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# Angiogenesis-related genes and thalidomide teratogenesis in humans: an approach on genetic variation and review of past *in vitro* studies

Thayne Woycinck Kowalski[a,b], Lucas Rosa Fraga[a,b], Luciana Tovo-Rodrigues[b,c], Maria Teresa Vieira Sanseverino[a,b,d], Mara Helena Hutz[b], Lavínia Schuler-Faccini[a,b,d], Fernanda Sales Luiz Vianna[a,b,d,e,*]

[a] *INAGEMP—Instituto Nacional de Genética Médica Populacional, Porto Alegre, Brazil*
[b] *Post-Graduate Program in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil*
[c] *Post-Graduate Program of Epidemiology, Universidade Federal de Pelotas, Pelotas, Rio Grande do Sul, Brazil*
[d] *Teratogen Information Service, Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Brazil*
[e] *Service of Experimental Research, Genomics Medicine Laboratory, Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, Brazil*

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ABSTRACT

Thalidomide embryopathy (TE) has affected more than 10,000 babies worldwide. The hypothesis of antiangiogenesis as the teratogenic mechanism of thalidomide has been investigated in several experimental models; but, in humans, it has only been accessed by *in vitro* studies. Here, we hypothesized the effect of thalidomide upon angiogenesis-related molecules or proteins, previously identified in human embryonic cells, through the *in silico* STRING-tool. We also investigated ten polymorphisms in angiogenesis-related genes in 38 Brazilian TE individuals and 136 non-affected Brazilians. *NOS2*, *PTGS2*, and *VEGFA* polymorphisms were chosen for genotyping. The STRING-tool suggested nitric oxide and β-catenin as the central angiogenesis-related molecules affected by thalidomide's antiangiogenic property. We did not identify a significant difference of allelic, genotypic or haplotypic frequencies between the groups. We could not predict a risk allele or a protective one for TE in *NOS2, PTGS2,* or *VEGFA*, although other genes should be analyzed in larger samples. The role of nitric oxide and β-catenin must be further evaluated, regarding thalidomide teratogenesis complex etiology.



## 1. Introduction

Thalidomide is a teratogen synthesized in 1954 in Western Germany, which was used for a variety of ailments, including morning sickness during early pregnancy [1,2]. Teratogenic effects of thalidomide were discovered in the beginning of the 1960s, with the observation of an increased number of babies born with limb reduction defects (LRD) and other severe congenital anomalies [2]. Thalidomide was withdrawn from the world's market in 1962, but it is estimated that more than 10,000 babies have been affected by this embryopathy [1].

Due to the identification of immunomodulatory and antiangiogenic properties [3,4], thalidomide resurfaced and is now used to treat immunological conditions, such as erythema nodosum of leprosy (ENL), and different types of cancer, particularly multiple myeloma [5]. Despite the established knowledge about its therapeutic properties, teratogenic mechanisms of thalidomide are not fully understood. Among the most accepted theories, antiangiogenic proprieties of thalidomide have been linked to its teratogenic effect [4]; the drug's action toward many molecules involved in the blood vessel development is well described in the literature [6–9]. *In vitro* studies with human umbilical vein endothelial cells (HUVEC) and multiple myeloma cell lines have demonstrated that thalidomide inhibits angiogenesis in both cell types, although

*Abbreviations:* ABPST, Brazilian Association of People with Thalidomide Syndrome; AKT, protein kinase B; bFGF, basic fibroblast growth factor; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; ENL, erythema nodosum of leprosy; eNOS, endothelial nitric oxide synthase; FLK-1, kinase insert domain receptor; GO, gene ontology; HIF-1α, hypoxia inducible factor alpha subunit; HUVEC, human umbilical vein endothelial cells; IL-8, interleukin 8; IMiD, immunomodulatory drug; LD, linkage disequilibrium; LRD, limb reduction defects; MMP-2, matrix metalloproteinase 2; NF-kB, nuclear factor kappa-B; NOS2, nitric oxide synthase 2; NOS3, nitric oxide synthase 3; PCR, polymerase chain reaction; PTGS2, prostaglandin synthase 2; sGC, soluble guanylyl cyclase; SP1, transcription factor Sp1; TE, thalidomide embryopathy; TNFα, tumor necrosis factor alpha; UTR, untranslated region; VEGF, vascular endothelial growth factor; VEGFA, vascular endothelial growth factor A.

* Corresponding author at: Teratogen Information Service (SIAT), Serviço de Genetica Medica – Hospital de Clinicas de Porto Alegre, 90035-903 Porto Alegre, RS, Brazil.
*E-mail address:* fslvianna@gmail.com (F.S.L. Vianna).

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affecting angiogenesis gene expression differently [10,11]. Hence, thalidomide's antiangiogenic effect in tumorigenesis may differ from its effect in embryonic vascular development.

Angiogenesis stimuli are affected by genetic variation [12], and the candidate genes for this mechanism have been mostly identified by studying genetic diseases in which angiogenesis plays a major role [13]. Similarly, the outcome of teratogenic exposure is influenced by genetic variation [14]. Recently, our group showed that haplotypes in the endothelial nitric oxide synthase (eNOS) gene, which is important in angiogenesis, have different distributions between thalidomide survivors and unaffected people [15], suggesting these variants have a pre-transcriptional effect in the risk of developing thalidomide embryopathy [16]. Other genes in the angiogenesis pathway may also present a susceptibility effect to TE outcome, however this hypothesis has never been evaluated. Moreover, the functional effects of many polymorphisms in genes *NOS2, PTGS2* and *VEGFA* are already described in different clinical conditions associated with angiogenic mechanisms [12,17,18].

In the present study we performed a literature review in order to identify the main molecular mechanisms already associated with the thalidomide antiangiogenic effect in human embryogenesis. An *in silico* analysis of protein interactions was carried out to hypothesize how all the molecular pathways could be connected. Afterwards, we aimed to evaluate polymorphisms in *NOS2*, *PTGS2*, and *VEGFA* genes in individuals with TE, comparing with a reference sample.

## 2. Methods

### 2.1. Literature review

Review of scientific publications was performed in PubMed database (National Center for Biotechnology Information, NCBI, http://http://www.ncbi.nlm.nih.gov/pubmed). The terms "human AND angiogenesis" were always used in search, together with one of the main immunomodulatory drugs (IMiDs) applied in clinic: "thalidomide OR lenalidomide OR pomalidomide". To filter for assays using embryonic cells, one of the following was added: "embryo" or "embryogenesis" or "development" or "fetal" or "fetus". Studies performed in animal models were not included in the review. Articles were read and all the publications that did not perform molecular assays (any gene or protein name reported) were excluded.

### 2.2. In silico *analysis*

From the literature review, a list of proteins that have been shown to be affected by thalidomide was obtained. All these proteins were included in a hypothesis of protein interaction, which was performed with STRING tool (http://string-db.org). Gene names were inserted in "multiple proteins" option. If a protein was encoded by more than one gene, all the coding genes were included in the analysis. Only active interactions with experimental evidence were selected; minimum required confidence was set at 0.400 (default). Gene Ontology (GO) analysis used were also performed through STRING tool. After the analysis, functional polymorphisms were selected for genotyping in a TE sample.

### 2.3. Sample

Thirty-eight individuals with TE were recruited through Brazilian Association of People Affected by Thalidomide Syndrome (ABPST), all of whom were previously examined by experts in TE and differential diagnosis. A clinical evaluation of the congenital anomalies was performed in 28 of these individuals in order to confirm the compatible phenotype of TE [19]; the other individuals were evaluated through telephone calls, because they live far from ABPST, making it difficult to follow up in person. Saliva samples were collected and stored in DNA Oragene® Kits (Genotek). DNA extraction was performed according to the manufacturer's instructions.

A sample of 136 subjects without congenital anomalies from the Brazilian population was used as a control group. These subjects were selected according to similar time and place of birth of the individuals with TE. The DNA of these unaffected subjects are stored in the Genetics Department of our institution and are identified by gender, age and place of birth. These samples have been previously evaluated in other epidemiological studies [20,21]. This study was approved by the Ethics Committee in Research of Hospital de Clínicas de Porto Alegre, Rio Grande do Sul, Brazil, number 10-0422.

### 2.4. Genotyping

Ten polymorphisms in the *NOS2*, *PTGS2* and *VEGFA* genes were selected according to: (i) Minor Allele Frequency (MAF) > 0.1, according to the 1000 Genomes Project data for European population (http://ensembl.org); (ii) previous association of clinical situations with commitment of angiogenesis pathway or limb reduction defects; and (iii) functional description of the variants as modifiers of their gene expression and/or protein activity. Only variants that met all three criteria were selected for genotyping. Nine single nucleotide polymorphisms (SNP) in genes *NOS2* (2), *PTGS2* (3) and *VEGFA* (4), and one microsatellite of *NOS2* gene were selected for analysis [12,17,22]. Table 1 summarizes all the variants evaluated in the present study.

Allelic discrimination of eight SNPs was performed through the TaqMan® Genotyping Assay (Applied Biosystems®) method in StepOnePlus™ Real-Time PCR Systems (Applied Biosystems®). The polymorphism numbers and commercial assay codes are: rs2297518 (C__11889257_10) of *NOS2*; rs689465 (C___2517146_10) and rs689466 (C___2517145_20) of *PTGS2*; rs3025039 (C__16198794_10), rs1570360 (C___1647379_10), rs2010963 (C___8311614_10) and rs699947 (C___8311602_10) of gene *VEGFA*. A custom TaqMan® assay was used to genotype rs2779249 of gene *NOS2*, as described in Oliveira-Paula et al. [22]. All reactions were performed according to the manufacturer's protocol and the results were evaluated with StepOne Software v.2.2 (Applied Biosystems®). The polymorphism rs20417 of gene *PTGS2* was evaluated through sequencing, using the Sanger method. Primers 5′-GCATACGTTTTGGACATTTAG-3′ (forward) and 5′-GCTAAGTTGCTTTCAACAGAAGAAAT-3′ (reverse) were synthesized and amplified a fragment of 238bp through polymerase chain reaction (PCR). The samples were purified and sequencing was performed in Macrogen®. The results were analyzed in software CodonCodeAligner®, v.3.0.1 (Codon Code Corporation, USA). The GenBank sequence was used as a reference. The microsatellite (CCTTT)n (rs869282985) in the promoter region of gene *NOS2* was evaluated through fragment analysis, according to Oliveira-Paula et al. [22]. Short alleles from the microsatellite (8–11 repeats) were classified as small (S) and the ones with more than 11 repeats (12–17) were identified as long (L) alleles.

### 2.5. Statistical analyses

The Hardy-Weinberg Equilibrium test was used to test all polymorphisms in both sample groups. Allelic and genotypic frequencies were compared by Fisher's Exact Test using SPSS® v.18 (SPSS, IBM, USA) software. A two-tailed $p$-value < 0.05 was considered significant.