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Genetic Contribution of the Adrenergic, Cholinergic, and Serotonergic Systems to Leiomyoma Development and Treatment

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ABSTRACT

The link between the autonomic nervous system and tumor biology is being unfold. We aim to study the contribution of genes of the adrenergic (ADBR2 - rs1042713, NM_000024.6:c.46G>A, NP_000015.2:p.Gly16Arg), cholinergic (CHRNA5 - rs16969968, NM_000745.3:c.1192G>A, NP_000736.2:p.Asp398Asn), and serotonergic systems (SLC6A4 - 5-HTTVNTR-intron2, HTR2A - rs6313, NM_000621.5:c.102C>T, NP_001365853 .1: p. Ser 34=) to gynecological tumorigenesis and their treatment by embolization. A total of 517 DNA samples from women were analyzed. Samples were genotyped by PCR, PCR-RFLP and EndPoint genotyping. Results show a statistically significant association between the AA genotype of the ADBR2 gene and GG genotype of the CHRNA5 gene with leiomyoma (OR = 2.311; p = 0.003 and OR = 2.165; p = 0.001, respectively), and the epistatic interaction between genotypes increases the risk (OR = 2.458; p= 0.043). The GG genotype (CHRNA5) shows a lower reduction of the volume of the main leiomyoma after treatment (p=0.015). Combination of the genotypes 12/12-AA (SLC6A4 - ADBR2) increases the risk to leiomyoma (OR = 2.540, p= 0.030). TT genotype of HTR2A gene in combination with any of the two risk genotypes (of ADBR2 or CHRNA5) increases substantially the risk (OR = 5.266, p = 0.006; OR = 6.364, p=0.007, respectively). We conclude that ADBR2 and CHRNA5 genes have a relevant role that is enhanced by the epistatic relationship with the genes HTR2A and SLC6A4. CHRNA5 gene may also be a modulator of the success of embolization. We confirm the contribution of the genetics of Autonomous Nervous System to tumor biology.

Keywords: Leiomyomas, embolization, ADBR2, CHRNA5, HTR2A, SLC6A4

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Introduction

Leiomyomas are the most common benign gynecological tumor in premenopausal women (1-3). They are made up of monoclonal cells which arise from the smooth muscle of the uterus (2, 4, 5). Leiomyomas are generally diagnosed by the age of 50 (2, 3), and several factors are associated with their risk: African ancestry, early menarche, use of oral contraception before the age of 16 and an increase in body mass index. On the other hand, multiparity decrease the risk (2, 3). Indeed, leiomyomas are highly sensitive to the effects of steroid hormones, since they have an increased expression of estrogen and progesterone receptors (6, 3), that contributes to their growth, and also explains why they reduce their volume after menopause (7).

Patients show different clinical presentations, ranging from asymptomatic to recurrent and progressive symptoms that affect a woman's daily activities. The most common symptoms are pain, pressure, and abnormal vaginal bleeding (7, 5, 8). Location, size, and the number of leiomyomas are important determinants for the symptoms onset (1).

Treatment can be conservative or surgical, is personalized and based on symptoms, location, size of the leiomyomas and the patient's preferences regarding preservation of fertility (9, 10). Surgical treatment includes hysterectomy, open or laparoscopic myomectomy or hysteroscopic resection (11-13). Alternatively, other methods include myolysis, interstitial thermotherapy and pharmaceutical hormone therapy with the administration of gonadotropin-releasing hormone agonists, aromatase inhibitors, oral contraceptives (estrogen-progestogen combination or progestogen alone) or placement of hormone-releasing intrauterine devices (14-17). Also, uterine artery embolization (UAE) has been used as an alternative method. It involves a percutaneous puncture of a femoral artery, selective catheterization of both uterine arteries and injection of agents, such as polyvinyl alcohol (PVA) particles or acrylic microspheres, for permanent embolization (18, 19). The procedure leads to vascular hypoxia followed by size reduction or degeneration of leiomyomas (20-22).

Chronic stress can increase the activities of the hypothalamic–pituitary–gonadal axes and therefore induce secretion of sex steroid hormones (23, 24). Some studies had found an association of chronic stress with the risk of leiomyoma development (25-27).

Chronic stress is also associated to dysregulation of the hypothalamic-pituitary–adrenal (HPA) axis, leading to an increase in the production of cortisol with simultaneous elevations of epinephrine and norepinephrine (23). Catecholamines are among the first signaling molecules that respond to stress (28). Catecholamines cause general physiological changes and a general reaction of the sympathetic nervous system. β-Adrenergic receptors are the targets for the endogenous agonists' epinephrine and norepinephrine. Through the nicotinic cholinergic receptors, acetylcholine allows for the adrenal glands release of epinephrine and norepinephrine, and in the peripheral sympathetic ganglia, activation of the sympathetic system with the release of norepinephrine (29). Aberrant sympathoadrenal stress response is evident in depression, anxiety, and other diseases linked to serotonergic signaling (30-32) and some authors state that the serotoninergic system can actually modulate the sympathetic stress response (33, 34). Important players in this process are serotonin receptors and integral membrane protein (members of the solute carrier family) that transports the neurotransmitter serotonin from synaptic spaces into presynaptic neurons (33, 35).

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Tumorigenesis represents a dynamic process that induces cells to develop specific traits, including deregulated proliferation, evasion of apoptosis and immunosurveillance, and abnormal metabolism (36), (37). Tumors are embedded on a heterogeneous ecosystem that has been referred to as the tumor microenvironment to which they establish a dynamic molecular crosstalk (38-40). Growth factors and exosomes are released by tumoral cells and target the nerve terminals in the surrounding tissue, leading to axon budding within the tumor microenvironment (axonogenesis). Nerve terminals interact with the expanding mass through the release of neurotransmitters (e.g., epinephrine, norepinephrine, serotonin, dopamine, and acetylcholine) (41). Indeed, tumor microenvironment is densely infiltrated by nerves, and peripheral nerves display a major role in the first steps of tumorigenesis (42-46).

Since chronic stress induces the secretion of sex steroid hormones, important factors in the development of leiomyomas, but at the same time leads to an increase in catecholamines, the main agents of the stress response, we decided to study the involvement of the adrenergic, cholinergic and serotonergic systems, all part of the neurotransmitter systems of the nervous system and with interference in the catecholamine pathways, in the clinical aspects of leiomyomas. Therefore, we aimed to conclude about the contribution of candidate genes from the adrenergic (*ADBR2*), cholinergic (*CHRNA5*), and serotonergic systems (*SLC6A4*, *HTR2A*), to leiomyoma development and its treatment by UAE. Genes under analysis are: the β2-adrenergic receptor-related gene (gene: *ADBR2;* polymorphism: rs1042713), the nicotinic receptor subunit α5 gene (gene: *CHRNA5*; polymorphism: rs16969968), the Solute Carrier Family 6 Member 4 (gene: *SLC6A4:* polymorphism 5-HTTVNTR-intron2*)* and the serotonin receptor 2A gene (gene: *HTR2A*; polymorphism: rs6313). All polymorphisms were already associated with tumorigenesis, rs1042713 (*ADBR2)* was associated with pancreatic and breast cancer (47, 48), rs1042713 (*CHRNA5)* has been extensively associated with lung cancer (49)–(51), 5-HTTVNTR-intron2 (*SLC6A4)* was associated with prostatic cancer (52) and rs6313 (*HTR2A*) with oral potentially malignant lesions (53).

Materials and methods

All procedures were carried out in accordance with the principles of the Declaration of Helsinki. Approval of this study was given by the Hospital Saint Louis Ethics Committee (record number 4; date: May 12th, 2010). Informed consent was obtained from all the participants prior to their enrolment in the study.

Subjects

We studied a population of 198 women with leiomyomas with ages between 24 and 73 (median age of 39), that were subjected to UAE treatment (according with is described in Pisco et al., 2009), resulting in about 91% (median) of ischemia. They were clinically followed in Serviço de Intervenção Radiológica from Hospital de Saint Louis, in Lisbon. Inclusion criteria were at least 18 years of age and a confirmed diagnosis of uterine leiomyomas. Exclusion criteria was the presence of another pelvic pathology not associated with uterine leiomyoma. The following parameters were collected: number of tumors, location of the main tumor, uterus volume before and after UAE, volume of the main tumor before and after UAE. Volumes from the uterus and the main leiomyoma were collected using pelvic ultrasound and magnetic resonance imaging, before the UAE and, on average, 6 months after the procedure, by multiplying the 3

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diameters by 0.523, as it is an ellipsoid. The control group included 319 healthy women with ages between 19 and 85 (median age of 57), without any uterine pathology, or history of that. Since the median age was higher for the control group, and leiomyoma is common in reproductive age, there was no rationale for age adjustment. We were not able to obtain all the parameters from all the women (control and disease), as a result, N differs between analyses.

Genomic DNA isolation, quantification, and quality assessment

Whole blood samples from patients and controls were stored with EDTA at −20°C. Genomic DNA was isolated using a nonenzymatic method adapted from D.K. Lahiri and J.I. Nurnberger Jr. (1991) (54). Concentration of the extracted DNA and purity inference was obtained by spectrophotometry using a Thermo Scientific™ µDrop™ Plate for low-volume measure (Multiskan Sky Spectrophotometer High Microplate, Thermo Fisher Scientific®).

Genotyping

ADBR2 **-** The **Arg16Gly - rs1042713** polymorphism in the *ADBR2* gene was analyzed by a PCR-restriction approach. PCR was carried out with DreamTaq DNA polymerase and 2X DreamTaq Green buffer (ThermoFisher) in a 25 µL reaction volume, containing ≈ 200 ng of genomic DNA, 1 µL (10 pmol, STABVIDA) of each of sense (5′-CCTTCTTGCTGGCACCCCAT-3′) and antisense primers (5′-GGAAGTCCAAAACTCGCACCA -3′). The PCR program included a step of 94 °C, for 2 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 45 s. An additional extension step was performed in the final at 72 °C for 5 min. The resulting 308 bp fragment was then restricted with Speedy Nco I (Nzytech) in a 20 µL reaction volume with 2 µL of 10X NZY Speedy Buffer Orange and 8.5 µL of PCR product.

SLC6A4 **-** The **5-HTTVNTR** polymorphism in the *SLC6A4* gene was analyzed by a PCR approach. PCR was carried out with DreamTaq DNA polymerase and 2X DreamTaq Green buffer (ThermoFisher) in a 50 µL reaction volume, containing ≈ 200 ng of the genomic DNA template and 1 µL (10 pmol) of each of sense (5′- GTCAGTATCACAGGCTGCGAG-3′) and antisense primers (5′- TGTTCCTAGTCTTACGC CAG -3′). The PCR program included a step of 94 °C, for 2 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 45 s. An additional extension step was performed in the final at 72 °C for 5 min. The resulting fragment was 299 bp length for the 12 allele and 256 bp length for the 10 allele.

HTR2A **-** The **T102C - rs6313** polymorphism in the ***HTR2A*** gene was analyzed by a PCR-restriction approach. PCR was carried out with DreamTaq DNA polymerase and 2X DreamTaq Green buffer (ThermoFisher) in a 25 µL reaction volume, containing ≈ 200 ng of genomic DNA, 1 µL (10 pmol, STABVIDA) of each of sense (5′-GTCAGTATCACAGGCTGCGAG-3′) and antisense primers (5′-TGTTCCTAGTCTTACGCCAG-3′). The PCR program included a step of 94 °C, for 2 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 45 s. An additional extension step was performed in the final at 72 °C for 5 min. The resulting 344 bp fragment was then restricted with MspI (ThermoFisher) in a 25 µL reaction volume with 2.5 µL of 10X Buffer Tango and 10 µL of PCR product.

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CHRNA5* -** The **rs16969968** polymorphism in the ***CHRNA5 was analyzed by an EndPoint genotyping approach in a MyGo Pro PCR system (IT-IS Life Science Ltd.) using a TaqMan® Genotyping Master Mix and an Applied Biosystems™ TaqMan® SNP Genotyping Assay (C__26000428_20; ThermoFisher). About 50 ng of genomic DNA was added to a 10 µL reaction volume containing 5 µL of master mix and 0.5 µL of a 20x mix of primers plus probes. The reaction of 45 cycles was performed in a 2-step amplification, with an initial hold of 600 s at 95ºC and a second step of 60ºC for 15 s.

Statistical analysis

A chi-square ($\chi 2$) was used to test associations between genotypes and disease or other related parameters. For continuous variables, normality was tested through Shapiro-Wilk or Kolmogorov-Smirnov and for non-parametric continuous variables, Mann-Whitney was used to test association. For non-parametric paired sample analyses, Wilcoxon test was used. Odds Ratio (OR) was established to determine the risk associated with any genotypes or allele, with a 95% confidence interval (CI). Epistatic analyses were carried to test associations of disease or other parameters with interacting genes. All the mentioned tests were performed in IBM® SPSS® Statistics Version 26. Statistical significance was defined as a p-value < 0.05.

Results

Characterization of the women population with leiomyomas

Concerning the number of leiomyomas, most women (60.0%) had 4 or more (Table 1).

Table 1. Distribution of the number of leiomyomas per woman

Leiomyomas number	N (%)
≤3	62 (40.0)
≥4	93 (60.0)

Regarding the localization of the main leiomyoma, the majority were intramural (43.4%) (Table 2).

Table 2. Distribution of the main leiomyoma per localization

Intramural N (%)	Suberosal N (%)	Submucosal N (%)	Intramural/ Suberosal N (%)	Intramural/ Submucosal N (%)
69 (43.4)	58 (36.5)	17 (10.7)	7 (4.4)	8 (5.0)

After treatment, there was a significant reduction on the uterine and main leiomyoma volumes (Table 3).

Association of *ADBR2* and *CHRNA5* genes with leiomyomas

We were able to detect genotypes and alleles associations with two of our candidate genes, *ADBR2* and *CHRNA5*. *ADBR2* gene shows association of the A allele and AA genotype with leiomyoma (Table 4). *CHRNA5* gene shows association of the G allele and GG genotype with the leiomyoma (Table 5). Associated alleles and genotypes for each gene were evaluated as risk factors (Tables 4 and 5).

Once both risk genotypes were analyzed in epistasis, the risk increased (OR = 2.311 and 2.165 → 2.458; Table 6).

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Table 3. Uterine and main leiomyoma volumes reduction after UAE

Volume	N	Median cm^3 (max-min)	p-value*
Main leiomyoma			
pre-UAE	159	69 (1-1176)	<0.001
pos-UAE	108	38.5 (1-1000)	
Uterus			
pre-UAE	173	303 (59-1901)	<0.001
pos-UAE	117	217 (29-1449)	

* Wilcoxon´s test

Table 4. Distribution of Genotype and Allelic frequencies of the *ADBR2* gene between Leiomyoma and Control Population and risk analysis

Genotype frequency	Leiomyomas N (%)	Controls N (%)	p*	OR IC (95%)
AA	39 (42.9)	37 (24.5)		
AG	48 (52.7)	75 (49.7)	<0.001	-
GG	4 (4.4)	39 (25.8)		
AA	39 (42.9)	37 (24.5)		2.311
AG+GG	52 (57.1)	114 (75.5)	0.003	(1.324-4.032)
AA+AG	87 (95.6)	112 (74.2)		7.574
GG	4 (4.4)	39 (25.8)	<0.001	(2.607-22.002)
Allele frequencies	**Leiomyomas N (%)**	**Controls N (%)**	**p***	**OR IC (95%)**
A	126 (69.2)	149 (49.3)		2.310
G	56 (30.8)	153 (50.7)	<0.001	(1.569-3.403)

*χ^2 – test

Table 5. Distribution of Genotype and Allelic frequencies of the *CHRNA5* gene between Leiomyoma and Control Population and risk analysis

Genotype frequency	Leiomyomas N (%)	Controls N (%)	p*	OR IC (95%)
GG	61 (52.6)	62 (33.9)		
AG	46 (39.7)	90 (49.2)	0.003	-
AA	9 (7.8)	31 (16.9)		
GG	61 (52.6)	62 (33.9)		2.165
AG+AA	55 (47.4)	121 (66.1)	0.001	(1.345-3.484)
GG+AG	107 (92.2)	152 (83.1)		2.425
AA	9 (7.8)	31 (16.9)	0.023	(1.109-5.301)
Allele frequency	**Leiomyomas N (%)**	**Controls N (%)**	**p***	**OR IC (95%)**
G	168 (72.4)	214 (58.5)		1.865
A	64 (27.6)	152 (41.5)	<0.001	(1.307-2.659)

*χ^2 – test

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Table 6. Different distribution of genotypes frequencies in the epistatic interaction between *ADBR2* and *CHRNA5* genes in Leiomyoma and Control Population and risk analysis

Genotypes in Epistasis (*ADBR2 - CHRNA5*)	**Leiomyomas N (%)**	**Controls N (%)**	**p***	**OR IC (95%)**
AA - GG	21 (25.0)	8 (11.9)	0.043	2.458
Other interactions	63 (75.0)	59 (88.1)		(1.011-5.977)

*χ^2 – test

Association of the GG genotype of the *CHRNA5* gene with a lower reduction of the main leiomyoma volume after UAE

Once analyzing the previous mentioned clinical phenotypes of the leiomyoma population, we could find an association of the GG genotype from the *CHRNA5* gene with a lower reduction of the main leiomyoma volume (Table 7).

Table 7. Comparison of the main leiomyoma reduction between *CHRNA5* genotypes

Genotype frequency	**Leiomyomas N (%)**	**Median cm^3**	**(Min) – (Max) cm^3**	**p***
GG	34 (54.0)	11	(-40) – (274)	
AG+AA	29 (46.0)	50	(-20) – (683)	0.015

*Mann-Whitney - test

Epistatic interactions involving *SLC6A4* and *HTR2A* with *ADBR2* or *CHRNA5* genes

We observed an increased risk of the AA genotype (*ADBR2)* if in epistasis with the 12/12 genotype of the *SLC6A4* gene (OR=2.311→OR=2.540). TT genotype of the *HTR2A* gene increases substantially the risk of AA genotype (*ADBR2*) (OR=2.311→OR=5.266) and GG genotype (*CHRNA5*) (OR=2.165→ OR=6.364) (Table 8, 9).

Table 8. Epistatic interaction between *SLC6A4* and *ADBR2* genes and risk analysis.

Genotypes in Epistasis	**Leiomyomas N (%)**	**Controls N (%)**	**p***	**OR IC (95%)**
12/12-AA	14 (16.7)	10 (7.3)		
Other interactions	70 (83.3)	127 (92.7)	0.030	2.540 (1.072-6.017)

*χ^2 – test

Table 9. Epistatic interaction between *HTR2A* and *ADBR2* or *CHRNA5* genes and risk analysis

Genotypes In Epistasis	**Leiomyomas N (%)**	**Controls N (%)**	**p***	**OR IC (95%)**
HTR2A-ADBR2				
TT-AA	12 (13.2)	3 (2.8)		5.266
Other genotypes	79 (86.8)	104 (97.2)	0.006	(1.437-19.293)
HTR2A-CHRNA				
TT-GG	14 (12.4)	2 (2.2)		6.364
Other genotypes	99 (87.6)	90 (97.8)	0.007	(1.407-28.773)

*χ^2 – test

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Discussion

Leiomyomas are the most common benign tumor among women and one of the more frequent pathologies in the uterus. When symptomatic, leiomyomas affect a woman's life quality. Women can present a large array of symptoms such as enlarged uterus associated with abdominal distention, heavy and irregular menstrual bleeding (menorrhagia and metrorrhagia) associated with the development of iron-deficiency anemia, pelvic pressure associated with urinary and fecal frequency or obstruction and pelvic pain associated with degeneration of tissues and/or torsion of leiomyomas (2, 55-61). Regarding reproductive consequences, uterine leiomyomas have been associated with intercourse pain, pre-term births, miscarriages, and infertility (2, 56-60). Heavily symptomatic women with medium to large leiomyomas require strong measures, which can go from UAE to surgery, partial or total removal of the uterus (hysterectomy) (2, 56, 57) . Due to its unclear etiology, symptomatology and risk of recurrence, uterine leiomyomas request additional investigations. In this study we worked with a population of women with leiomyomas, treated by UAE. Most of the women had 4 or more leiomyomas and with an intramural localization. UAE treatment resulted in a significant reduction of the uterus and of the main leiomyoma.

Chronic stress has been related with tumor progression (62, 63) . The overall stress response involves activation of several body systems including the Autonomic Nervous System (ANS) and the HPA axis. The response is prompted by the production of mediators such as the catecholamines norepinephrine and epinephrine from the sympathetic nervous system (SNS) and the adrenal medulla. Norepinephrine, epinephrine and cortisol are considered the major stress hormones and are known to be elevated in chronic stress.

The effects of catecholamines are mediated through adrenergic receptors coupled to G-proteins, which mediates activation of the cAMP-dependent PKA system, resulting in downstream activation of several pathways, including the ones related with cellular growth (64, 65) . The β-adrenergic receptors 2 are the dominant adrenergic receptors, meaning that mediates the majority of cellular responses to external stimuli. They are cell membrane-spanning receptors codified by the *ADRB2* gene. They are found presynaptically and stimulate release of norepinephrine (66). β-blockers are beta-adrenergic blocking agents, that have been shown to have health benefits regarding several tumors (67). Since stress response is mediated by catecholamines, which main receptors are β-adrenergic receptors 2, we hypothesized that genetic variation in *ADBR2* gene might have implications in leiomyoma clinical aspects. Here we studied the association of *ADBR2* gene with leiomyoma disease, clinical parameters, and response to treatment. We found an association of the AA genotype and A allele with the disease, both (genotype and allele) representing a two-fold risk for leiomyomas development. The AA genotype is linked to a missense polymorphic mutation whereas Gly is replaced by Arg in codon 16 of the intronless *ADBR2* gene. In accordance with the "dynamic model of receptor regulation" proposed by Liggett´s group (68), the endogenous catecholamines dynamically desensitize the β-adrenergic receptors 2 in their basal state, and this occurs to a greater extent for the Gly than for the Arg variant. Also, the Gly variant was seen to undergo significantly enhanced down-regulation after isoprenaline exposure (69). Therefore, the AA genotype (Arg) may represent a stronger physiological response to stress than the GG genotype (Gly).

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Our results also show an association of GG genotype and G allele from the *CHRNA5* gene with the disease and a risk of around two-fold with their presence. Indeed, although we are dealing with the most frequent genotypes/allele in the control population, there is an increase in the presence of both in the leiomyoma population. *CHRNA5* gene codifies to a subunit of the nicotinic acetylcholine receptor. In the adrenal medulla, acetylcholine released by the sympathetic splanchnic nerves activates the acetylcholine receptors present on the membrane of chromaffin cells which release catecholamines into the bloodstream. As mentioned before, catecholamines are major mediators of the stress response. Rs16969968 is a missense variant that results in an amino acid substitution at codon 398 (D398N) of *CHRNA5*. *In vitro* functional studies demonstrated that nicotinic receptors containing this variant exhibit reduced response to agonists (70). Therefore, as the most frequent genotype/allele is functionally more active, it promotes a higher release of catecholamines, contributing to the maintenance of a chronic stress physiological response. Besides, the GG genotype was not so sensitive to UAE treatment as the other genotypes since a lower reduction of the main leiomyoma volume was associated with this genotype. We believe that a long period of a more efficient bloodstream release of catecholamines associated with the GG genotype may be difficult for the effectiveness of the UAE treatment on a more consolidated tumor.

SLC6A4 gene codifies to an integral membrane protein (SERT) whose primary function in the central nervous system involves the regulation of serotonergic signaling via transport of serotonin molecules from the synaptic cleft back into the pre-synaptic terminal for re-utilization. Loss of SERT function has been reported to increase the sympathetic stress response (71, 72). SERT is highly expressed in chromaffin cells of the adrenal medulla and acts locally within the adrenal gland to control the sympathetic stress response (34, 73). Indeed, there is evidence that SERT coordinates serotonergic regulation of catecholamine exocytosis via a 5-HT1A receptor-mediated inhibition of catecholamine secretion, playing its role is stress response. Although serotonin is not synthesized in adrenergic chromaffin cells, SERT allows its accumulation (73). For this gene, it was analyzed as a known polymorphic variation, 5-HTTVNTR, which consists in a variable number of tandem repetitions (VNTR) in this gene's second intron. Only the two most frequent alleles were detected in this study, the ones with 10 or 12 tandem repetitions of a 17bp sequence. In a study using single photon emission, there was a tendency in computed tomography for subjects who were homozygous for the 12-repeat allele to display lower SERT availability (74). Indeed, the 12/12 genotype (*SLC6A4*), although with no apparent relevance with the onset of the disease alone, can increase the risk of the AA- genotype (*ADBR2*) in a synergetic epistatic interaction. Thus, although the *SLC6A4* gene alone is not associated with the disease (data not shown) it can modulate the risk for the disease. We believe that *SLC6A4* genotype can have a mild effect on catecholamine secretion and thus slightly modify the association of the *ADBR2* with the phenotype.

Besides the above mentioned interaction between catecholamines and serotonin pathways, several studies show that serotonin and serotonergic drugs influence activity of several components of the HPA axis and stress response in a more general mode (75). *HTR2A* encodes a serotonin 2A receptor that affects serotonin's action by increasing excitability of the host neuron (35). The polymorphic change studied for this gene was T102C (rs6313), which consists of a substitution of a thymine (T) for a cytosine (C) in the receptor's 102nd position (76). Such alteration does not change the corresponding amino-acid (synonymous

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mutation), however according to some authors, these SNP results in a decrease of the promoter activity, thus the gene transcription (77, 78). It was interesting to verify that the presence of the TT genotype could modulate the risk of the AA genotype of the *ADBR2* and GG genotype of *CHRNA5*. Indeed, we could observe a substantial increase in the risk of both genotypes, once in epistasis with the TT genotype from *HTR2A*.

Although uterine leiomyomas studies of genome-wide association have been previously performed, different populations with divergent genetic structures were analyzed (79-82), not including the Portuguese population, that from our point of view is unique, given their strong connection with its former African colonies, which is presently continuous to bring new inputs to its genetic pool. To our knowledge, this is the first time, that this set of genes is studied in the context of leiomyomas development and treatment. Here, we show that these genes are associated with the disease, since genetic variation is modulating the final phenotype, this is no surprising since these genes codify proteins involved in Autonomous Nervous System functionality that are major players in neurotransmission mechanisms. Peripheral nerves display a major role in the first steps of tumorigenesis (42, 45). Studies on prostate cancer showed that denervation of adrenergic nerves or inhibition of adrenergic signaling delays tumor formation (83). In spontaneous pancreatic ductal adenocarcinoma, increased release of catecholamines by sympathetic neurons in the pancreas promotes the development of neoplastic lesions through the activation of the adrenergic receptor. Furthermore, surgical or pharmacological denervation of the stomach markedly reduces the incidence of gut cancer (84). The surgical ablation of sensory cutaneous nerves in hair follicles inhibits tumorigenesis in a spontaneous model of basal cell carcinoma, with tumors decreasing both in size (85). Nerve terminals interact, with the tumor microenvironment, through the release of neurotransmitters, such as epinephrine, norepinephrine, serotonin, and acetylcholine (41). In parallel tumors overexpress and release back neurotrophic factors (86-89). Indeed, inhibition or knockdown of NGF (Nerve Growth Factor) effects tumor growth (90), (91). NGF acts via receptor Tyrosine kinase A (TrkA), which is binding and activates several signaling cascades, ultimately promoting cell survival and proliferation (92). Increased expression of neurotrophic factors, including NGF was already verified in uterine leiomyomas (93).

For future studies, we believe that association studies in genes of neurotrophic factors, such as *NGF* (Nerve Growth Factor), *BDNF* (Brain Derived Neurotrophic Factor), *NTRK1* (Neurotrophic Receptor Tyrosine Kinase 1) and *NTRK2* (Neurotrophic Receptor Tyrosine Kinase 2) will increase our knowledge on this subject.

In terms of limitations of our work, we believe that a better characterization of the patient population will be beneficial, such as information on lifestyle and environment, and especially on therapeutics of the nervous system (e.g.: antidepressants, benzodiazepines). Long-term effects of UAE treatment on leiomyoma development and treatment were not studied because we lost track of the patients but would likely benefit our work. In addition, a multicenter study will improve the ability to generalize our results. Nevertheless, we used data from real clinical practice and obtained results that are complementary to previously published studies, thus strengthening the contribution of our findings.

Our work shows that genetic variation in genes from the adrenergic, cholinergic and serotonergic systems, plays a contribution for tumorigenesis development and treatment in leiomyomas. Through this

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study, we conclude that the *ADBR2* and *CHRNA5* genes have a relevant role in the development of leiomyoma. This role appears to be enhanced by the epistatic relationship with the genes *HTR2A* and *SLC6A4*. This study, confirms the involvement of the Autonomous Nervous System in tumor biology, along with the genetics of the mechanisms, involved in chronic stress. By highlighting and helping to elucidate the role of genes involved in the pathophysiology of leiomyoma disease and leiomyoma treatment, we believe that this research paves the way for the investigation of new therapies that interfere with the neurotransmitter systems of the nervous system and is a promising step forward in personalized medicine, by indicating individual susceptibility to the disease.

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