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Potential for pharmacogenetic use of FSH: a 2014-and-beyond view

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Abstract

Objective: To assess the potential pharmacogenetic use of FSH for infertility treatment.

Design: Review of the literature and genomic databases.

Methods: SNP assessed: rs6166 (c.2039A>G, p.N680S), rs6165 (c.919A>G, p.T307A), rs1394205 (c.-29G>A) in *FSHR* and rs10835638 (c.-211G>T) in *FSHB*. Literature search via PubMed. Blast analysis of genomic information available in the NCBI nucleotide database. Comparison of allele frequency and haplotype distribution using the http://spsmart.cesga.es tool.

Results: All these SNPs appear first in *Homo,* result in reduced FSH action and are present with variable frequencies and combinations worldwide. Stringent clinical studies demonstrate that the *FSHR* genotype influences serum FSH levels and gonadal response in both sexes. Serum FSH levels depend on the -211G>T SNP, influencing transcriptional activity of the *FSHB* promoter. Genotypes reducing FSH action are overrepresented in infertile subjects.

Conclusions: While the clinical relevance of the *FSHR* polymorphisms alone is limited, the combination of *FSHR* and *FSHB* genotypes has a much stronger impact than either one alone in both sexes. About 20% of people are carrier of the alleles associated with lower serum FSH levels/reduced FSHR expression or activity, possibly less favorable for reproduction. Prospective studies need to investigate whether stratification of infertile patients according to their *FSHR-FSHB* genotypes improves clinical efficacy of FSH treatment compared to the current, naïve approach. A relative enrichment of less favorable *FSHR-FSHB* genotypes may be related to changes in human reproductive strategies and be a marker of some health-related advantage at the costs of reduced fertility.

Introduction

Follicle-stimulating hormone (FSH) is fundamental for gamete maturation and is widely used in the treatment of hypogonadotropic hypogonadism and infertility in both sexes (1, 2). Both urinary FSH (in form of human Menopausal Gonadotropins, hMG, and highly purified FSH) and recombinant (r) FSH (rFSH, and long acting rFSH) are commercially available and currently in use with very good results, especially in assisted reproduction. Since unwanted infertility (defined as lack of conception after one year of unprotected intercourses) affects around 30% of couples, fertility treatment is very popular and the gonadotropin market florid, especially among gynecologists performing assisted reproduction technologies (ART) as well as endocrinologists and andrologists treating hypogonadism and male infertility.

With a steadily increasing demand of infertility treatment, therapies involving the use of FSH continue to evolve rapidly, mostly on empirical bases, so that ART protocols are often adjusted based on availability of new drug preparations despite the paucity of scientifically sound data. Systematic reviews (e.g. from the Cochrane collaboration) are regularly issued in order to compare different protocols but often fail to demonstrate the purported superiority of the new approaches (3-6). On the other hand new protocols of infertility treatment and ART are highly needed, especially considering the constantly increasing age of women in couples undergoing ART (7), a factor which decreases the efficacy of the treatment by affecting both pregnancy and abortion rate. In such a scenario, pharmacogenetic approaches are appealing and have been proposed (8).

FSH works through binding to its specific receptor, the FSHR (*FSHR*, Gene ID: 2492, location: 2p21-p16, OMIM: 136435) (9). In physiological conditions serum FSH levels are under hypothalamic and gonadal control, depend on age and, in women, on the menstrual cyclicity. Research of the last two decades revealed the role of common genetic variants of *FSHR* and FSH beta subunit (*FSHB*, Gene ID: 14308, location: 11p13, OMIM: 136530) in determining individual serum hormone levels and target organ response (10, 11). However, controversies exist concerning the impact of genetic polymorphisms of these genes on gonadotropin treatment and some contradictory findings have been

published. Several good reviews appeared recently in the literature summarizing the current knowledge, covering various aspects of this topic (12-17) and showing clearly that, currently, there is not enough evidence to provide practical clinical recommendations for the pharmacogenetic use of FSH.

In this article we take a different approach and try to give a coherent interpretation to a rich literature containing partially contradictory and fragmented data: starting with the analysis of evolutionary aspects, considering the biological consequences of the genetic variants *in vitro* and *in vivo* under various pathophysiological conditions, and examining the possible explanations for the controversies in the literature, we propose that the true pharmacogenetic potential of FSH use in therapy becomes evident only when the genetic complexity of the FSH-FSHR system is considered in its whole. Since the most studied genetic variants of *FSHR* and *FSHB* are associated with an overall lower FSH activity and appear first in the species *Homo,* we are facing an apparent evolutionary paradox, which suggests the opportunity of a wider view of the FSH role in life-time reproductive success. We suggest that the genotypes of *FSHR* and *FSHB* both influence the final biological activity of FSH and should be considered together in future pharmacogenetic studies dealing with infertility.

Genetic variants of *FSHR* and *FSHB*: Frequency, ethnic distribution and evolution

The receptor for FSH belongs to the family of G protein-coupled receptors. Its three-dimensional structure was resolved recently, and the mode of FSH-FSHR interaction illustrated (18). Fig. 1 shows the genomic location of the *FSHB* and *FSHR* genes along with the position of the single nucleotide polymorphisms (SNP) considered in this article (Table 1), chosen because much information about their possible role in the biological response to FSH exist in the literature. As shown in Fig. 1, these SNPs are selected genetic markers among many others belonging to complex genomic regions: the HapMap database (http://hapmap.ncbi.nlm.nih.gov) currently shows about 900 SNPs for *FSHR* and 24 for *FSHB*, respectively, organized in distinct linkage disequilibrium (LD) blocks, segments of the genome in which a given combination of alleles or genetic markers is inherited coordinately. The structure of *FSHR* and *FSHB* LD blocks in Caucasians is shown in Fig 1. LD blocks differ in their

structure between ethnic groups, resulting in various combinations of the different SNPs. This should be considered when assessing association studies obtained in different populations.

Progress in genomic research and studies on human diversity produce public databases, which can be consulted freely online. We interrogated these databases (HapMap, Release #28; Perlegen, complete data set; CEPH, U. Stanford HGDP; CEPH, NIH-U. Michigan HGDP; 1000 Genomes, Phase I May 2011) to analyze, in different ethnic groups, frequency, distribution and combinations of the SNPs considered in this article. The analysis was performed using the http://spsmart.cesga.es tool, freely available online.

FSHR

Concerning the *FSHR*, the most studied, non-synonymous SNP is rs6166 (c.2039A>G, p.N680S), which shows a high degree of LD with rs6165 (c.919A>G, p.T307A) (10) in many ethnic groups (Table 2). Both SNPs are located in exon 10 and cause an amino acid exchange: rs6166 exchanges asparagine for serine in the intracellular domain of the receptor, introducing a potential phosphorylation site; rs6165 replaces threonine by alanine, i.e. it results in a change from a polar (T) to a non-polar, hydrophobic (A) amino acid and removes a potential O-linked glycosylation site. Another common, but less studied SNP (rs1394205) exchanges a nucleotide in the promoter region of the *FSHR* (-29G>A). The analysis of these SNPs using the http://spsmart.cesga.es tool, extracting the genomic data present in the available databases[1], reveals differences in their frequency between human populations.

Looking at rs6166, its remarkable ethnic distribution is shown in Fig 2, freely available at http://hgdp.uchicago.edu/cgi-bin/alfreqs.cgi?pos=49043425&chr=chr2&rs=rs6166&imp=false, (19,

[1] HapMap, Release #28; Perlegen, complete data set; CEPH, U. Stanford HGDP; CEPH, NIH-U. Michigan HGDP; 1000 Genomes, Phase I May 2011.

20) depicting the allelic frequency in 52 populations and demonstrating important inter-population differences.

The ancestral A allele is most predominant in South Eastern Asia, while the derived G allele is highly prevalent in other populations and reaches its higher frequencies in Kalash (North-Western Pakistan), Yakuts (Siberia), Suruì Paiter (Mato Grosso, Brazil) and Melanesians (Oceania). It is interesting to notice that these ethnicities are geographically isolated and genetically well distinct, and posses a number of other genetic peculiarities. For instance, Melanesians display genomic signs of direct introgression of Neanderthal genome in modern humans after migration from sub-Saharan Africa as a consequence of strong positive selection (21); Yakuts are characterized by very low genetic diversity (22); Kalash and Suruì Paiter are very small populations (only a few hundreds individuals left) at extinction risk. In these ethnic groups the enrichment of the rs6166 G allele of the *FSHR* may be a consequence of insulation or the result of genetic drift.

Considering all genomic data available in the public databases together (Fig. 3A summarizing genomic data of 3228 individuals), the ancestral A allele has a minor allele frequency (MAF) of 0.6 in sub-Saharan Africans, which may be considered closer to the ancients human population. The G allele is enriched in Europeans, Middle East, Central-South Asia and Oceania, while it shows the lowest frequency in Far East Asia and North America. The fixation index (F_{ST}) value, a measure of the population differentiation due to genetic structure, calculated for rs6166, is high in East Asians (0.0525) compared to Europeans (0.0195) and Middle East (0.007) populations, a phenomenon seen with genetic variants in several other genes (the so-called East Asian sweep pattern) of uncertain meaning (23). In any case, these ethnic differences should be considered when we turn to genetic association- and clinical studies based on this SNP, as they might explain some heterogeneity in the results.

rs6166 is evolutionary recent. As shown in Supplementary Fig. 1, *FSHR* c.2039A>G (p.N680S) is not present in non-human primates and, in most animal genomes analyzed so far, the *Fshr* gene carries an Asn at the amino acid position corresponding to position 680 of the human *FSHR*. The analysis of the

Neanderthal genome (http://neandertal.ensemblgenomes.org) shows the presence of the G allele (Ser) in the three samples analyzed so far. This suggests that the new allele was already present in an extinct hominid branch very close to the modern human. Since, as it will be discussed below, the *FSHR* allele carrying a Ser at amino acid position 680 is functionally "resistant" to FSH both in women and in men, the evolutionary advantage of this allele is still unclear.

Population data about rs6165 are less abundant and show some difference compared to rs6166 (Fig 3B, summarizing genomic data of 2287 individuals). In particular the c.919A>G ancestral G allele is predominant in sub-Saharan African populations, with a MAF of 0.274, while the MAF is grossly similar to that of rs6166 in the other ethnic groups. This suggests that the two SNPs are not in LD in Africans and, to a minor extent, in other ethnicities as well (Table 2). In practically all other species sequenced so far the G allele is the rule and Ala is the amino acid occupying the position corresponding to 307 of the human *FSHR* (Supplementary Fig. 2). Therefore, both amino acids 307 and 680 are highly conserved across species but, interestingly, the non-human *FSHR* haplotype is Ala307-Asn680, which is rarely found in modern humans, with the notable exception of Africans. We consider this as that the ancestral haplotype and assume that it changed through two independent mutational events, one introducing rs6166, (c.2039A>G; p.N680S), with the ancient allele encoding Asn still predominating in most populations (Fig. 3); the other event, rs6165 (c.919A>G; p.T307A) now results in a predominance of the derived allele (encoding Thr) in all ethnic groups except Africans. As a result, these changes are now in LD in most people and form the two major exon 10 haplotypes, Thr307-Asn680 and Ala307-Ser680.

The third SNP in the *FSHR* considered in this article is rs1394205 (c.-29G>A), located in a separated LD block (Fig. 1) and found with different frequencies and independently of the exon 10 haplotype (24). Ethnic differences in the distribution of this SNP are known already for some time (25) and are confirmed by the current database collections (Fig. 4A, summarizing genomic data of 2288 individuals). Comparative alignment analysis suggests that the dominant G allele is the ancestral allele (Supplementary Fig. 3).

FSHB

As far as *FSHB* is concerned, a detailed populations genetics study identified two major haplotypes possibly influencing conception (26). Most clinical studies performed so far consider only the SNP rs10835638 (c.-211G>T) and genomic databases contain information about 1093 individuals (Fig. 4B). The MAF of the -211G>T varies between 0.028 (in Africans) and 0.145 (in Europeans). It appears that this SNP increased in frequency after migration out of Africa and is epidemiologically relevant only in Europe and in America, with 20-25% of people carrying at least one T allele. Although rare, this SNP has a significant functional importance (11), and it is located in an element of the *FSHB* promoter, which binds the LHX3 homeodomain transcription factor influencing gene transcription (27). Alignment of the corresponding genomic region in several species demonstrates a large predominance of G (Supplementary Fig. 4), which therefore represents the ancestral allele.

Genetic variants of *FSHR* and *FSHB*: *in vitro* effects

In clinical studies the variants of *FSHR* described above have been associated with changes in the sensitivity to FSH and the -211G>T SNP of the *FSHB* gene was associated to reduced FSH serum levels, as will be considered in the next chapter. Some *in vitro* studies were devoted to ascertain the molecular mechanism causing such changes in the levels/activity of FSH.

FSHR

Since a number of clinical studies suggest a different sensitivity to FSH of the two *FSHR* exon 10 haplotypes *in vivo* (see next chapter), some experiments were dedicated to clarify the possible mechanism at the molecular level. The molecular consequences of the combination of p.T307A and p.N680S (Ala307-Ser680) have been studied in transiently transfected cell lines and in human granulosa-lutein cells (hGLC) naturally expressing one of the two receptor variants (28-31). Intriguingly, no difference between the receptor activation depending on the *FSHR* haplotype could be demonstrated when very early events of cellular response, such as cAMP and IP3 production, were studied in various transiently transfected cell lines (28-31). hGLC homozygous for each *FSHR*

haplotype responded identically in terms of cAMP, progesterone and estradiol production, measured at saturation (31). Another experiment suggested that hGLC homozygous for Asn at amino acid position 680 only "tended" towards higher FSH-induced *FSHR* expression, whereas the expression of other, well-known *FSHR* dependent genes, such as *LHCGR* and *CYP19A1* was not affected (32).

We recently analyzed the effects of *FSHR* exon 10 SNPs *in vitro* more in depth, looking at the kinetics of response and at different signal transduction pathways. Our data show that the two *FSHR* variants, studied in hGLC, respond by activating the different signal transduction pathways with different kinetics, suggesting that the final biologic response involves different mechanisms. As an example of these novel experiments we show in Fig. 5 that ERK1/2 activation is blunted in hGLC naturally expressing the combination of p.T307A A and p.N680S S allele (Ala307-Ser680). These data, repeatedly confirmed in our lab (Casarini et al., manuscript in preparation), suggest for the first time that the Ala307-Ser680 FSHR is indeed less "active" in vitro, providing a molecular explanation for the clinical data.

Concerning the *FSHR* -29G>A, this SNP is located in the promoter region, in a consensus sequence for the cellular homolog to the viral E26 transformation specific sequence (cETS-1). Our early experiments did not show statistically significant difference in the activity of the promoter in the two different cell lines COS7 and SK11 (25). However, others could demonstrate, using CHO cells, that this single base exchange resulted in a significant, 56% decrease of the transcriptional promoter activity of the A allele (33). It has been shown that promoter activity by reporter assay can vary consistently depending from the cell line used (34), possibly explaining this discrepancy. The reduced promoter activity *in vitro*, as shown by Nakayama et al. (33), fits well with the clinical findings.

FSHB

The SNP rs10835638 (-211G>T) in the *FSHB* promoter falls in a binding element for the LHX3 homeodomain transcription factor, capable of influencing gene transcription. The -211G>T T allele decreased transcriptional promoter activity *in vitro* of about 50% in the LβT2 gonadotrope cell line

(27). This confirmed earlier studies *in vitro* showing a reduction of promoter activity varying from 46% (in JEG3 cells) to 58% (in TE671 cells) and 86% (in HEK293T cells) (34). The *FSHB* promoter region containing SNP rs10835638 is located in a putative hormone responsive element but recent experiments revealed that, unlike the murine *Fshb*, progestins and androgens are unable to induce *FSHB* transcription (27). Therefore, in the human, circulating progesterone levels are not expected to modulate serum FSH directly at the pituitary level *via* this mechanism.

In summary, the evolutionary more recent SNPs, both in the *FSHR* and in the *FSHB* gene, are associated *in vitro* with changes either in signal transduction (*FSHR* exon 10) or in transcriptional activity (*FSHR* and *FSHB* promoter) resulting in an overall reduced FSH action.

FSHR and _FSHB_ polymorphisms influence serum FSH levels and reproductive parameters: Studies in women

FSHR

The most popular model to study whether *FSHR* polymorphisms have any effect on FSH levels/action is represented by women with seemingly normal ovarian function undergoing ART for couple infertility due to male- or tubal factor. These women are treated with FSH to induce multiple follicle development. Classically they receive between 2000-5000 IU of FSH over 7-15 days of stimulation with remarkable inter-individual variability in ovarian response. In these women, the measurement of basal serum FSH levels, the amount of exogenous FSH needed to reach multi-follicular development and the levels of serum estradiol at the time of hCG administration for final follicular maturation can be taken as parameters of FSHR sensitivity.

The first observation that the *FSHR* haplotype consisting of the two SNP in exon 10 could be a determinant of serum FSH levels and ovarian response to FSH dates back to 2000 (10). In normoovulatory women undergoing ART we showed that the *FSHR* exon 10 haplotype p.T307A A and p.N680S S allele (Ala307-Ser680) was less sensitive to FSH, since these women had significantly higher basal serum FSH levels and required significantly more FSH to achieve multiple follicular

maturation (10). This observation immediately opened the questions whether *FSHR* genotype might be relevant for pharmacogenetic FSH therapy and for menstrual cycle physiology. A very stringent menstrual cycle monitoring study in young (mean age: 25 yrs), normally cycling, ovulatory women of European origin, indeed demonstrated the *FSHR* genotype to be instrumental in the determination of serum levels of FSH, menstrual cycle length and dynamics of reproductive hormones (35).

Following our original observation, a large number of studies have been published, mostly in women undergoing ART and reporting partially conflicting results. The reasons for these discrepancies will be analyzed here. In Suppl. Table 1 we summarize the main data of the studies published so far concerning *FSHR* genotype frequency distribution (rs6166), basal FSH levels and FSH dosage subdivided by genotype. In addition, from each study, we extracted the age of the study subjects and whether or not a cutoff of basal serum FSH levels was an inclusion criterion, two parameters crucial to interpret the results. In fact, age per se affects serum FSH levels in women and results obtained in young ovulatory women may well be different for (usually) older, infertile patients undergoing ART.

The data reported in Suppl. Table 1 show that some studies confirmed our original results (29, 36-40) but others did not, especially those conducted in women of advanced age (41-44). Serum FSH levels was not significantly different between *FSHR* genotypes in older women (41-44), and in conditions of very high FSH concentrations, such as in postmenopausal women (45) and in women with premature ovarian failure (46). Patient inclusion criteria are important as well, because studies excluding (young) women with basal FSH levels > 10 IU/L, potentially excluding p.N680S S carriers, did not reveal any genotype-related difference (37, 47, 48). Some studies included women with ovarian and/or unexplained infertility (e.g. 49). Finally most studies analyzed only rs6166 (p.N680S), assuming perfect LD with rs6165 (p.T307A) but, as shown in Fig. 3 this may not always be the case and represents another reason for inconsistency.

Many studies in women undergoing ART, tried to assess whether the *FSHR* genotype is useful to predict response and/or decide the FSH starting dose. This would result in personalized ovarian

stimulation with lower incidence of side effects (e.g. ovarian hyperstimulation syndrome) and saving of resources. These studies, reviewed recently in detail (12-17, 50) are extremely heterogeneous (Supplementary Table 1) and provided partly conflicting results. For instance, several studies were unable to confirm that women carriers of the *FSHR* c.2039A>G G allele (Ser680) have higher basal serum FSH levels compared to Asn carriers (42-44). Nevertheless, a recent meta-analysis of seven studies including 1421 patients undergoing ART confirmed a significant difference in basal FSH levels depending on the *FSHR* genotype: carriers of one or two c.2039A>G A alleles (Asn 680) showed significantly lower FSH levels, with a weighted mean difference of - 1.57 IU/L (C.L. -2.51/-0.64 IU/L) (51), therefore quantitatively small. Again, failure to detect increased basal FSH levels in c.2039A>G G allele carriers in individual studies involving women undergoing ART is likely to depend both on age (practically all ART patients are over 25 and several over 40, with a mean age varying in the individual studies between 30 and 38 yrs) and heterogeneity of patients included (Supplementary Table 1).

The importance of the woman's age in assessing the impact of the *FSHR* c.2039A>G on serum FSH levels has been recently proven by a very elegant study involving only fertile, young women (mean age of about 25 years) undergoing COH within an oocyte donation program (52). In this study, including 355 CHO cycles in 145 well characterized, healthy and homogenous oocyte donors, basal FSH levels, total FSH dose, antral follicle count and number of eggs retrieved were significantly different between the genotypes, with the homozygous c.2039A>G G allele confirmed to be less sensitive to FSH stimulation. This study demonstrated once more that, in young, ovulatory women, the *FSHR* polymorphism c.2039A>G is indeed a major determinant of ovarian sensitivity to FSH.

A conservative but reasonable conclusion from the studies performed so far is that the *FSHR* genotype is a physiological determinant of basal FSH levels evident in young, normo-ovulatory women. This is not necessarily the case in older women of infertile couples especially in the presence of non-optimal ovarian function and/or reduced reserve, which *per se* result in increase of serum FSH. Finally, lack of consideration of the *FSHR* -29G>A and/or *FSHB* -211G>T effect might also be one

reason why not all studies have been able to demonstrate *FSHR*-dependent differences in FSH levels in women.

Concerning the *FSHR* -29G>A, associated with reduced transcriptional activity in vitro (33) this SNP received less attention so far. This SNP was not associated with serum FSH levels in women (25, 41), but Indian carriers of the *FSHR* -29G>A A allele needed significantly more FSH for multiple follicle maturation in ART compared to carriers of the *FSHR* -29G>A G allele (41). This might be due to reduced FSHR expression: the less active -29G>A A allele was reported to result in significantly lower levels of *FSHR* mRNA and protein in granulosa cells obtained from women undergoing ART (53). The first attempt to assess the combined effect of *FSHR* c.2039A>G and *FSHR* -29G>A genotypes revealed that the amount of exogenous FSH required for ovarian stimulation and the frequency of poor responders was the highest in double homozygous carriers of the *FSHR* -29G>A A and *FSHR* c.2039A>G G (Asn) combination (54). This is intriguing because, while the *FSHR* -29G>A A allele is transcriptionally less active, the *FSHR* c.2039A>G G (Asn) is expected to be more sensitive to FSH. Most probably, a much higher number of subjects is needed to reach conclusive evidence when combined genotypes are considered. Therefore, these observations require independent confirmation in different ethnic groups, but the concept of genotype-dependent *FSHR* expression levels is interesting and increases the complexity of the *FSHR* genotype influence on FSH action.

FSHB

After the demonstration of a strong *FSHB* -211G>T effect on serum FSH levels in men (11, see below) the role of this polymorphism was studied in women. An intriguing retrospective study involving 365 normally cycling women undergoing ART suggested that the presence of T allele, i.e. that showing lower promoter activity *in vitro* and associated with the lowest FSH levels in men, was unexpectedly associated with significantly higher serum FSH concentrations in the follicular phase and lower progesterone levels in the luteal phase (55). Unable to explain this finding, the authors concluded for a gender-specific difference in the control of gonadotropin secretion. Granted that

gonadotropin regulation is different in the two sexes, some factors could have affected the results, such as luteal insufficiency in 20.5% of the patients, inclusion criteria (FSH levels comprised between 3-15 IU/L; no age limit), and, perhaps more importantly, the lack of stratification by the *FSHR* genotype. In fact, considering both *FSHR* and *FSHB* polymorphisms together, we observed in women the opposite results. In a prospective study involving a homogeneous group of 193 women with regular cycles, age < 40 yrs and normal antral follicle count and serum AMH levels, day 3 FSH concentrations were significantly lower in carriers of T allele, when stratified by the *FSHR* genotype. Interestingly, women carriers of the T allele did not show the age-related increase of serum FSH levels observed in GG homozygotes. This shows the importance of *FSHB* genotype in women, something deserving attention especially when FSH levels are evaluated as a marker of ovarian reserve (56). This study suggested that, in women, not considering the *FSHR* genotype might mask the *FSHB* polymorphism effect (56). In support to this concept, a very recent study in a longitudinal cohort of peripubertal girls confirmed that the combined effect of *FSHB* -211G>T G allele and *FSHR* c.2039A>G A allele was associated with a more effective FSH action, with a tendency to anticipate puberty entry and a significant reduction of serum AMH (57). More studies, however, are necessary in order to confirm the interaction between the two genotypes in women.

So far, no data are available about the possible role of the *FSHB* -211G>T polymorphism, alone or in combination with *FSHR*, on ART outcome.

Pharmacogenetic studies

Are these polymorphisms in *FSHR*/*FSHB* relevant for ART? Can we improve ART outcome using a pharmacogenetic approach based on their genotype? To date only one prospective, randomized, controlled study was conducted, in which women homozygous for the c.2039A>G of the *FSHR* were treated with fixed doses of FSH to assess ovarian response, measured as serum estradiol levels on the day of hCG triggering (47). This study demonstrated that homozygous *FSHR* p.N680S S women produced less estradiol compared to homozygous p.N680S N women treated with the same dose of FSH and this difference could be overcome by increasing the FSH dose. While demonstrating, in a

clean way, that the *FSHR* genotype effectively impacts ovarian response in an interventional, prospective, controlled design, the study did not answer the question whether this effect is clinically relevant for the success of an ART program. In fact, the study was not powered to assess possible effects on pregnancy rate and life births, i.e. the real, clinically relevant end points of each ART intervention. In addition, it completely disregarded heterozygous women, i.e. the majority of patients, and did not consider the other SNPs in the promoter of *FSHB* and of *FSHR*, the relevance of which was never studied in prospective, interventional studies. Answering the question whether a pharmacogenetic approach based on the *FSHR* and/or *FSHB* genotype may improve ART outcome in terms of life births and/or reduced side effects would require a very large, multicenter effort, involving thousands of well-selected women. Given the socio-economical aspects currently involved (pressure of the infertile couple to achieve a pregnancy, financial aspects) and pathophysiological variables (woman's age, male factor, ovarian reserve, unexplained infertility), it is unlikely that such a study will ever be performed and the question is whether it would be worth the trouble. There, is, however, a plethora of studies analyzing retrospectively the relevance of *FSHR* and other gene polymorphisms for ovarian response (variably defined) in ART programs based on different types of patients. To mention only the most recent ones, some studies confirm the impact of the *FSHR* c.2039A>G SNP (40), some do not (49) and this reflects faithfully the results of over a decade of literature summarized in recent reviews (12-17, 50). The heterogeneity of the study designs, patient characteristics and primary end points, often in the absence of power analysis, together with relatively advanced age of women in ART programs are the possible reasons for this inconsistency (Supplementary Table 1) In addition, other genes (e.g the *ESR2*, *AMH*, *AMHR2*, *MTHFR*, etc.) have been variably shown to be associated with ovarian response, to which they could contribute (15, 17).

FSHR and _FSHB_ polymorphisms influence serum FSH levels and reproductive parameters: Studies in men

FSHR

Several studies assessed the association of *FSHR* exon 10 polymorphisms and reproductive parameters in men. However, for long time, no effect of *FSHR* genotype on serum FSH levels could

be demonstrated (reviewed in 69). This was surprising because, meanwhile, many papers were published supporting the role of *FSHR* genotype in determining serum FSH levels women (12-14, 10, 35, 40, 59, 60) (Supplementary Table 1). As possible explanation, gender-specific differences in the feed-back regulation of FSH secretion were assumed.

The significant role of the *FSHR* p.N680S polymorphism in the male was very recently demonstrated by a study involving a very large number of Baltic men (61). Thanks to the large dimension of the study, performed on 1790 men, and to the meta-analytical approach it was possible to demonstrate for the first time the effects of the *FSHR* pN680S polymorphism alone on testis volume, serum FSH, inhibin B and testosterone levels. The effect was of small entity (effect of the Ser allele on testis volume: -1.40 mL) but significant. One reason why it was not demonstrated earlier could be that, apart from the sample size effect, it might have confounded by the *FSHB* -211G>T SNP effect in the previous studies based on much smaller subject numbers (62).

No data concerning the *FSHR* -29G>A SNP in normal males are available so far.

FSHB

The -211G>T SNP in the *FSHB* promoter was demonstrated to have a strong effect *in vitro* on transcription by luciferase reporter assay, with the T allele showing a relative activity which was only half of that of the G allele (34). Together with the evidence that the haplotype structure of the *FSHB* gene might be subjected to balancing selection (26) and, thereby, influence reproductive parameters, and the high evolutionary conservation across species of the promoter region including rs10835638, this induced the analysis of whether *FSHB* -211G>T polymorphisms could be associated with serum FSH levels. A seminal study in young male volunteers of Baltic origin demonstrated that this SNP indeed influenced serum FSH levels and other reproductive parameters. In particular FSH levels in homozygous *FSHB* -211G>T T carriers were significantly reduced compared to both heterozygous and homozygous G carriers (11). This was then amply confirmed in German and Italian men (63, 64). In accordance with the epidemiological and *in vitro* data reported above, homozygous carriers of the

minor allele represent only 1.5% of men with normal semen parameters but have serum FSH levels which are about 25% lower than homozygous, major allele carriers (11, 63, 65). In addition, this polymorphism is associated with lower testicular volume, lower sperm count, lower testosterone and higher LH serum levels (11, 63-66).

The association of *FSHB* -211G>T polymorphism with serum FSH and other reproductive parameters in men prompted to a reassessment of the (mild) effects of exon 10 *FSHR* polymorphisms in the male In an elegant study Tüttelmann et al (62) showed that when men are stratified by the *FSHB* genotype, significant differences in serum FSH levels and testicular volume between carriers of the different *FSHR* genotype become evident. This study demonstrated for the first time that the *FSHR* polymorphism effect is indeed present also in the male, although it can be masked by the *FSHB* polymorphism, suggesting, in addition, that the interplay between polymorphisms in hormone and receptor is of relevance under physiological conditions. Taking into account both *FSHR* and *FSHB* and considering their allelic frequencies, the authors suggested that carriers of the potentially "unfavorable" allele combination (i.e. those associated with lower testis volume and serum FSH levels) represent about 45% of all males, have smaller testes and could be at risk for reduced reproductive fitness (62).

In summary, the current evidence shows that both *FSHR* and *FSHB* genotypes are physiologically very relevant and interact with each other to determine gonadotropin levels in both sexes. So far only few studies assessed the effects of the combination of rs6166 in *FSHR* and rs10835638 in *FSHB* (62, 56, 57). This combination results in nine different genotype combinations, which have been demonstrated to be associated with significantly different serum FSH levels (56, 62). The matter is further complicated by the possible effects of the *FSHR,* -29G>A SNP, influencing levels of *FSHR* expression: only one study considered the combination of rs6166 and rs1394205 in women (54). The combination of these three SNPs would result in 27 combinations (Table 3). Among them, those predicted to be associated with the "less favorable" *FSHB/FSHR* genotype combinations (in terms of serum FSH levels and FSHR expression/sensitivity) are expected in about 20% of Caucasians. Very

large number of subjects will be necessary to address systematically the pathophysiological relevance of these allele combinations and to attempt a meaningful pharmacogenetic approach.

The *FSHR* and *FSHB* genotypes associated with lower FSH levels and/or less sensitive FSHR are enriched in human infertility

Ovulation disorders

Polycystic ovary syndrome (PCOS) represents an intriguing and frequent form of infertility and anovulation, object of intensive research. Given the role of gonadotropins in ovarian physiology, the association with the *FSHR* gene SNP c.2039A>G has been repeatedly investigated in several populations. Overall, there seems to be a selective enrichment of the c.2039A>G G allele in PCOS and a meta-analysis including 1028 PCOS and 3587 controls demonstrated a mild protective effect of the homozygous c.2039A>G A allele (67). There is evidence that the c.2039A>G G genotype is more frequent in ovulation disorders, infertility and poor or exaggerated FSH response in ART (36, 42, 59, 60, 68-74) (Supplementary Table 1). Interestingly, two genome-wide association studies in Chinese women identified, among other interesting candidates, the *FSHR* and the *LHCGR* as risk loci for PCOS (75, 76). Another, very carefully conducted, genetic association study based on selected, haplotype-tagging SNPs, confirmed these findings in women of European origin (77). In the case of the *FSHR* both a SNP 5.3 kb upstream of the gene, and rs6165 (p.T307A) were nominally strongly associated with PCOS. Therefore, evidence is accumulating that the genomic region of chromosome 2 encompassing *FSHR*, *LHCGR* and *GTF2A1L* (general transcription factor IIA, 1-like, involved in gametogenesis) confers susceptibility to PCOS and deserves further intensive, functional and genetic research. Experimentally, none of the existing mouse models recapitulate the whole spectrum of the human PCOS but, remarkably, overexpression of LH/hCG activity, androgen excess and reduced aromatization/estrogen action reproduce the ovarian cystic phenotype in mice (78). Together, these evidences identify (maybe not surprisingly) altered gonadotropin action as the most plausible determinant of the ovarian phenotype of PCOS. Women with PCOS may be treated with clomiphene or gonadotropins for ovulation induction. However, no prospective, pharmacogenetic trial on ovulation induction in PCOS based on the *FSHR* genotype has been performed so far.

Concerning the FSHR c.-29G>A an association was reported in Indian patients with poor ovarian response (41) as well as with primary and secondary amenorrhea (79). No data are available about the possible role of the *FSHB* c.-211G>T in female infertility. Understanding the molecular events related to the genetic *FSHR* (and *LHCGR*) variants will be essential for a pharmacogenetic approach to PCOS treatment.

Male infertility

Most studies failed to find any correlation between *FSHR* genotype and male infertility phenotype (reviewed in 58 and 80). Conversely, the rare, *FSHB* -211G>T homozygous T genotype was reported to be significantly overrepresented in infertile men (66). Whether the *FSHR* -29G>A SNP is of any relevance in the male remains controversial. A small effect of *FSHR* -29G>A A allele on testis volume was reported in a small group of infertile men (81) but not replicated so far. The original description of an association of *FSHR* haplotypes including -29G>A, 919A>G and 2039A>G with male infertility (82) has not been confirmed by the subsequent literature (80) and, for the time being, there is no evidence that the study of the -29G>A SNP adds any useful information to the *FSHB* -211G>T - *FSHR* c.2019A>G combination in the male.

Pharmacogenetic studies in men

Male idiopathic infertility remains a pathophysiological dilemma and a therapeutic challenge. In the ART era, most forms of male infertility are empirically resolved by intracytoplasmic sperm injection (ICSI) but, given the burden (not only economical) of the procedure, the question is whether sperm quality can be improved by a less invasive, medical intervention. Owing to the role of FSH in spermatogenesis, FSH treatment has always been tempting and is actively prescribed by many doctors in spite of the lack of evidence of its superiority compared to placebo. Several studies suggest that FSH treatment might be useful in a subgroup of normogonadotropic infertile men (83), purportedly those without spermatogenic blockade, but these men are impossible to identify beforehand. Could knowledge of the *FSHB* and/or *FSHR* genotype be helpful here for?

519

520 So far only two studies addressed the question whether a pharmacogenetic approach would be helpful
521 in identifying responders to FSH treatment. One study showed that patients with at least one *FSHR*
522 c.2039A>G G allele had a significant increase of total sperm count after 3 months of treatment with
523 rFSH (150 IU/thrice per week) (84). Another study considered the *FSHB* -211G>T genotype and
524 showed that TT homozygotes, representing 25% of men with oligozoospermia and low FSH levels,
525 could significantly benefit from FSH treatment (65). No study addressed the combination of the two
526 genotypes so far. Since both *FSHB* and *FSHR* genotypes affect testis volume and serum FSH levels
527 (62) it would be interesting to investigate whether normogonadotropic, oligozoospermic men carrier
528 of the less favorable SNP combinations could improve fertility upon FSH treatment. However, sperm
529 parameters are naturally quite variable within the same individual and the problem of the correct (and
530 robust) primary end point in such a study remains unresolved. Studies are ongoing assessing whether
531 sperm DNA fragmentation, believed to be ameliorated by FSH, changes upon FSH treatment in
532 dependence of the genotype (EudraCT 2010-020240-35).

533

534 **Conclusions and outlook**

535 In the light of the actual knowledge, is there any potential for the pharmacogenetic use of FSH in
536 infertility treatment (male and female)? Overall, the available *in vitro* an *in vivo* data support the
537 physiological relevance of the considered SNPs for FSH action. The genetic complexity thereof,
538 illustrated by the four SNPs considered here, is probably much higher than supposed so far and only
539 studies considering the *FSHB*/*FSHR* genotype combinations with numbers of subjects large enough in
540 dependence of the frequency of each genotype in a given ethnic group, will be able to assess this
541 issue. Efforts should be dedicated to identify subjects with the “less favorable” variant combination,
542 expected to be overrepresented among infertile patients, and assess whether they would be candidates
543 for FSH treatment and/or usage of higher FSH doses in case of ART or ovulation induction. This,
544 however, needs to be proven in large, prospective studies, objectively difficult to conduct in the ICSI
545 era. Nevertheless, FSH therapy might become an interesting medical option, especially for treatment
546 of selected cases of male infertility, in which the reduction of sperm parameters is combined with a

547 "sloppy" *FSHB/FSHR* genotype combination and the female partner has normal ovarian function and
548 a good reserve. Interventional trials should be started with such couples, because successful medical
549 treatment of the man would relieve the woman of carrying the burden of male infertility.
550
551 There is another interesting and intriguing aspect to consider. When epidemiological, functional and
552 clinical data are considered together with the evolutionary aspects illustrated above, it appears that an
553 overall trend towards a less efficient FSH-FSHR system emerges in the human species. This poses an
554 interesting evolutionary biology question: which environmental conditions select the evolutionary
555 more recent *FSHB* and *FSHR* genotypes and are there unrecognized advantages for life-time
556 reproductive success for such genotypes which, counter intuitively, reduce fertility/fecundity?
557 Another example of evolutionary paradox is PCOS, a common polygenic condition linked to both
558 infertility and metabolic disturbances, which is steadily increasing in epidemiological relevance in
559 spite of reduced fertility (85). Since evolution maximizes reproduction other aspects may come into
560 play.
561
562 In a recent evolutionary study, the *FSHR* gene was identified as a determinant of human birth timing
563 (86) suggesting its association in as yet unclear processes involved in shortening gestation time and
564 accelerating parturition in the human species. Although a driving evolutionary role of the less
565 "sensitive" *FSHR* c.2039A>G G allele was postulated (35), there is no evidence that specific
566 polymorphisms the *FSHR* gene influence duration of fertile life. The possible role in pregnancy
567 duration remains intriguing. Other studies described association of the *FSHR* and its variants with an
568 increasing number of non canonical and non obviously FSH-related pathophysiological events, such
569 as osteoporosis (45), vasculogenesis (87), hypertension (33), ovarian (88) and testis cancer (89). All
570 these suggestions would question the dogma of the unique action of FSH at the gonadal level. The
571 expression of the *FSHR* in extragonadal tissues is currently matter of vivid debate and the possible
572 role of FSH in non canonical target organ remains enigmatic. Should perhaps the *FSHR* gene be
573 viewed as a marker of health at large and these apparently weird associations reconsidered with a
574 wider, evolutionary vision?

575

576 *FSHR* exon 10 homozygous Ser680 genotype was significantly associated with lower risk of
577 developing sporadic Azheimer disease (90) and with surviving over the age of 90 years in women but
578 not in men (91), independently of previous fertility, age of menopause or other known risk factors,
579 suggesting a role for the *FSHR* genotype as determinant of longevity. In our study in normal
580 menstrual cycle (35) we observed differences in menstrual cycle length between carriers of the two
581 *FSHR* haplotypes, resulting in about one menstrual cycle less per year in homozygous p.N680S S
582 compared to homozygous p.N680S N carriers. Since no difference in the age of menopause was
583 demonstrated so far, women with the homozygous p.N680S S genotype would experience 30–40
584 cycles less than women with p.N680S N genotype during their reproductive life and would be
585 exposed to a lower incidence of pregnancies and related risks. In the absence of medical assistance,
586 maternity-related lifetime risk, still relevant in underdeveloped countries, must have been very high in
587 the earlier times of human evolution, possibly making pregnancy-related death an important factor in
588 determining a evolutionary selection of this genotype. We speculated that fewer menstrual cycles
589 during the reproductive life span might represent an evolutionary advantage, provided that the fertility
590 of the species is maintained (35). Medical progress, better nutrition, contraception, and the recent
591 dramatic changes in women' role in modern society are modifying the current human reproductive
592 strategies, so that the less fertile genotype becomes epidemiologically relevant, especially in couples
593 attempting to conceive in their late thirties.

594

595 In conclusion, the current evidence suggests that the combination of *FSHR* and *FSHB* genotypes is
596 predicted to have a much stronger impact than either one alone, both on male and female gonadal
597 function, since about 20% of people are carrier of the allele combinations associated with lower serum
598 FSH levels and lower FSHR expression/activity (Table 3). The advent of powerful, sensitive and
599 inexpensive techniques assessing several SNPs simultaneously will be very helpful in the
600 identification of the patients at infertility risk. Interventional, prospective studies are eagerly needed
601 to investigate whether stratification of patients according to their *FSHR-FSHB* genotype combination
602 is of any advantage in the treatment of male and female infertility compared to the current, naïve

approach. In the absence of this, every pharmacogenetic approach to the clinical use of FSH is empirical and there is no hard evidence that it would be superior in terms of better outcome, reduced side effects, and/or pharmaco-economic impact.

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Both authors selected and reviewed the literature and prepared the manuscript. LC performed the *in silico* analyses and the *in vitro* experiments.

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Legend to Figures

Figure 1

Intron-exon structure, SNP positions and LD blocks in the human *FSHR* and *FSHB* genes. The LD blocks (dark triangles) were calculated by the software Haploview (http://www.broadinstitute.org) in the selection of 121 samples obtained from Utah residents with Northern and Western European ancestry from the CEPH collection (CEU). The darkness of the squares indicates the strength of LD. The SNPs are indicated by black lines above the LD blocks (arrows). Gene name, chromosome number and coordinates of the shown region, specified in the white boxes within each panel, were obtained from the HapMap database (http://hapmap.ncbi.nlm.nih.gov). Panel A) 200 Kb genomic region of chromosome 2 including the human *FSHR* gene. Exons are represented by boxes and the positions of SNPs rs6166 (c.2039A>G), rs6165 (c.919A>G) and rs1394205 (-29G>A) are indicated. B) 200 Kb genomic region of chromosome 11 including the human *FSHB* gene. The genomic region in the boxed area is enlarged in panel C. C) 5 Kb genomic region of chromosome 11 including the human *FSHB* gene and the position of SNP rs10835638 (-211G>T).

Figure 2

Geographic distribution of the rs6166 (FSHR c.2039A>G) allele frequencies. The world map with geographic coordinates show the distribution of the alleles A and G in different countries by pie charts, calculated by the HGDP Selection Browser (http://hgdp.uchicago.edu). The panel on the right side shows the allele frequencies by bar charts for each population sample from different regions of the earth. The data on which the

946 analysis is based are from the Stanford University SNPs selection from Human Genome
947 Diversity Project (http://www.hagsc.org/hgdp/files.html).
948
949 **Figure 3**
950 Allele and genotype frequencies of the *FSHR* exon 10 SNPs rs6166 (c.2039A>G; panel A)
951 and rs6165 (c.919A>G; panel B) in different geographic regions. The sample size is indicated
952 above each bar; data were analyzed by SPSmart (http://spsmart.cesga.es) using the databases
953 HapMap, 1000 Genomes, HGDP CEPH Stanford selection and Perlegen as data sources;
954 n.a.=data not avaiable.
955
956 **Figure 4**
957 Allele and genotype frequencies of the *FSHR* and *FSHB* promoter SNPs rs1394205 (-
958 29G>A; panel A) and rs10835638 (-211G>T; panel B) in different geographic regions. The
959 sample size is indicated above each bar; data were analyzed by SPSmart
960 (http://spsmart.cesga.es) using the databases HapMap, 1000 Genomes, HGDP CEPH
961 Stanford selection and Perlegen as data sources; n.a.=data not avaiable.
962
963 **Figure 5**
964 Kinetics of phospho-ERK1/2 activation in the absence (left panels) and in the presence (right
965 panels) of 50 nM r-FSH (Gonal-F, Merck-Serono S.p.A., Rome, Italy) in p.N680S
966 homozygous -N or -S human primary lutein granulosa cells (hGLC), detected by Western
967 blotting. Cell preparation was performed as previously described (31) and specified in
968 supplementary methods. One representative of three independent experiments is shown.
969 hGLC carrying the p.N680S N homozygous genotype shown potent and rapid ERK1/2 FSH-
970 dependent phosphorylation. hGLC homozygous for the p.N680S S genotype response to FSH

971 was much lower and delayed in terms of ERK1/2 activation. PMA indicates the ERK1/2
972 activator phorbol myristate acetate (positive control); U0126 is a MEK (MAPK kinase)
973 inhibitor (negative control).
974

Table 1: Nomenclature of genes and polymorphisms discussed in this article (http://www.ncbi.nlm.nih.gov/pubmed).

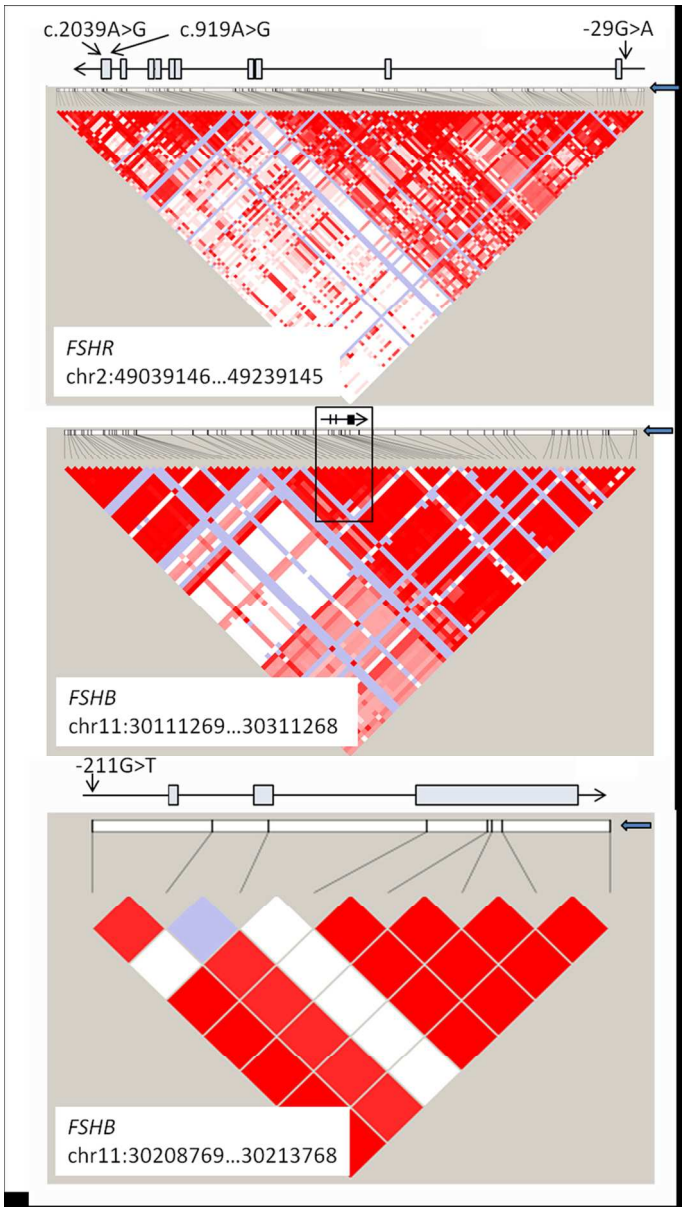
Gene	refSNP	DNA Nucleotide	Single nucleotide variation	NCBI nucleotide ref. sequence	Protein	NCBI protein ref. sequence
FSHR	rs1394205	-29G>A	g.49381585C>T	NT_022184.15		
FSHR	rs6165	c.919G>A	g.49191041C>T	NT_022184.15	p.A307T	P23945.3
FSHR	rs6166	c.2039A>G	g.49189921T>C	NT_022184.15	p.N680S	P23945.3
FSHB	rs10835638	-211G>T	g.30252352G>T	NT_009237.18		

Table 2: Pairwise linkage disequilibrium (LD) parameters between the polymorphisms rs6165 and rs6166 in HapMap populations calculated by the software Haploview (http://www.broadinstitute.org). Population names and their abbreviation (in brackets), sample size and minor allele frequencies (MAF) of rs6166 (*FSHR* c.2039A>G) and rs6165 (*FSHR* c.919A>G) were taken from the HapMap database (http://hapmap.ncbi.nlm.nih.gov). The reference allele is placed before each MAF value. The two LD values *D'* and r^2 are shown. *D'* is calculated by Haploview as $D' = D/D_{max}$, where D is the deviation of the observed from the expected. r^2 is the correlation coefficient between pairs of loci. The maximum values of *D'* and r^2 is 1.000, which indicates complete LD or pairwise correlation between the loci, respectively. *D'* and $r^2 = 0.000$ indicates random coupling of the SNPs.

HapMap populations	Sample size	rs6166 MAF	rs6165 MAF	*D'*	r^2
African ancestry in Southwest USA (ASW)	53	G 0.411	A 0.325	1.000	0.362
Luhya in Webuye, Kenya (LWK)	110	G 0.350	A 0.209	1.000	0.143
Maasai in Kinyawa, Kenya (MKK)	156	G 0.374	A 0.436	0.979	0.445
Yoruban in Ibadan, Nigeria (YRI)	153	G 0.493	A 0.224	0.961	0.251
Utah residents with European ancestry (CEU)	121	G 0.403	G 0.403	1.000	1.000
Tuscan in Italy (TSI)	102	G 0.475	G 0.470	1.000	0.977
Gujarati Indians in Houston, Texas (GIH)	101	A 0.490	A 0.490	1.000	0.978
Han Chinese in Beijing, China (CHB)	139	G 0.281	G 0.312	1.000	0.865
Chinese in Metropolitan Denver, Colorado (CHD)	109	G 0.303	G 0.321	1.000	0.924
Japanese in Tokyo, Japan (JPT)	116	G 0.348	G 0.363	0.974	0.902
Mexican ancestry in Los Angeles, California (MEX)	58	G 0.328	G 0.316	0.956	0.876

Table 3: Expected genotype frequencies in Caucasians of the 27 allele combinations resulting from rs6166 (FSHR p.N680S), rs1394205 (FSHR -29G>A) and rs1083563 (FSHB -211G>T) frequencies collected from the HapMap database (http://hapmap.ncbi.nlm.nih.gov) based on 121 CEU subjects. Shadowed areas indicate the *FSHB*/*FSHR* genotype combinations associated with lower serum FSH levels and lower FSHR expression and activity.

rs6166		**AA**			**GA**			**GG**		
rs1394205		**GG**	**GA**	**AA**	**GG**	**GA**	**AA**	**GG**	**GA**	**AA**
rs10835638	**GG**	13,82	10,26	2,85	19,01	14,12	3,93	6,21	4,61	1,28
	GT	4,15	3,08	0,86	5,71	4,24	1,18	1,86	1,38	0,38
	TT	0,20	0,15	0,04	0,27	0,20	0,06	0,09	0,07	0,02



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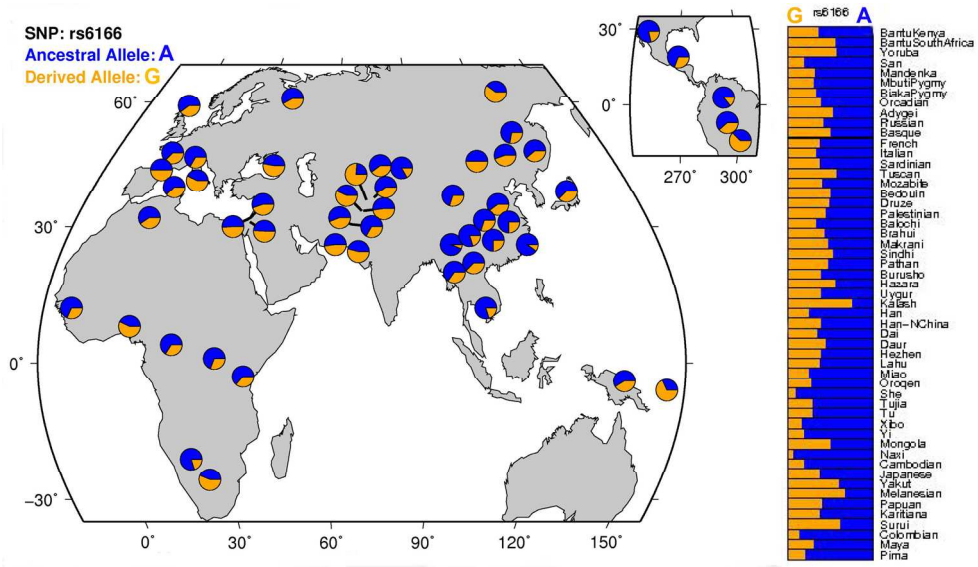

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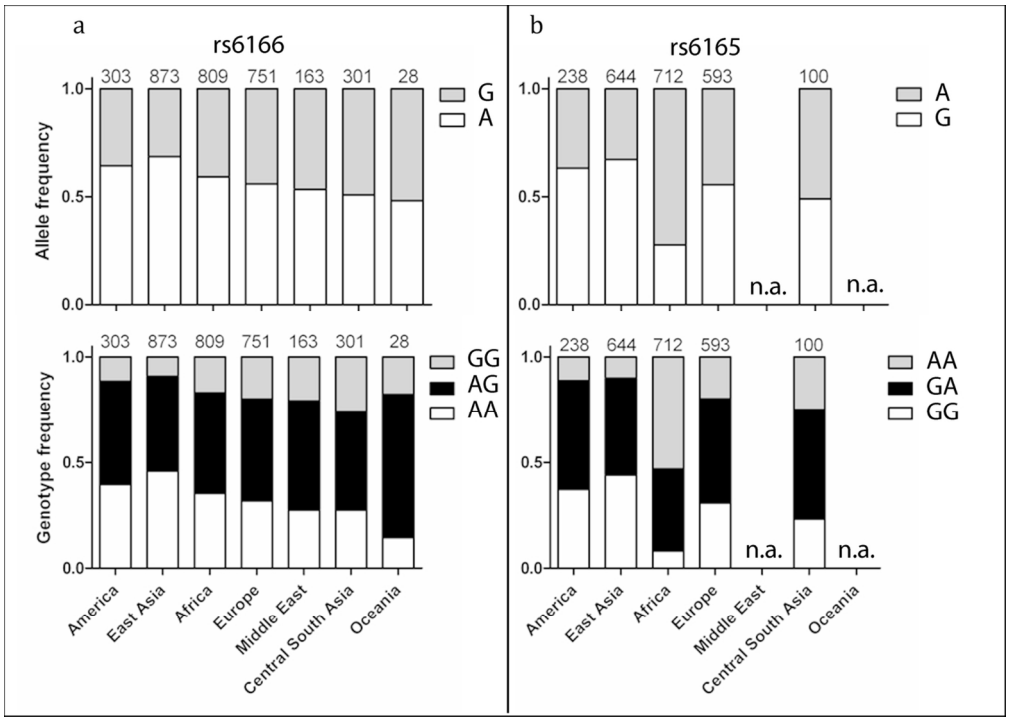

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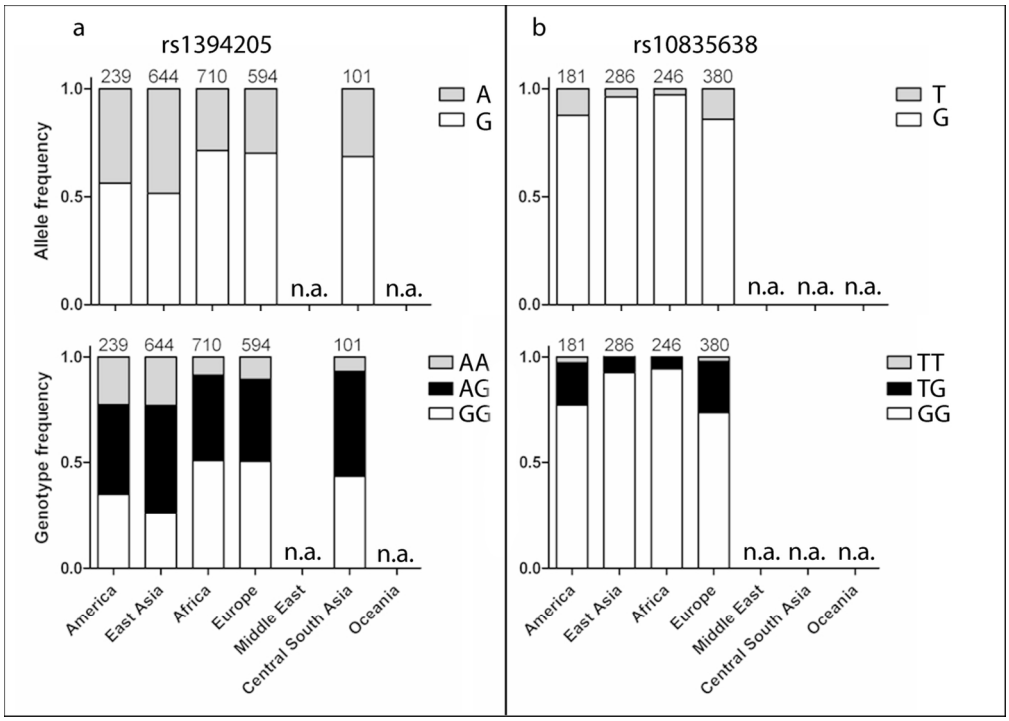

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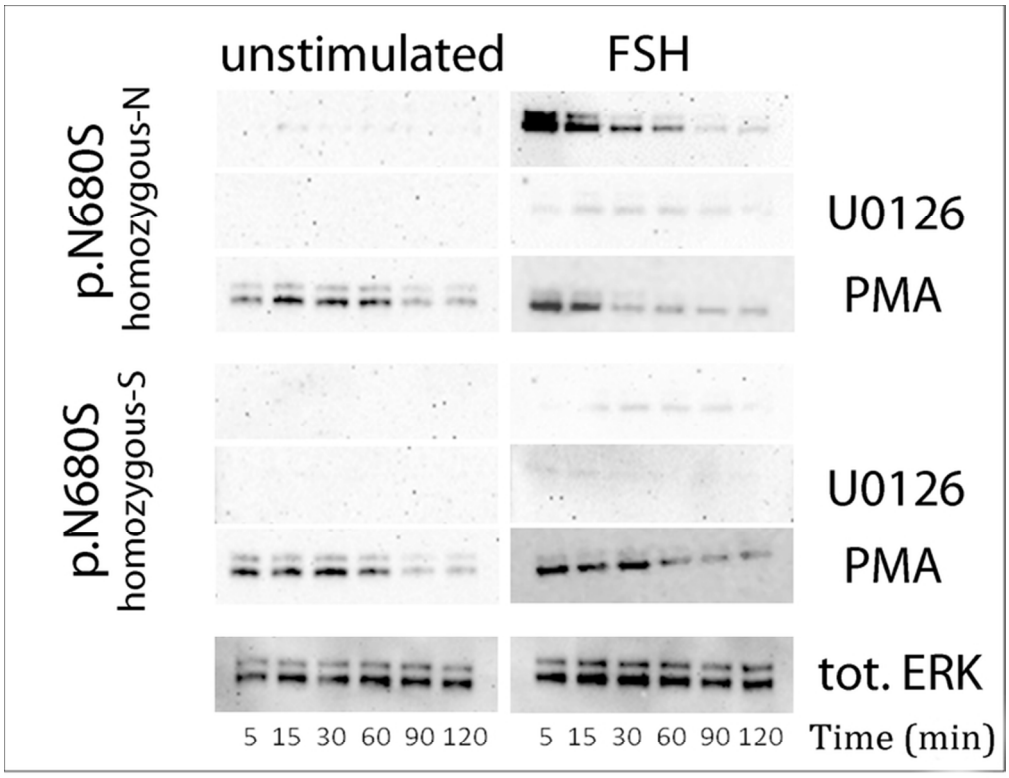

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