# Biomarker Analysis in a Phase II Study of Sunitinib in Patients with Advanced Melanoma

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**Abstract.** *Aim: To investigate the efficacy of sunitinib in patients with advanced melanoma and to correlate angiogenic biomarkers with response and survival. Patients and Methods: We performed a phase II study in patients with advanced pre-treated melanoma. The primary endpoint was tumor response. Blood samples for biomarker analysis including vascular endothelial growth factor (VEGF), and its receptors VEGFR1 and -2, placental growth factor (PlGF) and circulating endothelial cells (CEC) were collected at baseline and during the first cycle. Results: Four out of 39 patients (13%) achieved a partial response and eight (26%) stable disease. Time to progression was at least six months in seven patients. High baseline VEGFR1 levels and high baseline PlGF levels were both associated with a non-significant worse survival (p=0.08 for both). Conclusion: Sunitinib demonstrates limited activity in unselected patients with refractory advanced melanoma, but a minority of patients experienced long-term disease control. Identification of these patients remains a challenge.*

Up to 2011, treatment options for advanced malignant melanoma were limited. Dacarbazine-based chemotherapy resulted in response rates (RR) of 5-20% and a median overall survival (OS) of seven months (1). Since 2010, new treatment strategies have become standard-of-care for patients with advanced malignant melanoma. Firstly, immunotherapy with ipilimumab, an IgG1 monoclonal antibody against T-lymphocyte-associated antigen, and nivolumab, a IgG4 programmed death 1 (PD1) antibody, have been shown to improve survival in metastatic melanoma and monoclonal antibodies targeted against PD1 (nivolumab, pembrolizumab) are active and approved for the treatment of ipilimumab-refractory melanoma (2-4). Secondly, treatment with a v-raf murine sarcoma viral oncogene homolog B (BRAF) inhibitor and more recently combination of a BRAF plus mitogen/extracellular signal-regulated kinase (MEK) inhibitor increased survival in patients with *BRAF*-mutated melanoma (5, 6).

Angiogenesis is recognized as a hallmark of several types of tumors, including melanoma (7, 8). The process of angiogenesis is crucial for tumor development and metastasis (9, 10). One of the most important cytokines responsible for tumor-mediated angiogenesis is vascular endothelial growth factor (VEGF). VEGF is strongly expressed in melanoma (11) and plays a critical role in melanoma progression (9, 12). VEGF expression is up-regulated during melanoma tumorigenesis, progression and dissemination (9), suggesting that the VEGF pathway represents a potential target for melanoma treatment.

Sunitinib (Sutent®) is an oral, multi-targeted tyrosine kinase inhibitor of vascular endothelial growth factor receptors (VEGFR), platelet_derived growth factor receptors (PDGFR), v kit hardy-zuckerman 4 feline sarcoma viral oncogene homolog (*cKIT*), fms-like tyrosine kinase 3 (FLT3) and rearranged during transfection (*RET*) oncogene (13, 14).

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*Key Words:* Malignant melanoma, phase II study, sunitinib, biomarkers, PlGF.

Inhibition of these tyrosine kinases blocks signal transduction, affecting tumor growth, progression, metastases and angiogenesis. In addition to the anti-angiogenic effect, sunitinib was found to have activity against *cKIT*-mutant melanoma (15).

In this multicenter academic phase II study, we investigated the activity of sunitinib in patients with refractory advanced malignant melanoma, regardless of *cKIT* mutation status. Angiogenic biomarkers were measured at baseline and early during treatment in an attempt to identify potential biomarkers for response.

## Patients and Methods

*Patients*. Patients with locally advanced or metastatic malignant melanoma were eligible for the study if they met the following criteria: disease progression on prior dacarbazine-based chemotherapy, with multiple lines of chemotherapy allowed; measurable disease; Eastern Cooperative Oncology Group performance status (ECOG PS) of 0, 1 or 2; absence of prior history of thromboembolism; no anticoagulation therapy; no other malignancies or serious illness; signed and dated informed consent.

*Study design and treatment*. The study was a multicenter academic phase II trial in Belgium (EudraCT 2008-000756-27) and was approved by the leading ethical committee of the Universitair Ziekenhuis Brussel and by the Institutional Medical Ethics Review Boards (approval number 2008/076) of each participating center.

Sunitinib was administered in repeated six weekly cycles consisting of 50 mg taken orally once daily, for four weeks, followed by two weeks off (schedule 4/2). Dose reductions for toxicity were allowed to 37.5 mg/day or 25 mg/day. Treatment was continued until disease progression, unacceptable toxicity or patient refusal.

*Patient evaluation*. Baseline evaluation included a complete medical history, physical examination and assessment of ECOG performance status; a complete blood count, chemistry profile and thyroid function and radiological tumor assessment by computed tomographic or magnetic resonance imaging.

Objective response was assessed by the Response Evaluation Criteria in Solid Tumors (RECIST, v1.0) (16) after each cycle during the first two cycles and every two cycles thereafter until disease progression.

Toxicity was evaluated every two weeks during the first two cycles and every six weeks thereafter. Adverse events were graded in accordance with National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) Version 3.0 (17).

*Assessment of biomarkers*. Candidate predictive angiogenic biomarkers for response were investigated in a translational sub-protocol.

Circulating protein biomarkers including VEGF, VEGFR1 and -2, and placental growth factor (PlGF) were assessed at baseline and every two weeks during the first cycle. Heparin-containing plasma tubes and serum tubes were collected on ice for analysis of VEGF/PlGF and VEGFR1/2, respectively. Plasma was separated and stored at −80˚C. The analyses were performed using enzyme-linked immunosorbant assay (ELISA) kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The limit of detection and the measuring range were 9 pg/ml and 31.2-2000 pg/ml for VEGF, 7 pg/ml and 15.6-1000 pg/ml for PlGF, 13.3 pg/ml and 31.2-2000 pg/ml for VEGFR1 and 11.4 pg/ml and 78.1-5000 pg/ml for VEGFR2.

For analysis of circulating endothelial cells (CECs) and circulating endothelial progenitor cells (CEPs), plasma was collected at baseline and weekly during the first cycle. Samples were transported immediately after collection to the designated central laboratory and CECs and CEPs were measured the same day using 4-color flow cytometric analysis. Detection of CECs was based on cluster of differentiation (CD) 31bright/CD45$^{-}$/CD34$^{+}$/CD133$^{-}$ phenotype and detection of CEPs on CD133$^{+}$/CD34$^{bright}$/CD31$^{+}$/CD45$^{dim}$ phenotype (18). All antibodies were obtained from Miltenyi Biotec, Bergisch Gladbach, Germany. Cell suspensions were evaluated using the MACSQuant Analyser (Myltenyi Biotec).

*Mutation analysis*. *KIT* and *BRAF* mutation analysis were performed in patients with available tumor tissue. DNA was extracted from formalin-fixed paraffin embedded (FFPE) tumor tissue using the QiaAmp DNA FFPE kit from Qiagen, Hilden, Germany. Mutation analysis was performed for exons 8 (c.1232-c.1346), 11 (c.1648-c.1774), 13 (c.1880-c.1982) and 17 (c.2362-c.2484) of *cKIT* [Reference Sequence (RefSeq): NM_000222.2]; exons 11 (c.1315-c.1432) and 15 (c.1742-c.1860) of *BRAF* (RefSeq: NM_004333.4). All exons were sequenced on a Genome Sequencer Junior platform from Roche, Basel, Switzerland (amplicon based Next Generation Sequencing (NGS), primers available on request). NGS analyses were performed in duplicate and aim at obtaining at least 2x500 reads for each exon, which allows in principle to detect mutations present in 1% of the obtained reads. Data analysis was done using the Amplicon Variant Analysis software from Roche.

*Study endpoints and statistical considerations*. The primary endpoint of the study was objective response rate as defined by RECIST v1.0 (16).

Sample size was determined using a Simon's Minimax two-stage design (19) to test the null hypothesis that the response rate was ≤5% *versus* the alternative hypothesis that the response rate was ≥20% ($\alpha$ level of 10%). In the first stage of accrual, 18 patients evaluable for response were to be accrued. If at least one response was documented, an additional 14 evaluable patients would be accrued in the second stage. At the end of the study, sunitinib would be warranted for further investigation if four or more responses were observed in the final sample of 32 evaluable patients.

Secondary endpoints were time to time to progression (TTP), overall survival (OS) and toxicity.

Analyses were performed using SPSS statistics version 22, IBM Corporate, Armonk, NY, USA. Time to event variables were estimated using the Kaplan–Meier method and two-sided 95% confidence intervals (CI) for the median were provided for each variable.

Correlations between efficacy endpoints and plasma biomarkers were analyzed with Student *t*-tests, Kaplan–Meier method and log-rank test. Values of $p \leq 0.05$ were considered statistically significant.

## Results

*Patients' characteristics*. Between July 2008 and July 2009, 41 patients with advanced melanoma were treated with sunitinib. Two patients (one with uveal and one with mucosal

melanoma) were excluded from the final analysis in order to limit the analysis to all patients with cutaneous melanoma. Baseline patient characteristics are summarized in Table I.

*Efficacy*. Thirty-one out of 39 patients (79%) were evaluable for response. Eight patients discontinued treatment because of unacceptable toxicity before the first planned radiological evaluation of tumor response.

No complete responses were observed. Partial response (PR) was observed in four patients (13%) and stable disease (SD) in eight (26%).

At the time of analysis in December 2014, all patients were off treatment. The median PFS (Figure 1a) and OS (Figure 1b) for the overall population was 1.3 (95% CI=1.2-1.4) months and 4.3 (95% CI=1.0-7.6) months, respectively. Patients with PR and SD had median TTP of 8 (95% CI=2.7-13.3) months and 2.7 (95% CI=2.6-2.8) months and median OS of 10 (95% CI=0-42.1) months and 8 (95% CI=0-23.7) months, respectively.

*Toxicity*. Toxicity was assessable in all 39 patients. Treatment interruption was necessary for 16 patients (41%) due to intolerance and dose reductions were performed for six (15%) patients. Eleven patients discontinued treatment before apparition of disease progression because of excessive toxicity (n=10) and withdrawal of consent (n=1).

Almost all patients experienced adverse events during treatment (95%) and 64% of patients had at least one grade 3 or 4 adverse event. The most frequent grade 3 or 4 adverse events were asthenia (28%), thrombocytopenia (15%), neutropenia (15%) and anorexia (10%).

*Biomarker analysis*. Thirty patients (77%) signed an informed consent form for the translational sub-protocol.

*Analyses of plasma VEGF, VEGFR1, VEGFR2 and PlGF levels:* At baseline, patients with an ECOG PS of 0 had significantly lower plasma VEGF, VEGFR1 and PlGF levels than patients with PS 1 or 2 (mean 89.9 *vs*. 270.6 ng/l, respectively, $p$=0.037; 80.8 *vs*. 119.5 ng/l, $p$=0.018; 32.2 *vs*. 59.0 ng/l, $p$=0.017).

During treatment with sunitinib, VEGF and PlGF increased in 90% and 100% of patients, respectively, and VEGFR1 and -2 decreased in 82% and 100% of patients, respectively.

No significant correlation was observed between response and baseline VEGF, VEGFR1 and VEGFR2 levels. When compared to patients with PR, patients with progressive disease (PD) had a non-significantly higher baseline PlGF level (mean 47.7 *vs*. 22.0 ng/l; $p$=0.073).

For each biomarker, patients were divided into two categories: high baseline *vs*. low baseline VEGF, VEGFR1, VEGFR2 and PlGF. The cut-off value was defined as the mean plasma level for each biomarker: 186.3 ng/l for VEGF, 101.4 ng/l for VEGFR1, 8920.6 ng/l for VEGFR2 and 46.5 ng/l for PlGF. Both high baseline plasma VEGFR1 and PlGF levels were associated with a non-significantly worse survival when compared to low baseline plasma VEGFR1 and PlGF levels [1.5 (95% CI=0.4-2.6) months *vs*. 7.5 (95% CI=4.1-10.9) months, $p$=0.08 for VEGFR1 and 2.2 (95% CI=1.4-3.0) months *vs*. 7.6 (95% CI=5.6-9.6) months, $p$=0.08] (Figure 2). No correlation between survival and baseline VEGF or VEGFR2 was observed.

Table I. *Patients' characteristics at baseline (n=39).*

| Characteristic | |
|---|---|
| Median age (range), years | 55 (31-84) |
| Gender, N (%) | |
| Male | 21 (54%) |
| Female | 18 (46%) |
| ECOG PS, N (%) | |
| 0 | 22 (56%) |
| 1 | 15 (39%) |
| 2 | 2 (5%) |
| Stage, N (%) | |
| M1a | 5 (13%) |
| M1b | 8 (20%) |
| M1c | 26 (67%) |
| Previous treatments for metastatic disease, N (%) | |
| Chemotherapy | 38 (97%) |
| Interferon alpha-2b | 9 (31%) |
| Vaccination protocol | 3 (8%) |

ECOG PS: Eastern Cooperative Oncology Group performance status.

*Analyses of CECs and CEPs:* During treatment with sunitinib, an increase in CECs and a decrease in CEPs were recorded on day 8 of the first cycle.

No correlation between response and baseline CEC/CEP or changes in CEC/CEP was observed (data not shown).

*Mutation analysis*. Tumor tissue was evaluable for mutational analysis in 21 patients, including three patients with PR and two patients with SD longer than 3 months.

No KIT mutation was observed and a *BRAF* mutation was detected in 13 out of 21 samples (62%). In this subset of 21 patients, no significant difference between the presence or not of a *BRAF* mutation and PFS, OS or baseline biomarker levels was observed.

## Discussion

Although the current phase II trial of sunitinib monotherapy in patients with refractory advanced melanoma met its primary endpoint of at least four objective responses, the observed activity was modest with an objective response rate (ORR) of 13% and a disease control rate (PR plus SD>6 months) of 18%.

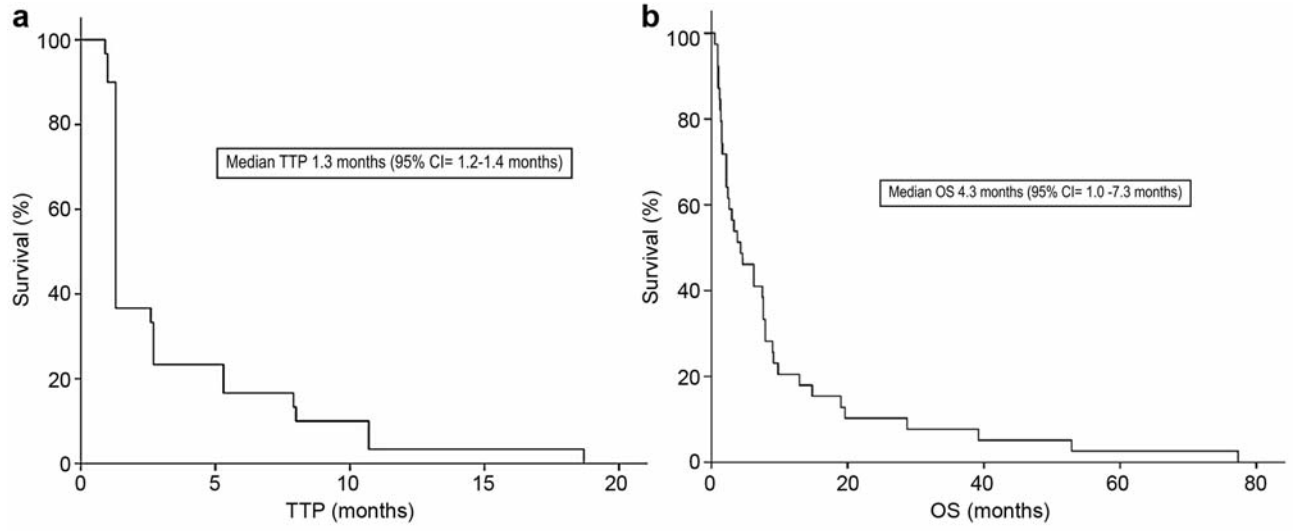


Figure 1. *Time to progression (TTP) (a) and overall survival (OS) (b) for the overall population.*

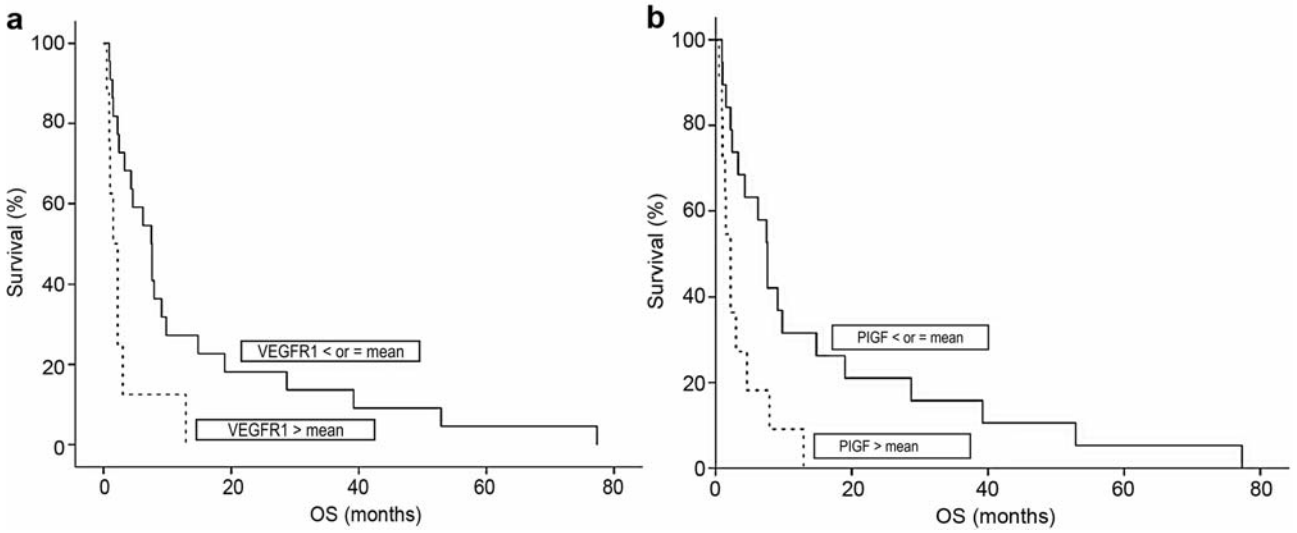


Figure 2. *Overall survival (OS) according to baseline plasma vascular endothelial growth factor receptor (VEGFR) 1 (a) and placental growth factor (PlGF) (b) levels. High baseline VEGFR1 levels (above the mean) were associated with a worse survival when compared to low baseline VEGFR1 levels (at or below the mean), although not reaching significance: median=1.5 months [95% confidence interval (CI)= 0.4-2.6 months] vs. 7.5 months (95% CI=4.1-10.9 months), p=0.08. Similarly, high PlGF levels (above the mean) were associated with a worse survival when compared to low PlGF levels (at or below the mean), although not reaching significance: median=2.2 months (95% CI=1.4-3.0 months) vs. 7.6 months (95% CI=5.6-9.6 months), p=0.08.*

Other multi-targeted tyrosine kinase inhibitors of VEGFR, such as sorafenib, that is also a BRAF inhibitor (20, 21) and vatalanib (22), have shown no single-agent activity, with response rates of 3% and median TTP ranging from 1.8 to 2.1 months. Axitinib, a selective second-generation inhibitor of VEGFR1, -2 and -3, demonstrated more promising results in patients with stage IV melanoma, with an ORR of 19% and SD for more than 16 weeks in 19% (23).

In the current trial, seven patients had a TTP lasting 6 months or longer, including the four patients with PR, indicating that a sub-group of patients with melanoma may

benefit from treatment with sunitinib. In order to identify such patients at baseline or early during treatment, a biomarker analysis was performed in a translational sub-protocol. During the first cycle, circulating protein biomarkers VEGF, VEGFR1, VEGFR2 and PlGF levels, as well as CECs and CEPs were measured. In the vast majority of patients, an increase in VEGF and PlGF and a decrease in VEGFR1 and 2 levels were observed, reflecting the pharmacodynamic effects of the anti-angiogenic activity of sunitinib. These observations are consistent with similar findings in renal cell carcinoma (RCC) and breast cancer (24, 25). In addition, in the present study an increase in CECs and a decrease in CEPs were observed, which is also consistent with observations in RCC, as well as other tumor types, and compatible with vascular damage and antiangiogenic activity respectively (26).

In the current trial, both high baseline plasma VEGFR1 and PlGF levels correlated with worse survival compared to low baseline VEGFR1 and PlGF levels, suggesting that these biomarkers may be prognostic markers in advanced melanoma. This observation agrees with the observations in RCC (27), oral squamous cell carcinoma (28) and colorectal cancer (29), where plasma PlGF level was an independent prognostic factor. Because of the small sample size, our findings did not reach significance and should be confirmed in a larger sample of melanoma patients. If confirmed it may warrant the exploration of anti-PlGF as a therapeutic strategy, perhaps especially in patients with high PlGF levels. Others have found a similar correlation for VEGF levels and prognosis in melanoma (30), but this was not confirmed in our study possibly due to the smaller sample size.

We did not observe any correlation between tumor response and baseline VEGF, VEGFR1, 2 or CEC/CEP. However, definite conclusions are difficult due to the low number of samples and further study of these biomarkers remain of interest. A significantly higher baseline PlGF was observed in patients with PD, probably reflecting the bad prognosis of these patients. In RCC, De Primo *et al*. observed significantly larger proportional changes in VEGF and VEGFR2 levels in patients with an objective tumor response compared to patients with SD and PD (24), while Gruenwald *et al*. reported a correlation between longer TTP to sunitinib and an early increase of CECs (31).

Since sunitinib also targets KIT, and sunitinib has demonstrated activity in *cKIT*-mutant melanoma, tumor tissue that was available in five out of seven patients with a major clinical benefit was analyzed. However, no mutation was detected in this patient population. This indicates that observed responses may be due to antiangiogenic effect of sunitinib, rather than directly targeting KIT.

Finally, the toxicity of sunitinib was a major issue in the present trial: 64% of patients developed a grade 3 or 4 adverse event, 41% of patients needed a treatment interruption due to intolerance and 26% of patients experienced a serious adverse event. When compared to the first-line treatment of RCC with sunitinib (32), more grade 3/4 adverse events were reported, especially more asthenia (28% in the current trial and 12% in the RCC trial). However treatment interruptions were similar (41% in the current trial *vs*. 38% in the RCC trial).

In conclusion, the present trial reveals VEGFR1 and PlGF as possible prognostic markers for survival in advanced melanoma and their role as a prognostic and predictive biomarkers and therapeutic targets should be further explored. The current observed activity of sunitinib in a sub-group of patients, however, provides a proof of principle for therapeutic targeting of angiogenesis in advanced melanoma using VEGFR tyrosine kinase inhibitors, but combination therapies may be more beneficial. The toxicity of sunitinib at the investigated dosing regimen would probably not be optimal for combination with immunotherapy (*e.g*. CTLA4 or PD1 blockade). Based on phase I data (33), however, further exploration of immunotherapy with an anti-antiangiogenic drugs may be an attractive option and alternative (less toxic) dosing regimens of sunitinib may be of potential interest for such combinations.

Predictive biomarkers that would allow selection of patients that do benefit, either at baseline or early in the treatment, are of utmost importance.

## Conflicts of Interest

This study was supported by a research grant to Professor Jacques De Grève and medication from Pfizer.

Professor Bart Neyns received a scientific grant from Pfizer, not related to the current study.

Professor Sylvie Rottey is a member of advisory boards for Pfizer, speaker for Pfizer and received a scientific grant from Pfizer. All remaining authors have declared no conflicts of interest.

## Source of Funding

This study was supported by a research grant and medication from Pfizer.

The biomarker sub-study was supported by a grant from the Wetenschappelijk Fonds Willy Gepts (WFWG) of the UZ Brussel, Brussels, Belgium.

## Acknowledgements

We thank the data management of Ellen De Wandeler and Gerda Lauwers for their hard work collecting all data files. We also thank all of the investigators and their site research teams. We would like to thank Pfizer for providing an unrestricted grant and trial medication, and Ludwig Van den Hove, Ph.D., Pfizer Belgium, for his support and critical review of the manuscript. We would also like to thank the Wetenschappelijk Fonds Willy Gepts of the UZ Brussel for the financial support of the translational sub-protocol.

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*Received September 10, 2015*
*Revised October 13, 2015*
*Accepted October 14, 2015*