variants in the CGB8 gene was associated with RM.

Conclusion(s): Carriage of particular variants in the promoter of the CGB5 gene seems to protect against RM. No common genetic variants in CGB5 and CGB8 were associated with increased RM susceptibility in the studied North European populations.

Key Words: Recurrent miscarriage, hCG beta coding genes, association study, CGB5 promoter polymorphisms

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/rullk-cgb5-promoter-polymorphisms-recurrent-miscarriage/

Received November 26, 2012; revised and accepted February 8, 2013; published online March 15, 2013.

K.R. has nothing to disclose. O.B.C. reports expenses paid to travel to Estonia to discuss results of this study; he also reports travel and accommodation paid by European Society of Human Reproduction and Embryology (ESHRE) in connection with meetings relating to activity as an executive committee member of the ESHRE early pregnancy special interest group. L.N. has nothing to disclose. R.S. has nothing to disclose. T.M. has nothing to disclose. M.L. has nothing to disclose.

This work was supported by the grants to M.L.: Wellcome Trust International Senior Research Fellowship in Biomedical Science in Central Europe (070191/Z/03/A), Estonian Science Foundation grant ETF7471, ETF9030, and Estonian Ministry of Education and Science core grant (SF018002212). K.R. has been personally supported by an Estonian Women in Science Award financed from European Commission grant no. 205419 (ECOGENE) to Estonian Bio-centre. T.M. has been supported by grant SF0180026s09 from the Estonian Ministry of Education and Research and by the European Union through the European Regional Development Fund through the Estonian Centre of Excellence in Genomics.

Reprint requests: Maris Laan, Ph.D., Department of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu, Riia St. 23, 51010 Tartu, Estonia (E-mail: maris.laan@ut.ee).

Fertility and Sterility® Vol. 99, No. 7, June 2013 0015-0282/$36.00
Copyright ©2013 American Society for Reproductive Medicine, Published by Elsevier Inc. Open access under CC BY license.
http://dx.doi.org/10.1016/j.fertnstert.2013.02.019
In human pregnancy, the production of hCG, a placental hormone, is indispensable. Its classical function is considered to maintain the production of steroid hormones in the corpus luteum. In addition, hCG enhances blastocyst implantation, uterine vascularization, and angiogenesis, as well as regulates maintenance of uterine quiescence and immunological adaptation during pregnancy (1–3). Low levels of hCG during the first trimester of pregnancy are related to miscarriage and extrauterine pregnancy (4–6). Abnormal circulating levels of hCG and alterations in the hormone’s glycosylation patterns have been described in several pathologies (trisomies, gestational trophoblastic diseases, malignant tumors, etc.) and implied in clinical diagnostics (3, 7, 8).

A clinical condition that may develop from low hCG is recurrent miscarriage (RM), defined as three or more consecutive pregnancy losses before 22 gestational weeks (9). Apart from the known risk factors for RM (parental chromosomal anomalies, maternal thrombophilic, anatomical, endocrine, or immunological disorders), >50% of the RM cases remain classified as idiopathic (10). As the prevalence of miscarriage among the first-degree relatives of the women with RM is increased (11), a notable fraction of unexplained RM cases is expected to represent carriers of genetic risk factors involved in RM pathogenesis. Due to an irreplacable role of hCG in normal gestation, genetic variants in genes encoding hCG subunits may affect gene expression and consequently the optimal levels of hormone production as well as pregnancy success.

HCG is a heterodimeric glycoprotein consisting of two dissimilar subunits, α and β. The α-subunit gene is shared among gonadotropins (hCG, LH, FSH) and TSH, whereas the β-subunit is hormone specific. In humans, the β-subunit of hCG is coded by four duplicated and highly homologous (97%–99% DNA identity) chorionic gonadotropin beta (CGB) genes (12–15). All CGB genes encode identical hCG β-subunit proteins, which are critical to the level of intact circulating hCG (16). Still, the transcriptional activity among gene duplicates varies greatly, and there is also a large interindividual variation in the hCGbeta transcript levels (6, 17, 18). The majority, up to 82%, of the total pool of hCGbeta transcripts is provided by two genes, CGB8 and CGB5 (6, 18).

We have recently conducted a clinical resequencing study of CGB5 and CGB8 genes among Estonian and Finnish patients with RM and fertile controls (19). The study identified three rare variations in the protein-coding exons resulting in amino acid changes in the hCG-beta protein (Val56Leu in CGB5; Arg8Trp and Pro73Arg in CGB8), and they may therefore be potential risk factors for the occurrence of RM. The subsequent detailed functional and structural analysis of these mutations concluded that only substitutions with neutral or mild functional consequences for hCG action might be tolerated in the major hCG-beta coding genes CGB5 and CGB8 (20). Additionally, the resequencing described six single nucleotide polymorphisms (SNPs) in the CGB5 and CGB8 genes located outside the exons with significantly lower frequency among RM patients compared with the control group and thus exhibiting a protective effect towards RM (19). These polymorphisms included four linked SNPs (c5-155G→C/c5-147G→del/c5-144T→C/c5-142T→A) in the upstream of the CGB5 gene (up to 350 bp relative to mRNA start site), which form the two main CGB5 promoter haplotypes that are composed of the combination of either major or minor alleles of these SNPs (Fig. 1). Association with RM susceptibility was also detected for two intronic SNPs in the CGB5 (c5+1038C→T) and CGB8 genes (c8+1045C→T) (Fig. 1).

This study aimed [1] to confirm the effect of the CGB5 (c5-155G→C, c5-142T→A, c5+1038C→T) and CGB8 (c8+1045C→T) polymorphisms on the susceptibility to RM by genotyping an independent sample set from Denmark and by an extended meta-analysis across the three study populations (Estonians, Finns, Danes); [2] to resequence the promoter region of the most actively transcribed hCG-beta-coding gene CGB8 in the Danish RM cases and controls to discover novel potential genetic risk variants to RM. The meta-analysis confirmed a modest but significant effect of the CGB5 promoter variants c5-155C and c5-142A in reducing the risk to RM. Other investigated SNPs in the CGB5 and CGB8 genes exhibited no effect on RM susceptibility.

SUBJECTS AND METHODS

Study Subjects

Subjects recruited in the study were admitted to the Fertility Clinic, Rigshospitalet, Copenhagen, and the Department of Obstetrics and Gynaecology, Aalborg Hospital, Aalborg, from all over Denmark for investigation and treatment. The study sample set included 450 Caucasian patients diagnosed with RM (three or more pregnancy losses confirmed by the hospital records). The group of Danish idiopathic RM cases consisted of 199 couples and 52 single female patients. Because maternally and paternally derived gene variants contribute equally to the function of the fetal genome in placenta, the patient group included both the women and their partners who had experienced RM. In the Estonian-Finnish discovery study (19) as well as in the current Danish follow-up study, the control group was designed under the assumption that fertile women with no history of miscarriage are carrying gene variants supporting successful pregnancies. The male partners were not investigated among the control group because detailed reliable information on their reproductive history is challenging to collect. The Danish control group comprised 119 Caucasian age-matched fertile women from couples with no history of miscarriage and at least two normal pregnancies. None of the recruited female patients had uterine abnormalities found by hysteroscopy, uterine hydrosonography, or hysterosalpingography, and all RM patients and their husbands had normal karyotypes. All women were regularly menstruating with a cycle length of <35 days, and all had normal plasma thyroxin levels (detailed in [21, 22]). The study was approved by the Ethics Committees of the Capital Region, Denmark.

The subsequent meta-analysis combined the Danish data set from the current study with the discovery data of the Estonian-Finnish sample (19). The patient group of unexplained RM comprised 35 couples and 29 single female
patients from Estonia and 40 couples and five single female patients from Finland. For the RM patients, the recruitment criteria in the three study centers were identical. The Estonian–Finnish control group was formed from age-matched fertile women with no history of miscarriage and consisted of 95 Estonians and 100 Finns (19). The definition of fertile female controls in the discovery study was based on at least one (Finnish) or three (Estonian) successful deliveries (the detailed description is in reference 19).

**Genotyping and Resequencing**

DNA was extracted from peripheral blood using an in-house protocol or Puregene DNA Isolation Kit (Gentra Systems), which are both based on the salting-out method for DNA extraction. The CGB5 (~1.7 kb fragment) and CGB8 (long-range polymerase chain reaction [PCR] ~8.3 kb; nested PCR ~2.5 kb fragment) genomic regions were amplified using previously described primers and PCR conditions (15, 19) (Fig. 1A, Supplemental Table 1).

As the four SNPs (c5-155G→C; c5-142G→T; c5-144T→C; c5-142T→A) forming the alternative CGB5 promoter variants are in strong linkage disequilibrium (LD) (r² = 0.9–1.0; Fig. 1A), only two of them (c5-155, rs72553901; c5-142, rs72553901) were selected for genotyping as the marker SNPs for the major CGB5 promoter haplotypes. For these two polymorphisms capturing the core CGB5 promoter variation and for the two intronic SNPs located at the identical position within CGB5 (c5-1038, rs4802541) and CGB8 (c8+1045, rs4802541), the genotypes were assessed by restriction fragment length polymorphism (RFLP) analysis. Detailed information and restriction analysis scheme are shown in Supplemental Figure 1 and Supplemental Table 2.

The 5’-upstream gene regulatory region of CGB8 was subjected to full resequencing, covering from 350 bp upstream relative to the mRNA start site to the end of exon 1 (at +400 bp). Primer design for the additional PCR amplification and sequencing primers was implemented using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/ primer3 www.cgi). Sequences were resolved using ABI 3730 XL DNA Analyzer (Applied Biosystems) and analyzed by the Phred, Phrap and Consed package (23), which facilitates base calling from sequencing trace files, sequence quality assessment, and assembly. Polymorphisms were identified using the PolyPhred program (ver. 6.02.) (24) and confirmed by manual checking. A genetic variant was called only if it was observed in both forward and reverse orientations. The nomenclature of the polymorphisms was based on the
following GenBank reference sequences: NM_033043.1 Gl:15451747 for CGB5; NM_033183.2, Gl:146229337 for CGB8.

Data Analysis

Allele frequencies were estimated, and conformance to Hardy-Weinberg equilibrium (HWE) in the full sample as well as in patient and control subgroups was calculated by Fisher’s exact test implemented in the GenoPOP software package (http://genepop.curtin.edu.au/index.html) (25). The statistical tests for population differentiation comparing allele and genotype frequencies of all studied SNPs among the three populations (Danish, Estonian, Finnish) were performed using GenoPOP (25).

Association with the diagnosis of RM as a binary trait was assessed by the Cochran–Armitage test for trend. Association tests and calculation of LD between SNP pairs (r²) were performed with the PLINK software, version 1.04 (http://pngu.mgh.harvard.edu/~purcell/plink/). The LD r²-statistic represents the square of the correlation coefficient between the alleles at addressed loci.

For a meta-analysis including data from three recruitment centers, the inverse-variance method was implemented under a fixed-effects model using R, version 2.7.2 (R Development Core Team, http://www.r-project.org/). Odds ratios (OR) with 95% confidence intervals (CI) were calculated to show the strength and direction of the association. P < .05 was considered statistically significant.

Haplotypes within the resequenced region of CGB8 (−350 bp to +400 bp relative to mRNA start) were determined based on all but singleton SNPs. Singleton polymorphisms carried in heterozygous status by one single individual were excluded from haplotype calculations as their location on either of the chromosomes cannot be reliably phased. Haplotypes were inferred from unphased genotype data using the Bayesian statistical method in the program PHASE 2.1.1 (http://www.stat.washington.edu/stephens/), applying the model allowing recombination (26). The running parameters were number of iterations = 1,000, thinning interval = 1, and burn-in = 100; the −10 parameter was used for increasing the number of iterations of the final run of the algorithm. The relationship between inferred haplotypes was analyzed with NETWORK 4.6.1.0. software (http://www.fluxus-technology.com) using the Median-Joining network algorithm (27). Haplotype networks for CGB8 were calculated using SNPs covering the promoter region up to the end of the first exon.

## TABLE 1

| Polymorphisms identified in CGB5 and CGB8 in the Danish sample set in comparison with individuals from Estonia and Finland. | MAF (%) in sample set |
|---|---|
| SNP, relative to mRNA start site | Allele major/minor | Danish (n = 569) | Estonian/Finnish (n = 379) | P for population comparison |
| **Genotyping data** |  |  |  |  |
| CGB5 |  |  |  |  |
| c5−155 | G/C | 5.94 | 9.92 | .001 |
| c5−142 | T/A | 5.94 | 10.58 | <.001 |
| c5−1038 | C/T | 7.45 | 11.38 | .004 |
| CGB8 |  |  |  |  |
| c8−1045 | C/T | 0.52 | 1.09 | .137 |
| **Resequencing data (from −350 bp to +400)** |  |  |  |  |
| CGB8 |  |  |  |  |
| c8−287 | T/C | 29.97 | 25.21 | .021 |
| c8−226 | A/del | 1.16 | 0 | N/A |
| c8−196 | G/A | 3.23 | 0 | N/A |
| c8−186 | G/T | 26.61 | 39.67 | <.001 |
| c8−4 | T/A | 0 | 0.41(Pa) | N/A |
| c8−105 | G/C | 3.23 | 2.45 | <.001 |
| c8−108 | C/T | 26.10 | 39.54 | .004 |
| c8−135 | G/A | 5.94 | 39.67 | <.001 |
| c8−276 | G/C | 5.94 | 39.54 | <.001 |
| c8−301 | T/A | 3.23 | 5.84 | .021 |

Note: N/A = not applicable.

a An SNP code includes gene name and position of the polymorphism relative to mRNA transcription start according to GenBank reference: NM_033183.2 Gl:146229337 for CGB8.

b Alleles at the coding strand.

c Data from discovery study (19).

d The full genotyped sample comprising females and males from couples with RM and fertile female controls; allelic distribution of all investigated CGB5 and CGB8 polymorphisms was in HWE in the full samples as well as in the subsamples of RM patients and controls. S: singleton SNP carried by one heterozygous individual; Pa: detected only among RM patients; Co: detected only among fertile controls with no miscarriages.

Rull. CGB5 promoter haplotype and recurrent miscarriage. Fertil Steril. 2013.

RESULTS

Frequencies of CGB5 and CGB8 SNPs and Haplotypes Vary among North Europeans

The CGB5 SNPs subjected to genotyping by RFLP (promoter: c5−155, c5−142; intron II: c5+1038; Fig. 1A) exhibited significantly (Fisher’s exact test, P ≤ .002) lower allele frequency in Danes (n = 569; minor allele frequency [MAF], 5.94%, 5.94%, and 7.45%, respectively), compared with the published Estonian–Finnish sample (9.92%, 10.58%, and 11.38%, respectively; Table 1) (19). Within the resequenced region of CGB8, the allele frequencies of common SNPs (MAF > 1%, c8−287, c8−186, c8+108; Fig. 1B) also differed significantly among the study samples (P < .05; Table 1). The genotyped
SNP in CGB8 intron II (c8+1045) was rare among Estonians-Finns (MAF 1.09%) and Danes (MAF 0.52%).

Among North Europeans, the resequenced CGB8 gene regulatory region is represented by three core haplotypes—H2, H8, and H11—determined by the allelic combinations of the two unlinked (LD $r^2 = 0.16–0.23$) common polymorphisms, c8–287 and c8–186 (Fig. 1B, Fig. 2A). In total, approximately 91% of individuals in the Danish and Estonian–Finnish study samples carried the H2, H8, or H11 core haplotypes, although their distribution was statistically different among populations ($P<.002$; Supplemental Table 3). Notably, the position c8–186 is in strong LD ($r^2>0.8$) with the SNP c8+108 located in 5’UTR of CGB8 exon 1 (Fig. 1B). It is also noteworthy that haplotype c8–287C/c8–186T combining the minor alleles of these SNPs was missing among the genotyped individuals (n = 948), although the expected carrier frequency estimated from the observed allele frequencies is ~9%.

**Susceptibility to RM Is Modulated by CGB5 Promoter Polymorphisms**

In the Danish sample set, both genotyped SNPs in the CGB5 promoter region (c5–155; c5–142) exhibited a higher minor allele frequency in Danish fertile women (n = 119; MAF 7.14%) compared with RM patients (n = 450; 5.62%). However, the difference was not statistically significant ($P=.367$). To increase statistical power, the genetic data of the Danish, Estonian, and Finnish recruitment centers were combined in a meta-analysis across the three study samples (total number of 948 individuals; 634 RM patients and 314 fertile female controls; Table 2). The carrier status of the minor alleles of the CGB5 promoter SNPs exhibited a modest but significant protective effect against RM occurrence ($P=.021$; c5–155: OR = 0.64; 95% CI, 0.44–0.94; and c5–142: OR = 0.66; 95% CI, 0.45–0.94; Table 2). This result enhanced and confirmed the outcome of the original report (19). The meta-analysis including only Danish, Estonian, and Finnish female RM patients (n = 349) compared with fertile female controls (n = 314) showed the same direction and magnitude of the effect as the analysis in the full sample, but it did not reach statistical significance owing to the smaller sample size (c5–155: $P=.116$; OR = 0.71; 95% CI, 0.46–1.08; c5–142: $P=.089$; OR = 0.68; 95% CI, 0.44–1.06). Overall, both male and female partners of RM couples had a lower prevalence of the minor alleles of the studied CGB5 promoter SNPs (c5–155 and c5–142) compared with fertile controls (Supplemental Table 4). The allele frequencies of the genotyped intronic polymorphisms (CGB5: c5+1038; CGB8: c8+1045) did not differ between the Danish RM cases and fertile controls (MAF, 7.14% vs. 7.42%, $P=.52$; 0.55% vs. 0.43%, $P=.83$, respectively; Table 2).

**Genetic Variation in CGB8 Promoter Does Not Affect RM Risk**

The allelic distribution of SNPs (excluding singletons) in the resequenced CGB8 gene regulatory region (from –350 bp to +400 bp from mRNA start site) did not differ between the Danish RM patients and fertile controls (Supplemental Table 5), confirming the discovery analysis in the Estonian–Finnish sample (19). Concordantly, no statistical difference was detected in the CGB8 haplotype distribution between RM patients and fertile controls either (Fig. 2B; Supplemental Table 3). We conclude that common genetic variants in the proximal regulatory region of CGB8 have no substantial effect on the susceptibility to RM.

**DISCUSSION**

Previously, we showed a significant association between six SNPs located in the promoter region or introns of the CGB5 and CGB8 genes and reduced susceptibility to unexplained RM among Estonians and Finns (19). The present study set out to confirm this finding in another European population (Danes) and in a meta-analysis across the three study samples (19).
TABLE 2

| Sample size (RM cases/fertile controls) | MAF (%) | MAF (%) | OR (95% CI) | P value* | OR (95% CI) | P value*
|---------------------------------------|---------|---------|-------------|----------|-------------|----------|
| Estonians, n = 194 (99/95)           | 13.16   | 8.08    | 0.54 (0.27–1.11) | 0.083    | 0.54 (0.27–1.11) | 0.083    |
| Finns, n = 185 (85/100)              | 11.50   | 6.55    | 0.58 (0.29–1.19) | 0.129    | 0.58 (0.29–1.19) | 0.129    |
| Danes, n = 569 (450/119)             | 7.14    | 5.62    | 0.66 (0.45–0.94) | 0.369    | 0.66 (0.45–0.94) | 0.369    |

* meta-analysis performed using the inverse-variance method implemented under the fixed-effects model; estimation of the combined OR and statistical significance of association takes into account the sample size of each individual contributing study.

As a major outcome, this study confirmed the effect of the CGB5 promoter haplotype on modulating the susceptibility to RM. The carrier status of the minor alleles of the two SNPs (c5-155, c5-142) investigated in the present study as the genetic markers for the CGB5 promoter haplotypes significantly reduced the risk of RM (meta-analysis, P = 0.021, OR = 0.64 [0.44–0.94]). This RM-protective CGB5 promoter haplotype consists of the minor alleles of four SNPs (c5-155G→C; c5-147G→del; c5-144T→C; c5-142T→A) and is completely identical to the homologous region in the CGB8 gene, exhibiting no genetic variation in these positions (Fig. 1).

All humans have the CGB8 promoter haplotype c8-155C/c8-147del/c8-144C/c8-142A, which seems to provide the most optimally functioning promoter because CGB8 is responsible for up to 40% of hCG production in pregnancy (6). Most probably, originally humans had the CGB5 gene with a slightly less efficient main promoter variant c5-155G/c5-147G/c5-144T/c6-142T (Fig. 1A). The detected CGB5 RM-protective haplotype c5-155C/c5-147del/c5-144C/c5-142A originates from the CGB8 gene via a meiotic gene conversion event between the two promoter regions (15). We speculate that in some pregnancies, where the trophoblast growth is impaired (due to genetic, thrombophilic, immunological, or other reasons), the placenta with the most efficient CGB5 promoter haplotype (originating from and identical to CGB8) may have a better capacity for extra hCG production that may eventually rescue the threatened fetuses. Subsequently, this CGB5 promoter haplotype is expected to become increasingly prevalent among humans and to exhibit a higher prevalence in couples with normal fertility than in those with RM. This is in agreement with the results of this study. We also suggest that the current CGB8 gene with the c8-155C/c8-147del/c8-144C/c8-142A promoter haplotype has already reached maximum efficiency. Therefore the detected common variations in this gene have neither evolutionary advantage nor effect on pregnancy success, and balancing selection is expected to rapidly eliminate new, less fit variants (19).

In conclusion, despite the essential role of hCG in human pregnancy, no common SNP or haplotype variants in the main hCGbeta coding genes (CGB5, CGB8) were associated with increased risk of RM among the analyzed North European samples. Instead, the evolution in human lineage seems to have favored the spread of CGB genetic variants (e.g., by gene conversion), which support a more efficient gene expression and may reduce the risk of pregnancy loss even in critical situations. Recent studies have suggested that apart from SNPs, the expression of CGB genes might be modified by populations. The two discovery samples, representing neighboring populations of Estonians and Finns, had exhibited similar allelic distributions of SNPs in the CGB5 and CGB8 genes, whereas the allele frequencies of the Danes appeared to be statistically different from the Estonian-Finnish sample. A recent large-scale study showed that the geography of European populations is also reflected in its genetic structure, where Scandinavians cluster together with western Europeans and the Estonian population is genetically closest to Finns (28). Thus, meta-analysis rather than pooling the samples across studies is a preferred approach for increasing study power.
epigenetic mechanisms (29, 30). A pilot study reported polymorphic DNA methylation in the CGB5 promoter region exclusively in placentas from RM cases leading to expression silencing of the paternal alleles (29). Future larger studies have to target epigenetic modifications and also other non-SNP variations (e.g., copy number variations, gene deletions/duplications) in the CGB genes, which may have clinical importance in modulating susceptibility to pregnancy loss.

Acknowledgments: The authors thank all the patients who participated in the study; Dr. Margus Putku and Dr. Siim Söber, Estonia for assistance in statistical analysis; and Dr. Astrid Lindmäe-Kukk and Hendrik Rull for technical assistance.

REFERENCES

1. Srisuparp S, Strakova Z, Fazleabas AT. The role of chorionic gonadotropin (CG) in blastocyst implantation. Arch Med Res 2001;32:627–34.
2. Tsampalas M, Gradelet V, Berndt S, Foidart JM, Geenen V. Perrier d’Hauterive S. Human chorionic gonadotropin: a hormone with immunological and angiogenic properties. J Reprod Immunol 2010;85:93–8.
3. Cole LA. Hyperglycosylated HCG. a review. Placenta 2010;31:653–64.
4. Gerhard I, Runnebaum B. Predictive value of hormone determinations in the first half of pregnancy. Eur J Obstet Gynecol Reprod Biol 1984;17:1–17.
5. Buyalos RP, Glassman LM, Rifka SM, Falk RJ, Macarthy PO, Tyson VI, et al. Serum beta-human chorionic gonadotropin, estradiol and progesterone as early predictors of pathologic pregnancy. J Reprod Med 1992;37:261–6.
6. Rull K, Laan M. Expression of beta-subunit of HCG genes during normal and failed pregnancy. Hum Reprod 2005;20:3360–8.
7. Stenman UH, Tiitinen A, Alfthan H, Valmu L. The classification, functions and clinical use of different isoforms of HCG. Hum Reprod Update 2006;12:769–84.
8. Montagnana M, Trenti T, Aloe R, Cervellin G, Lippi G. Human chorionic gonadotropin in pregnancy diagnostics. Clin Chim Acta 2011;412:1515–20.
9. Berry CW, Brambati B, Eskes TK, Exalto N, Fox H, Geraeds JP, et al. Serum beta-human chorionic gonadotropin, estradiol and progesterone as early predictors of pathologic pregnancy. J Reprod Med 1992;37:261–6.
10. Li TC, Makris M, Tomus M, Tuckerman E, Laird S. Recurrent miscarriage: aetiology, management and prognosis. Hum Reprod Update 2002;8:463–81.
11. Christiansen OB. A fresh look at the causes and treatments of recurrent miscarriage, especially its immunological aspects. Hum Reprod Update 1996;2:271–93.
12. Maston GA, Ruolo M. Chorionic gonadotropin has a recent origin within primates and an evolutionary history of selection. Mol Biol Evol 2002;19:320–35.
13. Nagirnaja L, Rull K, Uusküla L, Hallast P, Grigorova M, Laan M. Genomics and genetics of gonadotropin beta-subunit genes: unique FSHB and duplicated LHB/CGB loci. Mol Cell Endocrinol 2010;329:4–16.
14. Hallast P, Laan M. Evolution of the chorionic gonadotropin beta genes in pri mates. In: Encyclopedia of Life Sciences (ELS). Wiley-Blackwell; 2009:1–12.
15. Hallast P, Nagirnaja L, Margus T, Laan M. Segmental duplications and gene conversion: human luteinizing hormone/chorionic gonadotropin beta gene cluster. Genome Res 2005;15:1535–45.
16. Huth JR, Mourtjoy K, Perini F, Ruddon RW. Intracellular folding pathway of human chorionic gonadotropin beta subunit. J Biol Chem 1992;267:8870–9.
17. Bo M, Boime I. Identification of the transcriptionally active genes of the chorionic gonadotropin beta gene cluster in vivo. J Biol Chem 1992;267:3179–84.
18. Miller-Lindholm AK, LaBenz CJ, Ramey J, Bedloes E, Ruddon RW. Human chorionic gonadotropin-beta gene expression in first trimester placenta. Endocrinology 1997;138:5459–65.
19. Rull K, Nagirnaja L, Ulander VM, Kelgo P, Margus T, Kaare M, et al. Chorionic gonadotropin beta-gene variants are associated with recurrent miscarriage in two European populations. J Clin Endocrinol Metab 2008;93:4697–706.
20. Nagirnaja L, Venclovas C, Rull K, Jonas KC, Peltoheko H, Christiansen OB, et al. Structural and functional analysis of rare missense mutations in human chorionic gonadotrophin α-subunit. Mol Hum Reprod 2012;18:379–90.
21. Christiansen OB, Rasmussen KL, Jersild C, Grunnet N. HLA class II alleles confer susceptibility to recurrent fetal losses in Danish women. Tissue Antigens 1994;44:225–33.
22. Kruse C, Steffensen R, Varming K, Christiansen OB. A study of HLA-DR and -DQ alleles in SBP patients and 562 controls confirms that HLA-DRB1*03 is associated with recurrent miscarriage. Hum Reprod 2004;19:1215–21.
23. Gordon D, Abajian C, Green P. Consed: a graphical tool for sequence finishing. Gen Res 1998;8:195–202.
24. Bhangale TR, Stephens M, Nickerson DA. Automating resequencing-based detection of insertion-deletion polymorphisms. Nat Genet 2006;38:1457–62.
25. Rousset F. Genepop’007: a complete reimplementation of the Genepop software for Windows and Linux. Mol Ecol Resources 2008;8:103–6.
26. Stephens M, Smith N, Donnelly P. A new statistical method for haplotype reconstitution from population data. Am J Hum Gen 2001;68:978–90.
27. Bandelt HJ, Forster P, Rohl A. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 1999;16:37–48.
28. Nelis M, Esko T, Mägi R, Zimprich F, Zimprich A, Toncheva D, et al. Genetic structure of Europeans: a view from the North-East. PLoS One 2009;4:e4572.
29. Uusküla L, Rull K, Nagirnaja, Laan M. Methyltion alleles in chorionic gonadotropin (CGB) in two European populations. J Clin Endocrinol Metab 2011;96:E199–207.
30. Grigoriu A, Ferreira JC, Choufani S, Baczyk D, Kingdom J, Weksberg R. Cell genetic structure of Europeans: a view from the North-East. PLoS One 2009;4:e4572.
31. Hollenberg AN, Pestell RG, Albanese C, Boers ME, Jameson JL. Multiple promoter elements in the human chorionic gonadotropin beta subunit genes distinguish their expression from the luteinizing hormone beta gene. Mol Cell Endocrinol 1994;106:111–9.
SUPPLEMENTAL FIGURE 1

RFLP analysis to detect polymorphisms in CGB5 and CGB8. (A, B) The PCR product of CGB5 promoter (2243 bp) is digested with (A) FastDigestStyI (Thermo Fisher Scientific Inc./Fermentas). The substitution C/G at position −155 from the transcription start of CGB5 gives an additional fragment of 1,449 bp; lane 1, marker 100 bp DNA Ladder (Solis Biodyne); lane 2, minor homozygote; lane 3, heterozygous individual; lane 4, major homozygote. (B) FastDigestBanI (Thermo Fisher Scientific Inc./Fermentas). The polymorphism T/A at position −142 from the transcription start of CGB5 has an index fragment of 806 bp; lane 1, marker Gene Ruler, 100 bp DNA Ladder (Thermo Fisher Scientific Inc./Fermentas); lane 2, heterozygous individual, lanes 3 and 4, major and minor homozygotes, respectively. (C, D) The polymorphisms located in the same position in CGB5 and CGB8 (1038 bp and 1045 bp from transcription start) were addressed by digestion of PCR product of CGB5 (1757 bp) and CGB8 (2544 bp) with FastDigestNciI (Thermo Fisher Scientific Inc./Fermentas). In both graphs, lane 1 represents marker 100 bp DNA Ladder (Solis Biodyne); the index fragments of 498 bp and 308 bp allow the discrimination of the major homozygote CC (C, lane 2; and D, lane 3), heterozygous variant CT (C, lane 4; and D, lane 2), and minor homozygote TT (C, lane 3). Nomenclature is based on GenBank references: NM_033043.1 GI:15451747 for CGB5; NM_033183.2 GI:146229337 for CGB8; and alleles represent the nucleotides on the coding strand. The detailed restriction schema is given in Supplemental Table 2.

Rull. CGB5 promoter haplotype and recurrent miscarriage. Fertil Steril 2013.
**SUPPLEMENTAL TABLE 1**

| Primer | Sequence | Product size | Fig.1 label | Original name of the primer
|--------|----------|--------------|-------------|-----------------------------|
| **Primers for PCR** | | | | |
| **CGB5 promoter** | 5'-TTTAGTAGAGACAGGGATTCACCA-3' | 2243 bp | 1F | CGB5_3F |
| CGB5pr_F | 5'-AGACCACGGTGAAAGTCAG-3' | 1R | CGB5_2R |
| CGB5pr_R | 5'-CGCTCGAGCAGTTTTCTATTT-3' | 2F | CGB5_3F |
| **CGB5 gene** | 5'-CAGGAAAGCCTAAGTAGAGGAG-3' | 1757 bp | 2R | CGB5_2R |
| CGB5_F | 5'-CGCTCGAGCAGTTTTCTATTT-3' | 2F | CGB5_3F |
| CGB5_R | 5'-CACGCCCTGTAATTGTCGGAGGCTGT-3' | 8384 bp | 3F | CGB5/7_8kb_F3 |
| **CGB8 long-range PCR** | 5'-GAAAGAGAGTAGAGATGGGGACGAC-3' | 3R | CGB5/7_8kb_R3 |
| CGB8_F | 5'-CCCAGATAACTTTTCGTATTATA-3' | 2544 bp | 4F | CGB2_2R |
| CGB8_R | 5'-TCCTCAAGATCAACTCCTGATGAT-3' | 4R | CGB5/7_3nestR |
| **CGB8 nested PCR** | | | | |
| CGB8n-F | 5'-CCCTGCAGTCTTACCTGGAA-3' | | | |
| CGB8n_R | 5'-TGCTTGAGAAAGTCTGACACATCC-3' | | | |
| **Primers for resequencing** | | | | |
| **CGB8 promoter and 5’UTR** | | | | |
| cgb8prom_seqF | 5'-CCCTGCAGTCTTACCTGGAA-3' | | | |
| cgb8prom_seqR | 5'-TGCTTGAGAAAGTCTGACACATCC-3' | | | |
| cgb8_1F | 5'-GGCCTTTGAAGAAGGAGA-3' | | | |
| cgb8_1R | 5'-GCCCTAGGTGAGGTGCAA-3' | | | |

* Reference 15.

Rull. CGB5 promoter haplotype and recurrent miscarriage. Fertil Steril 2013.
### SUPPLEMENTAL TABLE 2

List of addressed single nucleotides with applied restriction enzymes and fragment length.

| SNPa | rs No. b | Allelec major/minor variant | Restriction enzyme | Fragments present in all variants; length in base pairs | Specific fragments according to addressed nucleotide; length in base pairs |
|------|----------|----------------------------|------------------|--------------------------------------------------------|---------------------------------------------------------------|
| CGB5 promoter (PCR product 2245 bp) | c5-155 | rs72553898 | G/C | FastDigestStyl (Eco130I) | 486 | 1,759 | 310, 1,449 | 310, 1,449, 1,759 |
|      | c5-142 | rs72553901 | T/A | FastDigestBanl (BshNI) | 63, 156, 425, 492 | 1,109 | 303, 806 | 303, 806, 1,109 |
| CGB5 gene (PCR product 1757 bp) | c5+1038 | rs4802541 | C/T | FastDigestNcil (BcnI) | 7, 28, 79, 204, 305, 636 | 120, 378 | 498 | 498, 120, 378 |
| CGB8 gene (nested PCR product 2544 bp) | c8+1045 | rs4802541 | C/T | FastDigestNcil (BcnI) | 2, 7, 12, 28, 79, 204, 306, 583, 825 | 120, 378 | 498 | 120, 378, 498 |

* SNP code includes gene name (e.g., c5 = CGB5) and location relative to mRNA start site; GenBank references: NM_033043.1 GI:15451747 for CGB5, NM_033183.2 GI:146229337 for CGB8.

b rs number according to NCBI SNP database.

c Alleles on the coding strand. All restriction enzymes were provided by Thermo Fisher Scientific Inc/Fermentas.

*Ref. CGB5 promoter haplotype and recurrent miscarriage. Fertil Steril 2013.*
### SUPPLEMENTAL TABLE 3

The distribution of haplotypes covering the promoter and 5’ untranslated region of CGB8 among the patients with RM and fertile controls in Danish (n = 569) and Estonian-Finnish sample sets (n = 379).

| Position relative to transcription start site | Estonians/Finns | Danes |
|---------------------------------------------|----------------|-------|
| Haplotype -287 -226 -186 -4 +105 +108 +301 | RM cases       | All   | RM cases       | All   | P valuea |
| 1   c A G T G C A                         | 4.25           | 7.33  | 5.85           | 3.14  | 2.52     | 2.95 | .00569 |
| 2*  c A G T G C T                         | 16.71          | 16.75 | 16.74          | 23.62 | 23.53    | 23.59 | .00091 |
| 3   c A G T G t T                         | 0.37           | 0.42  | 0.38           | 0.42  | 0.38     | .09237 |
| 4   c A G T c C T                         | 2.27           | 2.36  | 2.31           | 3.14  | 3.36     | 3.20 | .29044 |
| 5   c A G a G C T                         | 0.85           | 0.41  | 0.85           | 0.41  | 0.41     | .07409 |
| 6   c del G T G C T                       | 0.18           | 0.42  | 0.26           | 0.18  | 0.42     | .16953 |
| 7   T A G T G C A                         | 0.42           | 0.42  | 0.42           | 0.42  | 0.42     | .33153 |
| 8*  T A G T G C T                         | 36.83          | 33.51 | 35.10          | 42.62 | 42.02    | 42.44 | .00343 |
| 9   T A G T g t T                         | 0.37           | 0.42  | 0.38           | 0.42  | 0.38     | .09237 |
| 10  T A T T G C T                         | 0.92           | 2.1   | 1.28           | 0.92  | 2.1      | .00207 |
| 11* T A t T G t T                         | 39.09          | 40.05 | 39.59          | 24.72 | 23.53    | 24.36 | <.00001 |
| 12  T del G T G C T                       | 0.18           | 0.13  | 0.13           | 0.18  | 0.13     | .33153 |
| 13  T del t T G t T                       | 0.74           | 1.26  | 0.90           | 0.74  | 1.26     | .01005 |

Note: The major variant of a polymorphism is marked with a capital, and the minor variant with a lowercase letter. The haplotypes are in concordance with haplotype networks (Fig. 2).

a The difference between the Danish and Estonian-Finnish (Est/Fin) sample sets is calculated by the \( \chi^2 \)-test.

* Three core haplotypes.

Rull. CGB5 promoter haplotype and recurrent miscarriage. Fertil Steril 2013.
**SUPPLEMENTAL TABLE 4**

Frequencies of the minor alleles of genotyped SNPs in the *CGB5* and *CGB8* genes in the subgroups of male and female partners of the couples with RM compared with fertile female controls in the Danish (n = 569) and Estonian (n = 194) and Finnish sample sets (n = 185).

| Polymorphism | Fertile controls, n = 95 | RM patients, n = 99 | Fertile controls, n = 100 | RM patients, n = 85 | Fertile controls, n = 119 | RM patients, n = 450 |
|--------------|--------------------------|---------------------|--------------------------|---------------------|--------------------------|---------------------|
|              | Females, n = 64 | Males, n = 35       | Females, n = 45 | Males, n = 40       | Females, n = 240 | Males, n = 210       |
| c5-155       | 13.16                 | 8.59                | 11.50                    | 5.56                | 7.69                    | 7.14                | 6.60                | 4.52                |
| c5-142       | 11.50                 | 8.59                | 13.00                    | 5.56                | 7.69                    | 7.14                | 6.60                | 4.52                |
| c5+1038      | 14.47                 | 8.59                | 14.00                    | 5.43                | 10.00                   | 7.56                | 8.97                | 5.71                |
| c8+1045      | 0.53                  | 0.78                | 3.13                     | 0                   | 0                       | 0.43                | 0.72                | 0                   |

*Ruši, CGB5 promoter haplotype and recurrent miscarriage. Fertil Steril 2013.*
## SUPPLEMENTAL TABLE 5

Common variants in *CGB8* promoter and 5' untranslated region in Danes (n = 569).

| SNP     | MAF (%) | Fertile female controls, n = 119 | RM patients, n = 450 | P value |
|---------|---------|----------------------------------|----------------------|---------|
| c8-287  | 30.74   | 29.52                            | .85                  |
| c8-226  | 1.79    | 0.94                             | .34                  |
| c8-186  | 26.84   | 26.38                            | .83                  |
| c8+105  | 3.46    | 2.77                             | .83                  |
| c8+108  | 25.97   | 26.19                            | .94                  |
| c8+301  | 2.88    | 3.14                             | .83                  |

Note: Association P values were calculated by the Cochran-Armitage test for trend.

Rull. *CGB5* promoter haplotype and recurrent miscarriage. Fertil Steril 2013.