**LETTER TO THE EDITOR**

**VEGF, VEGFR2 and GSTM1 polymorphisms in outcome of multiple myeloma patients treated with thalidomide-based regimens**

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Angiogenesis (AG) abnormalities are crucial in pathogenesis and prognosis of multiple myeloma (MM).\(^1\) Increased microvessel density (MVD) in bone marrow (BM) is an unfavorable prognostic factor in disease,\(^1\) supporting the use of inhibitors of vascular endothelial growth factor (VEGF) in patients’ treatment.\(^2\)

VEGF and its VEGF type 2 receptor (VEGFR2),\(^3\) and hypoxia-inducible factor-1 alpha (HIF-1α)\(^4\) were described as key regulators of AG, and glutathione S-transferases mu1 (GSTM1) and theta1 (GSTT1) promote AG by effecting the HIF-1α pathway.\(^5\) The wild-type alleles of VEGF c.-2595C>A (rs699947),\(^5\) c.-1154G>A (rs1570360),\(^5\) c.-634G>C (rs2010963),\(^6\) c.*237C>T (rs3025039),\(^7\) and VEGFR2 c.-906T>C (rs2071559)\(^8\) and c.889G>A (rs2305948)\(^9\) single-nucleotide polymorphisms (SNPs) were associated with a higher production of VEGF or higher transcriptional activity and binding efficiency for VEGF than the respective variant alleles. On the other hand, GSTM1 and GSTT1 genes may be homozygous deleted in healthy individuals, having lack of respective active angiogenic proteins as a consequence.\(^9\)

None of genotypes or haplotypes of VEGF SNPs (rs699947, rs833061, rs2010963 and rs3025039) have influenced in response to thalidomide of relapsed MM patients in a previous study.\(^10\) However, only the ACG haplotype of rs699947, rs833061 and rs2010963 loci, previously associated with higher production of VEGF,\(^5\) altered negatively the time of thalidomide failure in those patients.\(^10\) GSTM1 and GSTT1 genes were previously described as unimportant in response and survival to vincristine, doxorubicin and dexamethasone (VAD) and high-dose melphalan in newly MM patients previous studied.\(^11\) However, worse disease-free survival and overall survival (OS) were related with the GSTM1 present and GSTT1 null alleles in Hodgkin lymphoma patients.\(^12\)

We investigated herein the roles of VEGF c.-2595C>A, c.-1154G>A, c.-634G>C, c.*237C>T, VEGFR2 c.-906T>C, c.889G>A SNPs, and GSTM1 and GSTT1 genes, in outcome of MM patients treated with thalidomide-based regimens.

 Newly diagnosed MM patients (N=102) were included in the study from June 2005 to June 2013, after local institutional review board guidelines approvals. Therapeutic regimens consisted in thalidomide combined with steroids and/or chemotherapy, followed or not by autologous stem cell transplantation (ASCT)\(^2\) (Supplementary Table S1). Fragments of BM available from diagnosis (N=21) served for immunohistochemistry analysis using anti-CD34 (QBEnd/10). Slides were scanned at ×20 magnification in Aperio Scanscope XT to assess MVD, in a blinded fashion.

Response was evaluated at the end of treatment using the International Myeloma Working Group guidelines, and classified as complete response (CR), very good partial response (VGPR), partial response (PR), stable disease (SD) or progressive disease (PD). Event-free survival (EFS) and OS encompassed time from diagnosis until relapse, progression, death due to tumor effects or last follow-up, and time from diagnosis until death by any cause or last follow-up, respectively.

Genotyping was performed in DNA of patients’ peripheral blood. VEGF and VEGFR2 SNPs were analyzed by real-time polymerase chain reaction, using TaqMan SNP Genotyping Assays. Only the genotypes of VEGF c.*237C>T SNP, GSTM1 and GSTT1 genes were obtained by polymerase chain reaction plus enzymatic digestion and multiplex polymerase chain reaction, respectively.

The pairwise linkage disequilibrium was performed to ensure that markers were appropriate for inclusion in haplotype estimates. Two-tailed t-test was performed to investigate associations between genotypes and MVD. Logistic regression models assessed associations between genotypes and response. EFS and OS probabilities were estimated by Kaplan–Meier method and compared by log-rank test. The Cox hazards model was used to identify variables predicting EFS and OS. Variables with P<0.10 in univariate Cox analysis were included in multivariate Cox analysis. Significant results were validated using a bootstrap resampling study to investigate the stability of risk estimates (1000 replications). Differences were significant when P<0.05.

Linkage disequilibrium between VEGF and VEGFR2 SNPs were seen in study, and common haplotypes (>1%) of the genes were included in further analyses.

MVD was higher only in patients with VEGF c.-1154GG genotype compared to others (8.64×10^-4 vs 4.88×10^-4 vessels/μm², P=0.01) (Supplementary Figure S1). Patients treated with thalidomide-based regimens followed by ASCT had more chances of achieving better response to therapy than others, and for this reason the values of logistic regression data were adjusted by ASCT status. The VEGF c.-2595CC or CA isolated or associated with VEGFR2 c.-906TT or TC, and CGGC haplotype of VEGF c.-2595C>A, c.-1154G>A, c.-634G>C and c.*237C>T SNPs were also more common in patients with CR, VGPR or PR. Carriers of these genotypes or haplotype had 3.55, 9.91 and 3.86 more chances of obtaining better response to therapy, respectively (Table 1).

The median follow-up time of MM patients enrolled in study was 43 months. The estimated probabilities of 60-months EFS and OS were 24.5 and 48.1%, respectively. At the study end (February 2016), 50 patients were alive and 52 patients died.

In Kaplan–Meier estimates, the 60-months EFS and OS tended to be shorter in patients at ISS III (23.0 vs 25.2%, P=0.08; 41.3 vs 56.0%, P=0.08). At this time, both EFS and OS were shorter in patients who did not receive ASCT after chemotherapy (11.9 vs 42.4%, P<0.0001; 34.9 vs 65.1%, P<0.0001), with VEGFR2 c.889GG (17.0 vs 43.5%, P=0.004; 42.2 vs 62.3%, P=0.03), VEGF c.-634G4G plus VEGFR2 c.889GG (22.8 vs 50.8%, P=0.01; 43.7 vs 85.7, P=0.005), VEGFR2 c.889GG plus GSTM1 present (13.6 vs 31.6%, P=0.04; 30.7 vs 65.8%, P=0.01), respectively (Supplementary Figure S2). The VEGF c.-1154GG plus VEGFR2 c.889GG (18.8 vs 42.1%, P=0.04) and VEGFR2 c.-906TT plus c.889GG (13.3 vs 43.7%, P=0.001) predicted only worse EFS, and GSTM1 present (39.0 vs 58.3%, P=0.09) and VEGFR2 c.-906TT plus c.889GG (45.0 vs 56.4%, P=0.06) were marginally associated with shorter OS.
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Table 1. VEGF, VEGFR2, GSTM1 and GSTT1 polymorphisms in response rate of multiple myeloma patients

| Variable | Response rate (N = 97)* |
|----------|-------------------------|
|          | CR+VGPR+PR  | SD+PD  | P-value | OR (95% CI) |
|          | N (%)  | N (%)  |         |            |

|       | CR+VGPR+PR  | SD+PD  | P-value | OR (95% CI) |
|-------|-------------|--------|---------|-------------|
|       | N (%)  | N (%)  |         |            |

**ISS**
- I-II 40 (88.9) 5 (11.1) 0.25 Reference
- III 41 (80.4) 10 (19.6) 1.97 (0.60–6.44)

**ASCT**
- Yes 40 (93.0) 3 (7.0) 0.05 Reference
- No 42 (77.8) 12 (22.2) 3.74 (0.98–14.36)

**VEGF**
- c.-2595C > A
  - CC 38 (84.4) 7 (15.6) 0.69 1.25 (0.39–3.93)
  - CA+AA 44 (84.6) 8 (15.4) Reference
  - CC+CA 70 (88.6) 9 (11.4) **0.04** (1.03–12.20)

**GG**
- AA 43 (84.3) 8 (15.7) 0.71 1.23 (0.39–3.85)

**Vegf**
- C.-2595C > A
  - QQ 44 (84.6) 8 (15.4) Reference
  - CC 78 (85.7) 13 (14.3) 0.28 0.36 (0.05–2.30)

**GSTM1**
- Present 45 (81.8) 10 (18.2) 0.36 1.73 (0.53–5.67)
- Null 37 (88.1) 5 (11.9) Reference

**GSTM1**
- Present 45 (81.8) 10 (18.2) 0.36 1.73 (0.53–5.67)
- Null 37 (88.1) 5 (11.9) Reference

**c.-2595C > A+c.-906T > C**
- CC+CA+TT+TC 53 (88.3) 7 (11.7) **0.0007** 9.91 (1.85–52.85)
- AA+CC 3 (37.5) 5 (62.5) Reference

**c.-1154G > A+c.-889G > A**
- GG+GG 31 (86.1) 5 (13.9) 0.59 1.85 (0.19–17.90)
- GA+AA 12 (92.3) 1 (7.7) Reference

**c.-3434G > C+c.-889G > A**
- GG+GG 32 (84.2) 6 (15.8) 0.46 2.29 (0.24–21.51)

**c.-906T > C+c.-889G > A**
- TT+GG 19 (90.5) 2 (9.5) 0.31 0.37 (0.05–2.51)
- TC+CC+GA+AA 20 (83.3) 4 (16.7) Reference

**c.-889G > A+GSTM1**
- GG+Present 32 (82.1) 7 (17.9) 0.37 2.78 (0.29–26.31)
- GA+AA+Null 11 (91.7) 1 (8.3) Reference

**Abbreviations:** ASCT, autologous stem cell transplantation; CI, confidence interval; CR, complete response; Iss, International Staging System; N, number of patients; Ne, not evaluated; Or, odds ratio adjusted by Asct, Pd, progressive disease; Pr, partial response; Sd, stable disease; VEGF, very good partial response. Signiﬁcant differences between groups are presented in bold letters. The number of patients differed from the total quoted in the study, because it was not possible to obtain pertinent information in some cases. *PBootstrap = 0.02. **PBootstrap = 0.002. *Haplotype of VEGF c.-2595C > A, c.-1154G > A, c.-634G > A and c.-237C > T polymorphisms. **PBootstrap = 0.01. #Haplotype of VEGFR2 c.-906T > C and c.-889G > A polymorphisms.

In univariate Cox analysis, the signiﬁcance of differences between groups remained the same as of the above analyses, and for this reason the values of multivariate Cox analysis were adjusted by ISS and ASCT status. Patients at stage III, patients who did not receive ASCT and those with the VEGFR2 c.889GG, VEGF c.-1154GG plus VEGFR2 c.889GG, VEGF c.634GG plus VEGFR2 c.889GG, VEGFR2 c.906TT plus c.889GG, and VEGFR2 c.889GG plus GSTM1 present genotypes had 1.66, 3.43, 2.62, 2.78, 2.64, 3.48 and 2.80 more chances of disease relapse or progression, respectively. Patients who did not receive ASCT, and those with the VEGFR2 c.889GG, GSTM1 present, VEGF c.634GG plus VEGFR2 c.889GG and VEGFR2 c.889GG plus GSTM1 present had 3.29, 2.21, 1.85, 4.88 and 4.23 more chances of evolving to death, respectively (Table 2). We initially observed that carriers of VEGF c.-2595CC or CA genotype isolated or associated with VEGFR2 c.-906TT or CC genotype, and the CGGC haplotype (rs699947, rs1570360, rs2010963 and rs3025039) of all analyzed VEGF SNPs, previously associated with higher VEGF effects, 5–8 presented better response to thalidomide-based regimens. In contrast, genotypes and haplotypes of VEGF SNPs (rs699947, rs833061, rs2010963 and rs3025039) did not influence the response to thalidomide in a unique study conducted in relapsed MM patients. 10 Differences in response of tumors to thalidomide-based regimens may constitute a plausible explanation for the divergent results seen in both studies: only newly diagnosed MM patients were included in our study while that Andersen et al. 10 analyzed only MM patients at relapse. On the other hand, GSTM1 and GSTT1 genes did not alter response to thalidomide-based regimens in our newly MM patients, and also in those previously treated with VAD and high-dose melphalan. 11

Secondly, we found that carriers of VEGF c.-1154GG, VEGF c.-634GG, VEGFR2 c.-906TT, VEGFR2 c.889GG genotypes, and GSTM1 present, alone or combined, previously associated with higher VEGF effects, 5–8, 6, 8 had more chances of disease relapse/ progression and/or of evolving to death. The genotypes of VEGF SNPs (rs699947, rs833061, rs2010963 and rs3025039) had no influence in survival of relapsed MM patients after thalidomide treatment in a previous study, but patients with the ACG haplotype of VEGF SNPs (rs699947, rs833061 and rs2010963 loci) presented a shorter time of thalidomide failure. 10 On the other hand, no significant differences were observed in EFS and OS after

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| Variable | N of events/ N total | Univariate Cox analysis | Multivariate Cox analysis | N of events/ N total | Univariate Cox analysis | Multivariate Cox analysis |
|----------|----------------------|-------------------------|--------------------------|----------------------|-------------------------|--------------------------|
|          |                      | P-value                  | HR (95% CI)              | P-value              | HR (95% CI)              | P-value                  | HR (95% CI)              |
| ISS†     | I-II                 | 29/46                    | 0.08                     | Reference            |                         | 0.03†                    | 1.66 (1.03–2.70)         |
|          | III                  | 42/55                    | 1.52 (0.94–2.45)         | Reference            |                         | 19/46                    | 1.57 (0.89–2.77)         |
| ASCT     | Yes                  | 20/43                    | < 0.0001                 | Reference            | < 0.0001†               | 13/43                    | < 0.0001                 | < 0.0001†               |
|          | No                   | 51/59                    |                         |                       |                         | 39/59                    |                         |                       |
| VEGF c.2595C>A | C  | 32/49                  | 0.35                     | 0.08 (0.49–1.28)      | Reference               | 23/49                    | 0.43                     | 0.80 (0.46–1.39)         |
|          | A+AA                 | 39/53                    | 1.01 (0.54–1.90)         | Reference            |                         | 29/53                    | 0.24                     | 0.67 (0.35–1.30)         |
|          | AA                   | 12/18                    | Reference               |                         |                         | 12/18                    | Reference               |                         |
| VEGF c.1154G>A | G  | 39/55                  | 0.68                     | 1.10 (0.68–1.76)      | Reference               | 27/55                    | 0.92                     | 0.97 (0.56–1.68)         |
|          | A+AA                 | 32/47                    | Reference               |                         |                         | 25/47                    | Reference               |                         |
|          | AA                   | 68/96                    | 1.38 (0.43–4.39)         | Reference            |                         | 49/96                    | 0.79                     | 0.95 (0.26–2.75)         |
| VEGF c.-634G>C | G  | 37/54                  | 0.77                     | 1.07 (0.67–1.71)      | Reference               | 30/54                    | 0.20                     | 1.42 (0.82–2.49)         |
|          | C+GC                 | 34/48                    | Reference               |                         | Reference               | 22/48                    | 0.97                     | 0.98 (0.30–3.15)         |
|          | C                    | 68/97                    | 1.36 (0.42–4.39)         | Reference            |                         | 49/97                    | 0.74                     | 1.21 (0.37–3.95)         |
| VEGF c.-237C>T | C  | 54/76                  | 0.60                     | 1.15 (0.66–1.99)      | Reference               | 40/76                    | 0.62                     | 1.17 (0.61–2.25)         |
|          | T+TC                 | 17/26                    | Reference               |                         | Reference               | 12/26                    | 0.28                     | 0.46 (0.11–1.90)         |
|          | CC                   | 50/100                   | 1.65 (0.22–11.98)        | Reference            |                         | 50/100                   | 0.45                     | 0.57 (0.13–2.48)         |
| VEGFR2 c.-906T>C | C  | 22/28                  | 0.12                     | 1.52 (0.91–2.53)      | Reference               | 15/28                    | 0.45                     | 1.25 (0.68–2.30)         |
|          | T+TC                 | 54/75                    | 1.50 (0.86–2.60)         | Reference            |                         | 39/75                    | 0.70                     | 1.12 (0.60–2.11)         |
|          | CC                   | 17/27                    | Reference               |                         | Reference               | 13/27                    | 0.64                     | 1.15 (0.61–2.17)         |
| VEGFR2 c.889G>A | G  | 55/73                  | 0.006                    | 2.22 (1.26–3.91)      | Reference               | 41/73                    | 0.04                     | 2.00 (1.03–3.91)         |
|          | A+GA                 | 70/100                   | 1.62 (0.22–11.76)        | Reference            |                         | 51/100                   | 0.96                     | 0.95 (0.13–6.95)         |
|          | AA                   | 30/46                    | 1.05 (0.65–1.68)         | Reference            |                         | 19/46                    | 0.10                     | 1.60 (0.91–2.82)         |
| GSTM1    | Present              | 39/56                    | 0.82                     | 1.18 (0.74–1.89)      | Reference               | 33/56                    | 0.10                     | 1.60 (0.91–2.82)         |
|          | Null                 | 32/46                    | Reference               |                         | Reference               | 19/46                    | 0.10                     | 1.60 (0.91–2.82)         |
| GSTT1    | Present              | 55/80                    | 0.46                     | 1.23 (0.70–2.15)      | Reference               | 42/80                    | 0.30                     | 1.44 (0.72–2.87)         |
|          | Null                 | 16/22                    | Reference               |                         | Reference               | 10/22                    | 0.62                     | 1.19 (0.59–2.41)         |
| c.-2595C>A+c.-906T>C | C  | 47/65                  | 0.65                     | 1.23 (0.49–3.12)      | Reference               | 32/65                    | 0.42                     | 0.68 (0.26–1.76)         |
|          | AA+CC                | 5/8                      | Reference               |                         | Reference               | 5/8                      | 0.82                     | 0.89 (0.34–2.36)         |
| c.-1154G>A+c.-889G>A | G  | 30/39                  | 0.04                     | 2.29 (1.01–5.26)      | Reference               | 22/39                    | 0.31                     | 1.59 (0.63–3.95)         |
|          | A+GG                 | 7/13                     | Reference               |                         | Reference               | 6/13                     | 0.37                     | 1.52 (0.59–3.90)         |
| c.-634G>C+c.-889G>A | G  | 29/40                  | 0.02                     | 2.56 (1.14–5.73)      | Reference               | 22/40                    | 0.01                     | 4.79 (1.42–16.15)        |
|          | A+GG                 | 8/15                     | Reference               |                         | Reference               | 3/15                     | 0.01                     | 4.88 (1.42–16.70)        |
| c.-906T>C+c.-889G>A | T  | 20/24                  | 0.002                    | 3.34 (1.54–7.26)      | Reference               | 13/24                    | 0.06                     | 2.22 (0.94–5.24)         |
|          | TT+GG                | 14/25                    | Reference               |                         | Reference               | 9/25                     | 0.08†                    | 2.15 (0.90–5.14)         |
| c.889G>A+GSTM1 | G  | 31/40                  | 0.04                     | 2.21 (1.01–4.88)      | Reference               | 26/40                    | 0.02                     | 3.30 (1.14–9.51)         |
|          | A+GG+Null            | 8/13                     | Reference               |                         | Reference               | 4/13                     | Reference               |                         |
| VEGF     | CCGC                 | 15/77                    | 0.62                     | 1.15 (0.65–2.01)      | Reference               | 35/77                    | 0.17                     | 0.66 (0.37–1.19)         |
|          | Other haplotypes     | 16/25                    | Reference               |                         | Reference               | 16/25                    | 0.37                     | 0.76 (0.42–1.38)         |
VAD and high-dose melphalan in newly previously MM patients carrying or lacking the GSTM1 gene. The disparate results obtained in both studies may be attributed to different types and doses of treatment used, as the first-line therapeutic regimens with conventional doses of thalidomide and ASCT in our study and intensive treatment with VAD and high-dose melphalan in the other study. In fact, VEGF plays an important role in tumor AG, acting as a potent inducer of vascular proliferation and permeability, and thus may advantage the action of therapy in MM tumor cells with consequently better response. However, VEGF also increases interleukin-6 secretion by endothelial and BM stromal cells, which stimulates MM cell growth, with consequent relapse of disease.

In summary, our data present, for the first time, a preliminary evidence that VEGF c.-2595C>A, c.-1154G>A, c.-634G>C, c.-237C>T, VEGFR2 c.-906T>C and c.889G>A polymorphisms. In multivariate Cox analysis adjusted by ISS and ASCT. L Lopes-Aguiar1, MT Delamain1, ABC Brito1, GJ Lourenço1, EFD Costa1, GB Oliveira2, J Vassallo1, CA De Souza1,2 and CSP Lima3

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**CONFLICT OF INTEREST**
The authors declare no conflict of interest.

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**AUTHOR CONTRIBUTIONS**
LLA, ABCB and CSPL performed the study design. LLA, MTD, ABCB, GBO, JV and CADS performed the data acquisition. LLA, ABCB, EFDC and CSPL performed the data analysis and interpretation. LLA, GJL and EFDC performed the statistical analysis. LLA and CSPL drafted the manuscript. LLA, MTD, ABCB, GJL, EFDC, GBO, JV, CADS and CSPL made important contributions to conception of work. All authors approved the final manuscript.

**Table 2.** (Continued)

| Variable       | EFS (N = 102) | Multivariate Cox analysis | OS (N = 102) | Multivariate Cox analysis |
|----------------|--------------|--------------------------|--------------|--------------------------|
|                | N of events/ | Univariate Cox analysis  |              | Univariate Cox analysis  |
|                | N total      | P-value | HR (95% CI) | P-value | HR (95% CI) | P-value | HR (95% CI) | P-value | HR (95% CI) |
| **VEGFR2**     |              |          |            |          |            |          |            |          |            |
| TG<sup>a</sup> | 54/75        | 0.14    | 1.50 (0.86–2.60) | 0.05    | 1.79 (1.02–3.15) | 39/75 | 0.70 | 1.12 (0.60–2.11) | 0.64 | 1.15 (0.61–2.17) |
| Other haplotypes | 17/27        | Reference |            |         | Reference |          |            |          |            |

Abbreviations: ASCT, autologous stem cell transplantation; CI, confidence interval; EFS, event-free survival; HR, hazard ratio; ISS, International Staging System; N, number of patients; OS, overall survival. Significant differences between groups are presented in bold letters. The number of patients differed from the total quoted in the study, because it was not possible to obtain pertinent information in some cases. Bootstrap = 0.05. Bootstrap = 0.10. Bootstrap = 0.001. Bootstrap = 0.005. Bootstrap = 0.02. Bootstrap = 0.04. Bootstrap = 0.01. Bootstrap = 0.03. Bootstrap = 0.005. Bootstrap = 0.003. Bootstrap = 0.06.

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