Running title: 5-HT related gene variants and pregnancy

Genetic polymorphisms of serotonin transporter and receptor 1A could influence success during embryo implantation and maintaining of pregnancy

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CAPSULE

Exploratory genetic analysis of functional polymorphisms 5-HTTLPR and rs6295 reveals clinical implications during early pregnancy loss events in recipients undergoing IVF treatments using donated oocytes.

ABSTRACT

Objective: To explore whether serotonin-related gene polymorphisms influence clinical outcomes of in vitro fertilization (IVF) treatment in recipients using donated oocytes.

Design: Nested case-control study.

Setting: University-affiliated infertility clinic.

Patient(s): Two hundred forty-five women undergoing IVF treatment with donated oocytes.

Intervention(s): None.

Main Outcome Measure(s): Genotype and haplotype analysis of the serotonin transporter linked polymorphic region (5-HTTLPR), rs1800532, rs6295, rs6313 and rs3813929, between recipients grouped according to the results of the oocyte donation IVF treatment.

Result(s): No differences were found between genotype distribution of the Tryptophan hydroxylase 1 (TPH1), serotonin receptor 2A (5-HT2A) and serotonin receptor 2C (5-HT2C) polymorphisms. Recipients carrying LL genotype for 5-HTTLPR suffered lower clinical pregnancy (CP) rates and higher biochemical pregnancy loss (BPL) events. Lower implantation rates (IR) were found in CC carriers for 5-HT1A.rs6295 who also presented higher BPL rates. A lower incidence of CP was observed for *LC* haplotypes, corresponding to an increase in BPL rates.

Conclusion(s) A strong association was found between early pregnancy loss and recipients carrying the 5-HTTLPR and rs6295 genetic variants. Identifying biological processes involving serotonin and embryo implantation may help to understand the dynamics of the maternal-embryo dialogue.

Key Words: 5-HTTLPR, 5-HT1A, polymorphism, biochemical pregnancy loss, serotonin.

INTRODUCTION

Serotonin or 5-hidroxytryptamine (5-HT) comprises a highly connected molecular entity, involved in many different functions and widely detected in the reproductive tract of humans and other mammals (1–5). A serotonergic network has been described interacting between ovaries, oocytes and developing embryos (6,7). Animal models reflect that uterus could be sensitive to changes in 5-HT homeostasis, what could also affect embryo viability and maintenance of pregnancy (1,8–10). Furthermore, fine regulation is suspected since both high doses of subcutaneously administered serotonin and 5-HT deprivation interrupt decidualization and maintenance of pregnancy (11,12).

Many effectors of the 5-HT related pathways have been identified in the reproductive tract of mammals (7), what add a significant clinical value since many drugs are available for modifying and controlling 5-HT fluxes [Supplemental figure 1]. However, the study of molecular interactions surrounding the embryo-maternal interface in humans presents a great barrier because of obvious conditioning limitations for experimentation. The study of genetic variants related to 5-HT metabolism and their distribution between recipients, grouped according to reproductive outcomes obtained after an in vitro fertilization/intracytoplasmic sperm injection (IVF-ICSI) with donated oocyte, represents a suitable way to normalize other factors like quality of oocytes, embryos and sperm, isolating the potential effect of selected gene variants over 5-HT metabolism during the onset of the pregnancy.

The 5-HT transporter (5-HTT or SERT) is responsible for controlling extracellular levels of 5-HT and is expressed in the human endometrium (13). SERT could act during pregnancy since it has been shown that pregnant women treated with serotonin selective re-uptake inhibitors (SSRI) present an increased risk of spontaneous abortion (14,15). Although some evidence supports these observations, the effects of SSRIs on achievement of pregnancy may have passed unnoticed since these studies recruited women who were already pregnant or who had a spontaneous abortion. A candidate polymorphism to assess SERT implication is the 44 bp

insertion / deletion located at the 5-HTT gene-linked polymorphic region (5-HTTLPR), which modifies transcription rates of the solute carrier family 6 member 4 gene (SLC6A4) and therefore the activity of the transporter SERT in different cell types, like in the human placenta (16).

The polymorphism A(218)C (rs1800532) is associated with tryptophan hydroxylase-1 (TPH1) protein, the rate-limiting enzyme of tryptophan that is the direct precursor of 5-HT (6). TPH1 detected in the endometrium, fallopian tubes and ovaries (17), activity of which has been reported to be essential for maintaining the early embryo development (9), represents a potential source of 5-HT in the uterus.

As a functional 5-HT signaling system could be suspected from the wide range of serotonin specific receptor subtypes identified in the uterus, ovaries, oocytes and embryos (6,7,17–19), several candidate polymorphisms were selected in order to assess their implication. The polymorphism C(-1019)G in the 5-HT1A gene promoter seems to affect the expression of the 5-HT1A receptor and its binding with 5-HT (20). The polymorphism T(102)C has been related with different expression rates of the 5-HT2A gene and thus also with different densities of the 5-HT2A receptor (21). Finally, the functional polymorphism C(759)T in the gene 5-HT2C is involved in DNA-protein interaction, altering the self-expression of the receptor (6).

The genetic variants associated with 5-HT metabolism were used in this study to explore the influence on implantation and IVF results, focusing on pregnancy wastages at different stages using an oocyte donation model.

MATERIAL AND METHODS

Patients

We conducted a nested case-control study between July 2009 and February 2011 at the Instituto de Fertilidad Clinica Rincon, involving 245 female recipients who underwent a first IVF

treatment with donated oocytes. The inclusion criteria were not having received any previous embryo transfer (ET) cycle through oocyte donation, age 18 to 45 years, and receiving a transfer of at least two good quality embryos defined as four to five cells on Day 2 or eight on Day 3, symmetric blastomeres and less than 10 % of total fragmentation. Recipients were excluded from this study if they presented an abnormal endometrial thickness, hydrosalpynx, body mass index >30 kg/m^2, uterine disorder, or severe male factor like azoospermia or cryptozoospermia. The recipients provided signed informed consent to IVF treatment approved by the Spanish Society of Fertility and also for providing samples required. The consent for the genetic study was approved by the institutional ethics review committee of the Instituto de Fertilidad Clinicas Rincón and by the ethics committee of the university of Malaga, under whose auspices the present study was carried out.

Oocyte donors

The donors selected were healthy women with a good physical and mental state, younger than 35 years of age, with regular menstrual cycles, no prior family history of hereditary problems or chromosome abnormalities, a body mass index between 18 and 29 kg/m^2, and a favorable testing for HIV, cytomegalovirus, hepatitis B and C, and karyotype. Donors were not accepted if they had an abnormal ovarian response to gonadotropins, polycystic ovary syndrome, endometriosis, more than one previous miscarriage, or any medical or gynecological disorder that could compromise the success of the procedure. The donors were given an explanation and provided their specific consent for the oocyte donation before starting the procedure.

Ovarian stimulation, ovum pick-up, and ICSI.

Controlled ovarian hyperstimulation (COH) was developed under a protocol with gonadotropin-releasing hormone antagonists (GnRH). A fixed initial dose, between 150 and 300 IU, of human recombinant FSH (rFSH) (Gonal-F®; Merck-Serono, Barcelona, Spain) was used daily for the first 2-5 days, depending on age, body mass index, antral follicle count and ovary volume. The dose was then adjusted depending on the ovarian response and serum E_2. Once

the dominant follicle had reached a size of 14 mm, GnRH antagonists (Orgalutran; Organon, Barcelona, Spain) were also given, at a daily dose of 0.25 mg until the day of induction of ovulation. When at least three follicles reached 17 mm, ovulation was induced by the administration of a bolus of 0.4 mg Leuprolide Acetate (Procrin; Abbot S.A., Madrid, Spain). Oocyte retrieval was done exactly 35 hours after the administration of hCG in the donors. Decumulation was done by brief exposure to 40 IU/mL of hyaluronidase in Quinns Advantage® Medium with HEPES (SAGE, Trumbull, CT, USA) supplemented with albumin (SAGE, Trumbull, CT, USA), followed by mechanical disaggregation. After a maximum of 2 hours post-decumulation mature oocytes in metaphase II were inseminated by ICSI and were kept in culture individually in 35 µl droplets of equilibrated Quinn's Advantage cleavage medium (SAGE, Trumbull, CT, USA) covered with equilibrated mineral oil (SAGE, Trumbull, CT, USA), until day +2 or day +3. If the culture was prolonged beyond day +3 but not beyond day +5 the droplets were replaced by 35 µl Blastocyst (SAGE, Trumbull, CT, USA), also under equilibrated mineral oil (SAGE, Trumbull, CT, USA). The culture was stored in Minigalaxy A incubators (RSBiotech) at a hypoxic atmosphere of 5% O_2 and 6% CO_2.

Evaluation of the fertilization and embryo development

Fertilization was assessed 16-18 hours after ICSI. Zygotes were considered properly fertilized if two symmetric pronuclei were identified at the centre. Asymmetry, an excentric position or a great distance between the pronuclei were considered poor markers in the evaluation of the fertilization (22). Embryo quality was later assessed on day +2 and day +3, considering the rate of division, symmetry between blastomeres, and the extension and type of fragmentation, as well as the presence or absence of multinucleation. Embryos considered to be of good quality on day +2 were those that presented 2-4 symmetric cells and less than 15% fragmentation without multinucleation. Good quality embryos on day +3 were those that presented 6-8 cells and less than 20% fragmentation. Day +2 or day +3 ET and the surplus embryos were cryopreserved by vitrification on day +3 or at the blastocyst stage (23). The two

highest quality embryos were selected according to the criteria mentioned above for the transfer.

Endometrial preparation of the recipients

The endometrial preparation was undertaken as described previously (24). The suppression of the ovarian function in the recipients was done during the luteal phase with a single dose of GnRH agonist depot formulation (Decapeptyl; Ipsen Pharm, UK or Gonapeptyl; Ferring, Madrid, Spain). Ultrasound scan was performed to verify that ovarian suppression had taken place and then estradiol valerianate (EV) (Progynova; Schering, Madrid, Spain) was given, with each recipient receiving an increasing dose from 2 up to 6 mg daily. The luteal phase support in the recipients was achieved by the application of 600 mg/day of micronized progesterone vaginally (Progeffik, Effik Laboratories, Madrid, Spain), starting from the day of oocyte retrieval from the donor. The transfer was performed provided the endometrium had a typical trilaminar pattern and was at least 6 mm thick.

DNA preparation and genotyping

Genomic DNA was extracted from a buccal swab collected from the recipients and using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Different genotyping procedures were applied according to the nature of the gene markers, including the following SNPs: rs1800532, rs6295, rs6313, rs3813929 and the insertion/deletion 5-HTTLPR variant of the SLC6A4 gene. Each plate included replicate samples for quality control, which had to be in absolute concordance; negative controls were also assessed.

SNPs

Genotyping of the SNPs was performed by multiplex minisequencing preceded by multiplex PCR (25), using the following steps:

1) Amplification of regions flanking the SNPs was achieved by multiplex PCR reactions in a 2720 Thermal Cycler® (Applied Biosystems, UK) under programmed conditions of 94°C for 5

minutes, followed by 35 cycles for 30 s at 94°C, 30 s at 58°C, 30 s at 72°C, and a final extension for 7 min at 72°C. In order to eliminate the excess of primers and dNTPs, the PCR products obtained were digested by an 'ExoCiAP' mix consisting of 2 units/10 µL PCR E. coli exonuclease I (TAKARA BIO INC, Japan) and 5 units/10 µLPCR alkaline phosphatase (TAKARA BIO INC, Japan) incubated at 37°C for 60 minutes and a final step of enzymatic inactivation by heating at 80°C for 20 minutes.

2) A mini-sequencing primer with the 3'-end adjacent to the target SNP was designed to anneal specifically with the PCR product of each locus [Supplemental Table 1]. A reaction mix containing PCR product, the mini-sequencing primer mix and SNaPshot® multiplex kit (Applied Biosystems, UK) was subjected to the following conditions: 40 cycles at 10 seconds at 96°C, 7 seconds at 50°C, and 30 seconds at 60°C. 1 unit of alkaline phosphatase (TAKARA BIO INC, Japan) was added to purify the sample and incubated at 37°C for 1 hour and immediately after at 80°C for 20 minutes.

3) Analysis of purified mini-sequencing products was performed by capillary electrophoresis using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, UK) using LIZ120® size standard (Applied Biosystems, UK). The resulting data were analyzed with GeneMapper™ 4.1 Software (Applied Biosystems, UK).

Insertion/deletion 5-HTTLPR variant

To detect the 5-HTTLPR polymorphism a PCR assay was performed using Go Taq® Flexi DNA Polymerase (Promega, USA). Amplification was achieved in a 2720 Thermal Cycler® (Applied Biosystems, UK) with running conditions of 5 minutes at 94°C, followed by 25 cycles for 30 seconds at 94°C, 45 seconds at 58°C, 1 minute at 72°C, and a final extension for 7 min at 72°C.The fluorescently labeled products were resolved by capillary electrophoresis on an ABI PRISM 3130 Genetic Analyzer and GeneMapper™ ver. 4.1 software (Applied Biosystems, UK).

Clinical outcomes

Biochemical pregnancy was determined by quantifying the levels of human chorionic gonadotropin (β-hCG) in two consecutive measurements from day 11 or 12 after embryo transfer, depending on whether this had taken place on day +3 or day +2, respectively. Ultrasound observation of a gestational sac 5-6 weeks after embryo transfer together with the positive previous β-hCG test was considered to represent a clinical pregnancy (CP). The absence of ultrasound-identifiable pregnancy and a drop in the levels of β-hCG represented a situation of biochemical pregnancy loss (BPL). An ongoing pregnancy (OP) was defined as the presence of at least one viable fetus around week 12 of gestation. Miscarriages (M) were considered to be those gestational losses that occurred between weeks 4 and 20, having previously identified a gestational sac (26). The implantation rate (IR) was calculated as the ratio between gestational sacs found on ultrasound scans and the number of embryos transferred. Those recipients with no evidence of pregnancy after the treatment were defined as the non-pregnant group.

Statistical analysis

Recipient were grouped by genotype for each polymorphism, thus proportion of CP, OP and NP were calculated by embryo transfer cycle; BPL rates were calculated by pregnant recipient with positive β-hCG; proportion of M rates were calculated by CP. Chi square test was performed to determine the Hardy-Weinberg equilibrium. For each polymorphism recipients were pooled by genotype, then analysis of the variance (ANOVA) was performed to compare differences in age and Chi square test or Fisher's exact test were used to calculate statistical differences in reproductive outcomes between groups of recipients. A logistic regression model was used to compute the odds ratio (OR) with its 95% confidence interval, and thus the degree of association with each genotype to use as variables predicting the outcome of the treatment. In order to select the fittest interaction model between the genotypes, we considered the Akaike information criteria (AIC) and the Bayesian information criteria (BIC) in each

polymorphism, using the SNPstats bioinformatic tool (27). For the haplotype analysis, estimated relative frequencies of each haplotype were compared through a logistic regression model, similar to that employed with the genotype analysis.

RESULTS

The study included 245 Caucasian recipients aged 40.27 ± 4.09 years [Table 1]. The indications for oocyte donation were advanced age (56.1%), ovarian failure (23.4%), low response to gonadotropins (12.4%), and endometriosis (4.1%).

The genotype frequency distribution of different polymorphisms complied with Hardy-Weinberg equilibrium in each group, except in the NP recipients for the 5-HTTLPR polymorphism. Minor allele frequencies (MAF) were greater than 0.05 and no linkage disequilibrium was found between the polymorphisms studied. IR and rates of CP, BPL, M and OP did not vary between genotypes of recipients for TPH1-A218C, 5-HT2A-T102C and 5-HT2C-C759T polymorphisms [Table 2].

Different CP rates between 5-HTTLPR genotypes were found (P=0.014) [Table 2]. The dominant model for the S allele showed that the LL genotype was significantly increased in recipients who did not achieve a CP [Table 3].

The BPL rates presented strong differences for 5-HTTLPR marker distribution (P=5.82 × 10^{-4}) and for 5-HT1A-C1019G polymorphism (P=0.012), this latter also presented different distribution when comparing IR for different genotypes (P=0.011) [Table 2].

Logistic regression model corroborated a negative tendency of recipients carrying individually the LL or the CC genotype, what correlated with a strong risk of experiencing BPL as compared with the recipients who had the genotypes SL/SS or CG/GG respectively [Table 3].

The haplotype analysis of the 5-HTTLPR and 5-HT1A-C1019G polymorphisms showed significant differences when the proportion of women who achieved a CP was compared with those who did not; more specifically, the haplotype LC was associated with the condition of

not achieving a clinical pregnancy [Table 4]. A higher representation of recipients homozygous for both L and C alleles was found in the women who suffered BPL (30.77%) compared with the recipients who became clinically pregnant (3.97%). Thus, the haplotype LC was also strongly associated with experiencing BPL [Table 4].

These results agreed in both the genotype and the haplotype analysis, indicating a negative trend of the genotypes LL at 5-HTTLPR and CC at 5-HT1A-C1019G in reproductive outcomes in recipients undergoing oocyte donation IVF treatment and also for maintaining the pregnancy when this occurred.

DISCUSSION

The results of this study show a significant association of genotype variants at 5-HTTLPR and rs6295 with different results of IVF treatment with donated eggs. To our knowledge, this is the first time that gene variants involved in 5-HT metabolism have been shown to modify implantation potential. The alleles L of 5-HTTLPR and C of rs6295 are associated with lower CP rates and higher BPL rates, suggesting a negative effect on the earliest phases of embryo implantation. The strong association of the LL genotype at 5-HTTLPR with the risk of BPL highlights the importance of this gene during early implantation, which could thus lead to over-representation of this genotype in the group of NP recipients and the noncompliance with Hardy Weinberg equilibrium observed.

The increased expression rate of SERT induced by genotype LL (16) could hypothetically modify 5-HT levels in the embryo-maternal interface. As mentioned above, the dependence on a maternal supply of 5-HT has been reported during early embryo development (8–10) and later during the pregnancy (1). Two experimental procedures revealed an extreme decrease in the number of blastocysts recovered by embryo transfer when limiting maternal 5-HT biosynthesis through either blocking tryptophan production (9) or increasing its degradation (10). Furthermore, Doherty et al. (10) described a decreasing gradient of TDO gene expression from the inside of the stroma to the endometrial epithelium and lumen, which correlates with

the increase in 5-HT levels in the same direction. This non stochastic distribution of 5-HT suggests that regulatory elements could enhance embryo viability through secretion of 5-HT at the embryo-maternal interface and, by contrast, limit the interior tryptophan content to avoid immune rejection of the embryo (10). Monoamine oxidase A (MAO-A) activity, which also degrade 5-HT, is particularly intense in the endometrial epithelial cells during the mid-secretory phase of the menstrual cycle (28) and it has been associated with greater success in recipients receiving oocyte donation (29). From this, we hypothesize that an active regulatory system controls the 5-HT turnover in the endometrium.

Since SERT is expressed in the epithelial cells of the early decidua, it could represent the main agent responsible for the 5-HT clearance from the luminal side (13). The LL genotype would maintain an increased 5-HT turnover towards its assimilation into the endometrial cells, which could compromise 5-HT availability for the embryo (1). In fact, the pathological 5-HT depletion induced in mothers with phenylketonuria (PKU), which could represent a similar situation, is associated with a higher incidence of embryonic defects and spontaneous miscarriage (9), (30,31). The effect of SSRIs on SERT is expected to induce an increase of extracellular 5-HT levels, which would have a deleterious effect on the embryo (8) and, as mentioned above, could lead to a higher incidence of spontaneous abortions (14,15). Therefore, this could correlate with the necessity of a fine regulation of 5-HT levels to preserve the pregnancy during the early stages, or even sooner since SERT is active from the oocyte till the blastocyst stage (32).

The variant 1019 C>G of 5-HT1A seems to increase the binding affinity of the repressors Deaf1 and Hes5 to the DNA sequence when the C allele is present, hence reducing the expression of the receptor (33). Molecular interaction of the different allele combinations influences the number of 5-HT1A surface receptors, which in turn affects the secretion of 5-HT from the cell (34). Indeed, the CC genotype in the neuronal model seems to be associated with a lower density of the 5-HT1A receptor, resulting in a lower 5-HT induced response. In combination with the suggested effects of the *L/L* genotype in SERT, this could lead to a depression of

serotonergic activity during the implantation, when the embryo is most susceptible to the need for a maternal supply of 5-HT (9,10).

Interactions between polymorphisms have been reported to modify the regulatory activity between the two genes in 5-HTTLPR and in 5-HT1A in other tissues (35,36). Our results support the idea of synergic activity between the L and C alleles, as this haplotype was present in more than two thirds of the recipients who experienced BPL, whereas no differences were found in the haplotype distribution in those recipients who achieved CP. If the *L/L* and *C/C* genotypes are considered to be independent as far as their effect on pregnancy is concerned, the combination of both alleles is highly significant in the group of NP and BPL recipients, and they might have a synergic functional behavior that can be observed at the population level.

A possible relation between 5-HT polymorphisms and endometriosis can not be excluded because no distinction was performed between recipients; according with previous studies little effect could be expected for oocyte donation (37). Every recipient was recruited being less than 30 kg/m^2 of BMI, which is stated like a critical level from which previous studies reported increased risk of spontaneous abortions (38).

The high success of oocyte donation could underestimate the influence of the recipient's genetic profile. However, a strong interaction takes place between the expressed phenotypes of the recipient and the embryo during the onset of pregnancy that resembles an intense molecular dialogue involving immune-type molecular interactions and different functional couplings that alter the progress of the pregnancy (39).

Our results suggest a functional interaction between polymorphisms of SERT and 5-HT1A and the achievement or maintenance of early pregnancy. This interaction could be related with lower serotonergic interactivity due to an increased 5-HT re-uptake, which would reduce its intercellular concentration and could be enhanced by a decreased activity of the 5-HT1A receptor mediated by lower gene expression rates when the CC genotype is present. Although further research is still needed, the results open up a wide field of study and facilitate the

immediacy of a clinical trial, enabling other related studies about the role of serotonin in embryo-maternal tolerance.

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Table 1. Reproductive outcomes between recipients undergoing the study

Outcome	Patients	% (95% CI)

Number of cycles	245	-
β-hCG positive rate per cycle	164/245	66.9 (61.05–72.83)
Implantation rate	210/542	38.8 (34.64–42.85)
Clinical pregnancy rate per ET cycle	151/245	61.6 (55.54–67.72)
Ongoing pregnancy rate per ET cycle	126/245	51.4 (45.17–57.69)
Biochemical pregnancy loss rate per positive β-hCG	13/164	7.9 (3.79–12.06)
Miscarriage rate per clinical pregnancy	19/151	12.6 (7.29–17.87)
Ectopic pregnancy rate per positive β-hCG	7/164	4.3 (1.18–7.36)

Note: The overall group includes all women recruited for the study. CI = confidence interval.
%= percentage results. ET = embryo transfer.

Table 2. Age and clinical outcomes between recipients grouped by genotypes.

Marker	Genotype	Age (Y)	IR (%)	CP (%)	BPL (%)	M (%)	OP (%)
5-HTTLPR	S/S	41.1 ± 3.3	58/161 (36.0)	43/70 (61.4)	1/44 (2.3)	4/43 (9.3)	39/70 (55.7)
	S/L	40.2 ± 4.0	79/186 (42.5)	60/83 (72.3)	1/61 (1.6)	9/60 (15.0)	47/83 (56.6)
	L/L	40.1 ± 3.5	42/130 (32.3)	29/60 (48.3)	8/37 (21.6)	3/29 (10.4)	24/60 (40.0)
	P value	[c]0.227	0.165	[b]0.014	[a.b]5.82×10^{-4}	[a]0.739	0.103
TPH1 (rs1800532)	C/C	39.9 ± 4.1	66/188 (35.1)	51/86 (59.3)	3/54 (5.6)	7/51 (13.7)	41/86 (47.7)
	C/A	40.1 ± 4.4	107/259 (41.3)	73/114 (64.0)	4/77 (5.2)	7/73 (9.6)	64/114 (56.1)
	A/A	41.5 ± 3.0	29/77 (37.7)	20/35 (57.1)	3/23 (13.0)	3/20 (15)	17/35 (48.6)
	P value	[c]0.172	0.407	0.684	[a]0.367	[a]0.698	0.452
5-HTR1A (rs6295)	C/C	40.9 ± 3.4	37/118 (31.4)	28/55 (50.9)	6/34 (17.7)	5/28 (17.9)	22/55 (40.0)
	C/G	40.4 ± 3.8	95/261 (36.4)	71/116 (61.2)	3/74 (4.1)	7/71 (9.9)	61/116 (52.3)
	G/G	39.6 ± 5.0	72/149 (48.3)	47/66 (71.2)	1/49 (2.0)	6/47 (12.8)	40/66 (60.6)
	P value	[c]0.184	[b]0.011	0.073	[a.b]0.012	0.508	0.076
5-HTR2A (rs6313)	C/C	40.5 ± 4.3	55/150 (36.7)	38/67 (56.7)	5/43 (11.6)	3/38 (7.9)	34/67 (50.8)
	C/T	40.3 ± 4.3	100/267 (37.5)	72/119 (60.5)	4/76 (5.3)	9/72 (12.5)	61/119 (51.3)
	T/T	39.8 ± 3.6	45/107 (42.1)	34/49 (69.4)	1/36 (2.8)	5/34 (14.7)	27/49 (55.1)
	P value	[c]0.668	0.641	0.372	0.265	0.667	0.878
5-HTR2C (rs3813929)	C/C	40.1 ± 4.2	143/382 (37.4)	103/171 (60.2)	8/112 (7.1)	14/103 (13.6)	88/171 (51.5)
	C/T	40.9 ± 4.0	49/125 (39.2)	35/57 (61.4)	2/37 (5.4)	3/35 (8.6)	30/57 (52.6)
	T/T	40.3 ± 3.7	11/19 (57.9)	7/8 (87.5)	0.0	0/7 (0.0)	5/8 (62.5)
	P value	[c]0.445	0.200	[a]0.366	[a]1.00	0.564	[a]0.874

Note. Y=Years. Age values are represented by mean ± SD. IR=Implantation rates; CP=Clinical Pregnancy per ET cycle; BPL=Biochemical Pregnancy Loss per β-hCG positive; M=Miscarriage per CP; OP=Ongoing Pregnancy per ET cycle. Proportion of recipients that reached the outcome defined in rows are represented by each genotype (Percentage).

[a] *P* value by Fisher's exact test; otherwise by χ^2-test.

[b] *P* value < 0.05 obtained from either χ^2 test or Fisher's exact test.

[c] Analysis of variance (ANOVA)

Table 3. Genotype association for ins / del 5-HTTLPR and rs6295 with non clinical pregnancy outcome and biochemical pregnancy loss

				Non pregnancy vs Clinical pregnancy				Biochemical pregnancy loss vs Clinical Pregnancy			
Total	Gene Marker	Model	Genotype	NP	CP	OR (95% CI)	P-value	BPL	CP	OR (95% CI)	P-value
213	5-HTTLPR	Recessive	*S/S-S/L* *L/L*	50 (61.7%) 31 (38.3%)	103 (78%) 29 (22%)	2.20 (1.20-4.05)	0.010[a.b]	2 (20%) 8 (80%)	103 (78%) 29 (22%)	14.21 (2.86-70.60)	0.0003 [c.d]
237	5-HT1A.rs6295	Recessive	*G/G-C/G* *C/C*	64 (70.3%) 27 (29.7%)	118 (80.8%) 28 (19.2%)	1.78 (0.97-3.27)	0.063[a]	4 (40%) 6 (60%)	118 (80.8%) 28 (19.2%)	6.32 (1.67-23.91)	0.0077 [c.d]

Note: OR = odds ratio; CI = confidence interval; NP = non pregnant; CP = clinical pregnant; BPL = biochemical pregnancy Loss

[a] *P*-value from Chi-square test comparing genotype frequencies between non pregnant recipients and those with a clinical pregnancy

[b] *P* < 0.05

[c] *P*-value from Fisher's exact test comparing genotype frequencies between recipients suffering biochemical pregnancy loss and those with a clinical pregnancy

[d] *P* < 0.05

Table 4. Haplotype association between ins/del 5-HTTLPR and rs6295 markers and outcome

Markers		No pregnancy vs Clinical Pregnancy					Biochemical pregnancy loss vs Clinical Pregnancy				
5-HTTLPR	5-HT1A.rs6295	Total	Non pregnancy	Clinical pregnant	OR (95% CI)	*P*-value	Total	Biochemical pregnancy loss	Pregnant	OR (95% CI)	*P*-value
S	G	0.2831	0.2531	0.2988	1.00	---[a]	0.2888	0.0633	0.2988	1.00	---[a]
S	C	0.2407	0.2233	0.2547	1.12 (0.61 - 2.08)	0.7	0.2348	0.0937	0.2547	2.38 (0.20 - 25)	0.5
L	G	0.2393	0.2006	0.266	0.92 (0.48 – 1.75)	0.79	0.2533	0.1808	0.2660	4 (0.03 - 50)	0.26
L	C	0.2368	0.323	0.1805	1.92 (1.15 - 3.23)	0.012[b]	0.2231	0.6623	0.1805	14.23 (1.79 - 100)	0.013[b]

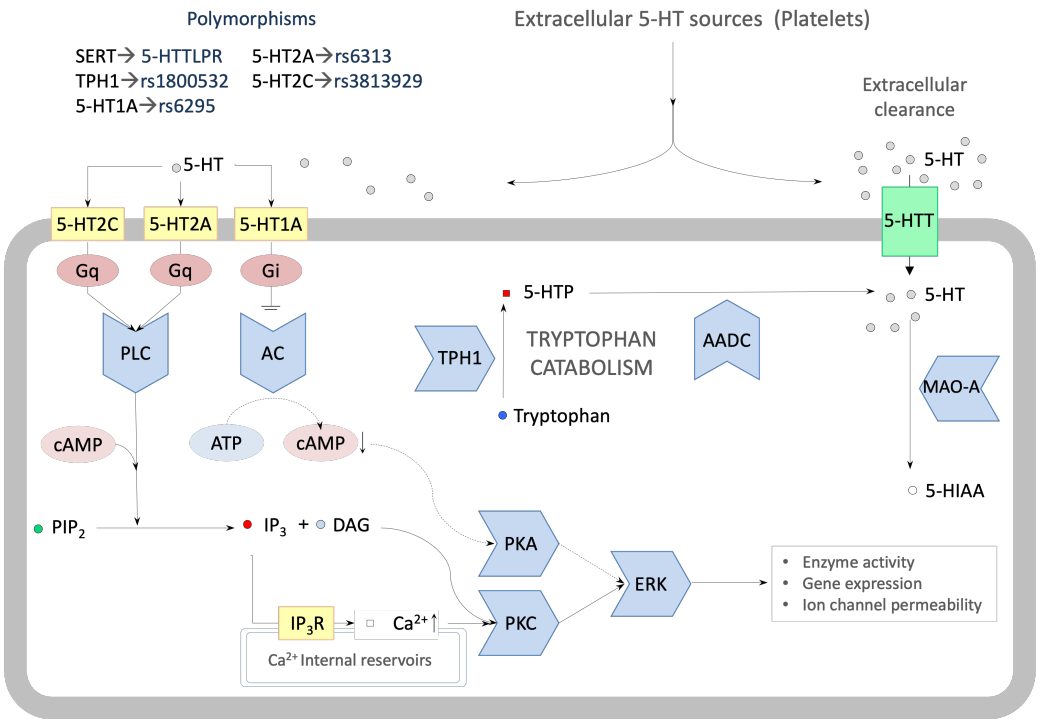
Note: OR = odds ratio; CI = confidence interval

[a] Reference category, the most frequent haplotype distribution is compared with the other haplotypes frequencies by groups

[b] P < 0.05

Supplemental table 1. Oligonucleotides used for the genotyping of the different genetic variants

Gene Name	Reference	Sense	Location	A1	A2	Forward Primers	Reverse Primers	Minisequencing Primers
SLC6A4	Ins / del 5-HTTLPR	F	5-HTTLPR	S	L	VIC-GCTGCTGCTCTACTGGGC	CCCGGCCGGTGATCTTGG	N/A
TPH1	rs1800532	R	Intron 7	A	C	CCATGCTCTATATGTGTTAGC	TCAGTGTTACATTCCCTATGC	(28t)AATTGACAACCTATTAGGTG
5-HT1A	rs6295	F	Promoter	C	G	TGCAATGGCGCGAGAACG	GCTAATTGATGGAAGAAGACC	(40t)AAGACCGAGTGTGTCTTC
5-HT2A	rs6313	F	Exon 1	C	T	TGAGCTCAACTACGAACTCC	ATTTTCAGAGTCGACTGTCC	(22t)CTACAGTAATGACTTTAACTC
5-HT2C	rs3813929	F	Promoter	C	T	TGCTGATTGGCTGCTCTTGG	AATCTGCACCACGCTCTTGG	(34t)GCTCCTCCCCTCATCC



Supplemental figure 1. Hypothesized scheme representing 5-HT interaction with different genes included in the study.

Monoamine oxydase A (MAO-A) degrading activity of serotonin (5-HT) would remove it in 5 Hydroxyndolacetic acid (5-HIAA); Trytophan can derive in 5-HT by Trytptophan Hidroxylase 1 (TPH1) and Aromatic amino acid decarboxylase (AADC) enzymes; 5-HT receptors 2A and 2C activate Phospholipase C (PLC) through G-protein (Gq) coupled subunit that promotes transformation of Phosphatidylinositol 4,5-bisphosphate (PIP_2) in Diacylglycerol (DAG) and Inositol 1,4,5-trisphosphate (IP_3); these second messengers activate protein kinase C (PKC) which in time may activate the extracellular signal-regulated kinases (ERK). 5-HT1A G-protein (Gi) coupled subunit inhibit the formation of cyclic adenylate monophosphate (cAMP) by adenylate cyclase (AC) what also modify ERK activity in a different way.