

# Cell Cycle

ISSN: 1538-4101 (Print) 1551-4005 (Online) Journal homepage: <http://www.tandfonline.com/loi/kccy20>

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**To cite this article:** Céline Pinheiro, Vera Miranda-Gonçalves, Adhemar Longatto-Filho, Anna LSA Vicente, Gustavo N Berardinelli, Cristovam Scapulatempo, Ricardo FA Costa, Cristiano R Viana, Rui M Reis, Fátima Baltazar & Vinicius L Vazquez (2016): The metabolic microenvironment of melanomas: prognostic value of MCT1 and MCT4, *Cell Cycle*, DOI: [10.1080/15384101.2016.1175258](https://doi.org/10.1080/15384101.2016.1175258)

**To link to this article:** <http://dx.doi.org/10.1080/15384101.2016.1175258>



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Accepted author version posted online: 22 Apr 2016.



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Prognostic value of MCT1 and MCT4 in melanoma

**The metabolic microenvironment of melanomas: prognostic value of MCT1 and MCT4**

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**Conflict of interest:** The authors declare no conflict of interest.

**ABSTRACT**

*BRAF* mutations are known drivers of melanoma development and, recently, were also described as players in the Warburg effect, while this reprogramming of energy metabolism has been identified as a possible strategy for treating melanoma patients. Therefore, the aim of this work was to evaluate the expression and prognostic value of a panel of glycolytic metabolism-related proteins in a series of melanomas. The immunohistochemical expression of MCT1, MCT4, GLUT1, and CAIX was evaluated in 356 patients presenting melanoma and 20 patients presenting benign nevi. Samples included 20 benign nevi, 282 primary melanomas, 117 lymph node and 54 distant metastases samples. *BRAF* mutation was observed in 29/92 (31.5%) melanoma patients and 17/20 (85%) benign nevi samples. *NRAS* mutation was observed in 4/36 (11.1%) melanoma patients and 1/19 (5.3%) benign nevi samples. MCT4 and GLUT1 expression was significantly increased in metastatic samples, and MCT1, MCT4 and GLUT1 were significantly associated with poor prognostic variables. Importantly, MCT1 and MCT4 were associated with shorter overall survival. In conclusion, the present study brings new insights on metabolic aspects of melanoma, paving the way for the development of new-targeted therapies.

### **Keywords**

Cancer, glycolysis, melanoma, monocarboxylate transporters, Warburg effect

## INTRODUCTION

Most solid tumors reprogram their energetic metabolism towards glycolysis, independently from oxygen levels, a phenomenon known as the *Warburg effect* or aerobic glycolysis<sup>1</sup>. Importantly, this metabolic reprogramming was recently identified as one of the hallmarks of cancer<sup>2</sup> and has been pointed out as a promising target for anti-cancer therapy<sup>3</sup>. Aerobic glycolysis results in a high production of free protons and lactate that must be shuttled to the extracellular milieu through many transporters<sup>4</sup>. The resultant microenvironment selects for cells with enhanced metastatic potential and is associated with evasion to immune destruction and resistance to radio- and chemotherapy<sup>5</sup>. In this context, monocarboxylate transporters (MCTs), specifically MCT1 and MCT4, play an essential role by contributing both for the hyperglycolytic and the acid-resistant phenotype of cancer cells, by mediating lactate and proton efflux to the extracellular milieu<sup>4</sup>. Accordingly, these transporters have been described as upregulated and associated with poor prognosis in many cancer types, with a high potential for exploitation as therapeutic targets<sup>6</sup>.

Melanoma is the most aggressive skin cancer, with an increasing incidence in the world<sup>7</sup>. In the last years, melanoma patient's handling have been changing thanks to an increase in the understanding of melanoma molecular heterogeneity<sup>8</sup>. In particular, studies have identified *BRAF* mutations in these tumors, being these mutations associated with an increase in the mortality rate of melanoma patients<sup>9</sup> and important for therapeutic decisions thanks to specific inhibitors targeting the signaling pathways involved<sup>10</sup>. Specifically, the V600E *BRAF* mutation is present in 40 to 60% of cutaneous melanoma and accounts for more than 80% of all *BRAF* mutations<sup>11-13</sup>, while *NRAS* mutations are found in 15 to 20% of melanomas<sup>13, 14</sup>. The

alterations in the MAP kinase cascade due to mutations have been targeted with clinical relevance. Treatment of V600E metastatic melanoma patients with BRAF inhibitors and, more recently, in association with MEK inhibitors, increases patient's survival rates and is approved and available for clinical use<sup>15-18</sup>.

Interestingly, the V600E *BRAF* mutation was recently shown to drive the Warburg effect<sup>19</sup>. In fact, previous studies in both melanoma and thyroid cancer have shown that this mutation increases the expression of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ )<sup>20, 21</sup>, the major driver of the Warburg effect in cancer cells<sup>22</sup>. Additionally, although mainly studied in the context of tumors different than melanoma, mutations in *RAS* family members, specifically *KRAS*, were shown to be associated with a glycolytic phenotype, with increased glucose uptake and lactate production<sup>23-26</sup>. Meanwhile, additional studies, both in human samples and *in vitro* models, have shown that melanoma cells exhibit the Warburg effect<sup>27, 28</sup> and that the progression to an invasive phenotype occurs under a metabolic switch from mitochondrial oxidative phosphorylation to glycolytic flux followed by lactate production<sup>29</sup>. As a result, melanoma cell metabolism has been pointed out as a promising strategy for melanoma treatment<sup>29-31</sup>.

Although the interest in the metabolic reprogramming of cancer cells is arising in the last years<sup>2</sup>, few studies focus on the metabolic profile of melanoma cells. Additional studies should especially evaluate the expression and clinical significance of the metabolism-related proteins, especially those that sustain the Warburg effect. Therefore, the aim of this study was to evaluate the expression and prognostic value of monocarboxylate transporters isoforms 1 and 4 (MCT1, MCT4), glucose transporter 1 (GLUT1) and the pH regulator carbonic anhydrase IX (CAIX), in

a series of melanocytic samples including benign nevi, primary tumors and both lymph node and distant metastasis.

## RESULTS

### Expression of MCTs, GLUT1 and CAIX in melanocytic samples

In benign nevi, with the exception of MCT1 expression that was observed in the plasma membrane, protein expression was almost exclusively found in the cytoplasm. In opposition, in malignant samples, MCT1, MCT4 and GLUT1 expression was almost exclusively found in the plasma membrane (Figure 1). CAIX exhibited cytoplasmic expression, alone or in combination with plasma membrane expression, however, with plasma membrane predominance. Therefore, and in accordance to the activity of the proteins herein studied, from now on, all results shown are based on plasma membrane expression.

Comparison of protein expression among samples from different origins (benign nevi, melanoma primary tumors, lymph node metastases and distant metastases) showed a significant difference in the overall expression frequency of MCT4, GLUT1 and CAIX ( $p=0.030$ ,  $p=0.015$  and  $p=0.009$  respectively), and no significant difference for MCT1 (Table 1). In the case of MCT4 and GLUT1, the differences observed were due to a significant increase in the transition from primary tumor to lymph-node metastasis, while, for CAIX, a significant increase in the expression frequency was observed in both the transition from benign nevi to primary tumor and lymph-node metastasis to distant metastases. Additionally, considering protein expression in all malignant samples, both MCT1 and MCT4 were significantly co-expressed with GLUT1 and CAIX (Table 2).

From the 92 patients with results for *BRAF* mutation status, 29 (31.5%) patients showed the V600E mutation in at least one tumor sample (primary tumor, lymph node metastasis or distant metastasis), while 17 of the 20 benign nevi (85%) showed the V600E mutation. Importantly, MCT1 and GLUT1 were significantly more expressed in *BRAF* mutated melanoma patients [17/28 (60.7%) and 9/29 (31.0%), respectively] than in *BRAF* wild-type melanoma patients [23/61 (37.7%) and 8/63 (12.7%),  $p=0.043$  and  $p=0.035$ , respectively].

From the 36 patients with results for *NRAS* mutation status, 4 (11.1%) patients showed mutation (Q61H, Q61R, Q61L and G12A) in at least one tumor sample (primary tumor, lymph node metastasis or distant metastasis), while 1 of 19 benign nevi (5.3%) showed the Q61R *NRAS* mutation. No significant associations were found between *NRAS* mutation status and expression of the proteins herein analyzed.

### **Clinicopathological significance of MCTs, GLUT1 and CAIX**

The clinicopathological significance of MCTs, GLUT1 and CAIX was analyzed considering plasma membrane expression in primary tumor samples (Table 3 and Table 4). MCT1 expression frequency was significantly associated with higher clinical stage ( $p<0.001$ ), higher pT ( $p=0.016$ ), higher pN ( $p=0.004$ ), and higher pM ( $p=0.012$ ). MCT4 expression frequency showed the highest number of significant results, being associated with male gender ( $p=0.045$ ), higher clinical stage ( $p=0.032$ ), nodular histological type ( $p=0.029$ ), trunk tumor location ( $p=0.038$ ), higher pT ( $p=0.002$ ), higher pN ( $p=0.008$ ), presence of locoregional recurrence ( $p=0.002$ ), higher Breslow's thickness ( $p=0.001$ ), and higher number of mitoses/mm<sup>2</sup> ( $p=0.015$ ). GLUT1 frequency of expression was significantly associated with higher pT

( $p=0.035$ ), higher Breslow's thickness ( $p=0.036$ ), and higher number of mitoses/mm<sup>2</sup> ( $p=0.049$ ), while CAIX showed no significant associations with the clinicopathological data.

### Survival analysis

The influence of MCTs, GLUT1 and CAIX on overall survival was analyzed considering plasma membrane expression in primary tumor samples (Figure 2 and Table 5). Overall survival analysis using Kaplan-Meier (Figure 2) showed that MCT1 and MCT4 expression is significantly associated with shorter overall survival ( $p=0.037$  and  $p=0.001$ , respectively). No significant results were observed for GLUT1 and CAIX expression (data not shown). The predictive prognostic values of the metabolism-related proteins were analyzed by means of Cox proportional hazards regression models (Table 5). Univariate analysis showed similar results to the ones obtained with Kaplan-Meier analysis, with significant values for MCT1 and MCT4 expression. However, when applying multivariate analysis, none of these proteins showed to be a stage-independent predictor of overall survival.

### DISCUSSION

Although recent advances have improved the management of patients with melanoma, with, for example, the use of specific inhibitors targeting mutated *BRAF*, resistance to the agents recently approved is a common event<sup>8, 32</sup>. Importantly, V600E *BRAF* mutation was shown to drive the Warburg effect<sup>19</sup>, suggesting a relevance of the glycolytic metabolism in melanoma and a possible relation with response/resistance to BRAF inhibitors. Therefore, the knowledge on the expression and clinicopathological significance of key metabolism-related proteins in melanomas may bring new insights for melanoma patients' handling.



In the present study, the immunohistochemical evaluation showed that the expression of the metabolism-related proteins in primary melanomas varied from around 10% for GLUT1 to around 35% for MCT1, while, in metastases, expression frequencies varied from around 20% for GLUT1 to around 45% for MCT1, with similar results amongst lymph node and distant metastases (exception for CAIX expression). To the best of our knowledge, only one study evaluated the expression of MCT1 and MCT4 in melanomas; however, median score values were used instead of expression frequencies, making comparisons difficult to establish <sup>28</sup>. Regarding GLUT1 expression, more studies are available and, although 2 previous studies, with small casuistic and no description of melanoma histological type or anatomical site, showed lack of GLUT1 expression in primary melanomas <sup>33, 34</sup>, a more recent study by Koch and collaborators showed an important GLUT1 expression in melanomas, with an expression frequency of 50% in primary tumors, 5 times the one found in the present study, and an expression frequency of 58% in metastases <sup>35</sup>, 3 times the one found in the present study. These higher expression frequencies may be due to the different immunohistochemical classification, as only staining intensity was evaluated (with weak results also considered positive) and distinct cellular locations were not discriminated <sup>35</sup>. In the case of CAIX, a previous study showed lack of expression in melanomas, which is not in agreement with the findings of the present study <sup>36</sup>. However, once again, the size of the casuistic was small (only 32 malignant melanomas analyzed), with no description of melanoma histological types or anatomical site, while the use of different protocols during immunohistochemical procedure and different parameters to score the positive reactions may also contribute to these discordant results.

Importantly, MCT4 and GLUT1 expression was significantly increased in the transition from primary tumor to lymph-node metastases, suggesting that the hyperglycolytic phenotype, determined by increased glucose uptake by GLUT1 and lactate efflux by MCT4, contributes to the invasive capacity of cancer cells, as previously suggested by others <sup>1</sup>. These results are partially in agreement with the work of Ho and collaborators, where a significant increase in MCT1 expression levels when comparing metastases with thin primary melanomas was observed, as well as a significant increase in MCT4 expression levels when comparing primary melanomas or metastases with nevi, with no differences between primary melanomas and metastases <sup>28</sup>. In the present study, we did not observe an increase in MCT1 expression frequency in metastatic samples; however, Ho and collaborators observed a significant difference in primary *versus* metastatic melanomas when considering only thin primary melanomas. This division among primary tumors was not performed in the present study and may explain similar results obtained for MCT1 among primary and metastatic melanomas. The different results obtained regarding MCT4, may be due to sample size, as Ho and collaborators analyzed MCT4 expression in 31 primary melanomas, subdivided in thin and thick melanomas, decreasing the power of the statistical analysis due to small sample size. Additionally, Koch and collaborators showed a significant increase in GLUT1 expression in metastatic samples when compared with primary melanoma <sup>35</sup>, which is in accordance with the present study. As mentioned above, both MCT4 and GLUT1 are involved in the Warburg effect; while both these proteins allow the continuous flow of the glycolytic pathway by providing, respectively, efflux of intracellular lactate and glucose uptake by cancer cells, MCT4 also acts as a pH regulator by promoting proton efflux. As a result, the increased expression of these proteins in metastatic samples

suggests a metabolic remodeling towards a hyperglycolytic and acid-resistant phenotype in the progression to an invasive phenotype, which is in accordance with evidence obtained from different approaches <sup>29</sup>.

In the case of CAIX, a significant increase was observed in 2 transitions along melanoma progression: from benign nevi to primary tumor and from lymph-node metastasis to distant metastases. CAIX is a HIF-1 $\alpha$ -induced pH regulator that contributes to the acid-mediated cancer cell invasive phenotype <sup>37-40</sup>, and has been associated with poor prognosis in a variety of neoplasias <sup>40</sup>. As mentioned above, only one additional study evaluated the expression of CAIX in melanoma samples, showing negative expression for this protein <sup>36</sup>. In the present study, the finding of an increased expression of CAIX along progression to malignancy is in accordance with the role of CAIX in mediating cancer cell invasive phenotype. Therefore, additional studies evaluating the expression of CAIX in malignant melanomas are warranted to further understand the actual contribution of this protein for melanoma progression.

Interestingly, both MCT1 and GLUT1 expression frequency was associated with *BRAF* mutation, in accordance with the observation that mutated *BRAF* drives the Warburg effect <sup>19</sup>. In the case of GLUT1, this association may be a result of HIF-1 $\alpha$  stabilization by mutated *BRAF* <sup>20, 21</sup>, as GLUT1 is induced by HIF-1 $\alpha$  activity as a transcription factor <sup>41</sup>, while the same mechanism is not able to explain MCT1 association, as this MCT isoform is not a direct target of HIF-1 $\alpha$  <sup>42</sup>. In fact, since MCT4 and CAIX are also targets of HIF-1 $\alpha$  <sup>38, 42</sup>, one would expect that these proteins would also be associated with *BRAF* mutation; however, regulation of these proteins is complex, with some mechanisms still to be described.

The metabolic reprogramming of cancer cells, besides contributing to cancer cell growth under intermittent hypoxia, is involved in cancer cell aggressiveness and therapeutic resistance, and, consequently, patients' poor prognosis<sup>43</sup>. In this context, lactate emerges as an important player, through modulation of the tumor microenvironment<sup>5</sup>. As a result, MCTs may be associated with cancer patients' poor prognosis<sup>6</sup>. In melanoma, no previous study evaluated the clinicopathological significance of MCTs; however, an *in vitro* study showed that inhibition of MCTs, in particular MCT1, may be an effective therapeutic approach for malignant melanomas<sup>44</sup>. In the present study, both MCT isoforms, but to a higher extent MCT4, were significantly associated with different variables of poor prognosis, including overall survival. This is in accordance with the biological role of MCTs and tumor microenvironment modulation by lactate. However, it is important to mention that, although MCTs were significantly associated with overall survival in Kaplan-Meier and univariate Cox regression analysis, multivariate analysis showed that none of these proteins have a stage-independent prognostic value. Nevertheless, these results suggest that MCTs are promising druggable targets for melanoma treatment. Importantly, a Phase I clinical trial using a MCT1 specific inhibitor in patients with advanced prostate cancer, gastric cancer or diffuse large B cell lymphoma is currently recruiting participants (CRUKD/12/004). GLUT1 retains an important role in cancer metabolic reprogramming, as the high glycolytic flow requires high glucose uptake rates, mostly provided by this glucose transporter in cancer cells<sup>45</sup>. In accordance, *in vitro* suppression of GLUT1 in melanomas cells reduced proliferation, apoptosis suppression, and migration, while *in vivo* suppression of GLUT1 reduced metastases formation<sup>35</sup>. Information regarding the prognostic value of GLUT1 using melanoma tissues is lacking; however, in the present study, we observed

that GLUT1, at a lesser extent than MCTs, was associated with poor prognostic variables, in agreement with the role of this protein in the metabolic shift. Finally, CAIX does not appear to have a significant role in the prognosis of melanomas.

In conclusion, the present study brings new insights on the metabolic alterations of melanoma in the progression to a metastatic phenotype, showing an increased expression of MCT4 and GLUT1 in melanoma metastases. Also, we show a clinicopathological value of MCT1, MCT4 and GLUT1 in melanoma. Since studies in this field are limited for melanomas, these results contribute to the characterization of melanoma molecular heterogeneity, paving the way for new options in the development of targeted therapies.

## **PATIENTS AND METHODS**

### **Melanoma patient characteristics**

The present series included 356 melanoma patients, treated from 1999 to 2012 at the Barretos Cancer Hospital, Barretos, SP, Brazil. Clinicopathological features included age at diagnosis (mean (SD): 58.3 (16.3) years), gender, clinical stage, Clark's level, Breslow's thickness (mean (SD): 4.1 (4.4) mm), histological subtype, anatomical site, number of mitoses *per* mm<sup>2</sup>, ulceration, peritumoral lymphocyte infiltration, intratumoral lymphocyte infiltration, tumor regression, pT, pN, pM, locoregional recurrence and overall survival. The main demographics and clinical data are demonstrated in Table 6. Patients were mainly treated with surgery (stages 0-III). Non-operable stage III/IV or recurrent disease was treated with cytotoxic chemotherapy (first line dacarbazine and second line carboplatin plus paclitaxel regimens). Bulky stage III operated tumors received adjuvant radiation at the physician description. Palliative radiation was indicated for central nervous system metastasis and symptomatic bone

metastasis. No patient received adjuvant or target/immune therapy.

### **Human samples**

Formalin-fixed paraffin-embedded (FFPE) melanoma samples (282 primary tumors, 117 lymph node metastases and 54 distant metastases; sample type distribution among patients is shown in Supplementary Table 1) as well as 20 benign nevi samples were retrieved from the files of the Pathology Department of Barretos Cancer Hospital. The Barretos Cancer Hospital's Ethics Committee approved the present study (548/2011).

### ***BRAF* and *NRAS* mutations**

DNA was obtained from FFPE tissue sections, as previously described<sup>46</sup>. Briefly, serially 10 µm thick unstained sections of paraffin blocks were sectioned and one H&E section was first evaluated by a pathologist to confirm the diagnosis and used for identification and selection of the areas of interest, which were macrodissected into a microfuge tube using a sterile needle (BD PrecisionGlide, BD, #305165). The macrodissected tissue was deparaffinized by a serial wash with xylol and ethanol (100%-70%-50%). DNA was extracted using Qiagen's QIAamp® DNA Micro Kit (Qiagen, #56304) following manufacturer's instructions and quantified by NanoDrop® 2000 (Thermo Scientific).

The analysis of *BRAF* V600E mutation was performed by PCR followed by direct Sanger sequencing, as previously described<sup>47, 48</sup>. PCR primers were as follows: 5'-AGTGGATTTCGCGGGCACAGA-3' (forward) and 5'-CAGCGCTGCCTGAAACTC-3' (reverse). PCR cycling conditions were as follows: initial denaturation at 96°C for 15 minutes, followed by 40 cycles of 96°C denaturation for 45 seconds, annealing temperature at 55.5°C for 45 seconds and 72°C elongation for 45 seconds, and 72°C final elongation for 10 minutes, in a

Verity PCR machine (Applied Biosystems).

The analysis of hotspot mutations of *NRAS* (codon 12/13 and 61) was performed by PCR followed by direct Sanger sequencing, as previously described<sup>47, 48</sup>. The specific primers designed to include the regions of interest were as follows: 5'-ATGACTGAGTACAACTGGT-3' (forward) and 5'-CTCTATGGTGGGATCATATT-3' (reverse) for codon 12/13, and 5'-TCTTACAGAAAACAAGTGGT-3' (forward) and 5'-GTAGAGGTAAATATCCGCAA-3' (reverse) for codon 61. The PCR cycling conditions were as follows: initial denaturation at 95°C for 15 minutes, followed by 40 cycles of 95°C denaturation for 45 seconds, annealing at 56.5°C for 45 seconds and 72°C elongation for 45 seconds, and 72°C final elongation for 10 minutes, in a Verity PCR machine (Applied Biosystems).

Amplification of PCR products was confirmed by gel electrophoresis. PCR products were purified using ExoSAP-IT (USB Corporation, #78200) and sequencing PCR was performed using a Big Dye terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems, #4337456) and the ABI PRISM 3500 xL Genetic Analyzer (Applied Biosystems). All mutated cases were confirmed with a second independent PCR followed by sequencing.

### **Immunohistochemistry**

For immunohistochemical analysis, samples were organized into tissue microarrays (TMA) containing cores of 1.0 mm diameter. Each case was represented in TMAs by three cores and control samples (placenta and liver) were also included for TMA orientation. MCT1 immunohistochemistry was performed according to the avidin-biotin-peroxidase complex method (R.T.U. VECTASTAIN Elite ABC Kit (Universal), Vector Laboratories, #PK-7200), as previously described<sup>49</sup>. Immunohistochemistry for MCT4, GLUT1, and CAIX was performed

according to the streptavidin-biotin-peroxidase complex principle (Ultravision Detection System Anti-polyvalent, HRP, Thermo Scientific, #TP-125-HL), as previously described<sup>50-52</sup>. Specificity of the antibodies was previously validated by siRNA followed by Western-blot<sup>53, 54</sup>. Negative controls were performed by the use of appropriate serum controls for the primary antibodies (Dako, #N1698 and #N1699). Colon carcinoma tissue was used as positive control for MCT1 and MCT4, head and neck squamous cell carcinoma was used for GLUT1, and normal stomach was used for CAIX. Tissue sections were counterstained with hematoxylin and permanently mounted. Please refer to Supplementary Table 2 for detailed information on each antibody used.

### **Immunohistochemical evaluation**

Protein expression was scored semi-quantitatively for plasma membrane expression in cancer cells as follows: 0: no immunoreactive cells; 1: <5% of immunoreactive cells; 2: 5-50% of immunoreactive cells; and 3: >50% of immunoreactive cells. Also, intensity of staining was scored semi-qualitatively as follows: 0: negative; 1: weak; 2: intermediate; and 3: strong. Figure 3 shows photomicrographs representative of staining intensity scores 1-3. The final score was defined as the sum of both parameters (extension and intensity), and grouped as negative (score 0 and 2) and positive (score 3-6), as previously described<sup>49</sup>. Two independent observers performed the immunohistochemical evaluation blindly and discordant results were discussed in a double-head microscope to determine the final score.

### **Statistical analysis**

Data were stored and analyzed using the IBM SPSS Statistics software (version 21, IBM Company). All comparisons were examined for statistical significance using Pearson's chi-



square ( $\chi^2$ ) test and Fisher's exact test (when  $n < 5$ ). Overall survival was defined as the time from the date of primary diagnosis to death related to melanoma or last follow-up and overall survival curves were estimated by the method of Kaplan-Meier and data compared using the log-rank test. Stage 0 (melanoma *in situ*) cases were excluded from survival analysis. Predictive factors of prognosis were identified using Cox proportional hazards regression models, which were used to estimate hazard ratios (HR) and 95% confidence intervals in univariate and multivariate analysis. For multivariate analysis, variables that reached a  $p$  value  $< 0.1$  at univariate analysis were included. The threshold for significant  $p$  values was established as  $p < 0.05$ .

#### **List of abbreviations**

Carbonic anhydrase IX (CAIX); glucose transporter 1 (GLUT1); hypoxia inducible factor 1 alpha (HIF-1 $\alpha$ ); monocarboxylate transporter (MCTs); tissue microarray (TMA).

#### **ACKNOWLEDGMENTS**

This work was supported by FAPESP grant to VLV (2012/04194-1). VMG received a doctoral fellowship (SFRH/BD/51997/2012) from Fundação para a Ciência e a Tecnologia (FCT).

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**Table 1.** Expression frequencies of MCT1, MCT4, GLUT1 and CAIX in benign nevi, melanoma primary tumors, lymph node metastases and distant metastases. Only plasma membrane expression was considered.

	<i>MCT1</i>			<i>MCT4</i>			<i>GLUT1</i>			<i>CAIX</i>		
	n	Positive(%)	<i>p</i>	n	Positive(%)	<i>p</i>	n	Positive(%)	<i>p</i>	n	Positive(%)	<i>p</i>
			0.442			0.019			0.007			0.009
<b>Benign nevi</b>	<b>20</b>	8 (40.0)		<b>20</b>	1 (5.0)		<b>19</b>	0 (0.0)		<b>20</b>	0 (0.0)	
<b>Primary tumor</b>	<b>221</b>	80 (36.2)		<b>233</b>	36 (15.5)		<b>232</b>	24 (10.3)		<b>227</b>	60 (26.4)	
<b>Lymph node metastases</b>	<b>109</b>	49 (45.0)		<b>111</b>	29 (26.1)		<b>111</b>	24 (21.6)		<b>110</b>	25 (22.7)	
<b>Distant metastases</b>	<b>53</b>	23 (43.4)		<b>53</b>	14 (26.4)		<b>54</b>	10 (18.5)		<b>53</b>	20 (37.7)	
<b>Benign</b>	<b>20</b>	8 (40.0)	0.735	<b>20</b>	1 (5.0)	0.20	<b>19</b>	0 (0.0)	0.14	<b>20</b>	0 (0.0)	<b>0.00</b>

nevi					4			0			8	
Primary tumor	221	80 (36.2)		233	36 (15.5)		232	24 (10.3)		227	60 (26.4)	
Primary tumor	221	80 (36.2)	0.125	233	36 (15.5)	0.018	232	24 (10.3)	0.005	227	60 (26.4)	0.463
Lymph node metastases	109	49 (45.0)		111	29 (26.1)		111	24 (21.6)		110	25 (22.7)	
Lymph node metastases	109	49 (45.0)	0.851	111	29 (26.1)	0.969	111	24 (21.6)	0.644	110	25 (22.7)	0.045
Distant metastases	53	23 (43.4)		53	14 (26.4)		54	10 (18.5)		53	20 (37.7)	

**Table 2.** Co-expression of MCT1 and MCT4 with GLUT1 and CAIX, in melanoma samples (primary tumors, lymph node metastases and distant metastases). Only plasma membrane expression was considered.

	<i>MCT1</i>			<i>MCT4</i>		
	n	Positive(%)	<i>p</i>	n	Positive (%)	<i>p</i>
<b>GLUT1</b>			<b>&lt;0.001</b>			<b>&lt;0.001</b>
Negative	<b>315</b>	110 (34.9)		<b>329</b>	49 (14.9)	
Positive	<b>58</b>	41 (70.7)		<b>57</b>	30 (52.6)	
<b>CAIX</b>			<b>0.008</b>			<b>0.003</b>
Negative	<b>272</b>	99 (36.4)		<b>279</b>	47 (16.8)	
Positive	<b>103</b>	53 (51.5)		<b>104</b>	32 (30.8)	

**Table 3.** Association of MCT1, MCT4, GLUT1 and CAIX plasma membrane expression with the clinicopathological parameters (categorical variables) in melanomas. Only expression in primary tumors was considered.

	<i>MCT1</i>			<i>MCT4</i>			<i>GLUT1</i>			<i>CAIX</i>		
	n	Positive (%)	<i>p</i>	n	Positive (%)	<i>p</i>	n	Positive (%)	<i>p</i>	n	Positive (%)	<i>p</i>
<b>Gender</b>			0.516			<b>0.045</b>			0.42			0.506
Male	<b>102</b>	34 (33.3)		<b>109</b>	22 (20.2)		<b>108</b>	13 (12.0)		<b>106</b>	31 (29.2)	
Female	<b>109</b>	41 (37.6)		<b>114</b>	12 (10.5)		<b>114</b>	10 (8.8)		<b>111</b>	28 (25.2)	
<b>Clinical stage</b>			<0.001			<b>0.032</b>			0.80			0.495
0 + I + II	<b>110</b>	26 (23.6)		<b>121</b>	12 (9.9)		<b>121</b>	14 (11.6)		<b>117</b>	29 (24.8)	
III + IV	<b>86</b>	42 (48.8)		<b>88</b>	18 (20.5)		<b>86</b>	9 (10.5)		<b>86</b>	25 (29.1)	

<b>Clark's level</b>		0.181	0.90	
	0.868		5	0.157
I + II + III	<b>48</b> 16 (33.3)	<b>54</b> 5 (9.3)	<b>50</b> 6 (12.0)	<b>51</b> 18 (35.3)
IV + V	<b>124</b> 43 (34.7)	<b>130</b> 22 (16.9)	<b>132</b> 15 (11.4)	<b>129</b> 32 (24.8)
<b>Histological subtype</b>		<b>0.029</b>	0.43	
	0.561		4	0.440
Acral lentiginous	<b>27</b> 10 (37.0)	<b>33</b> 1 (3.0)	<b>36</b> 4 (11.1)	<b>31</b> 6 (19.4)
Nodular	<b>74</b> 27 (36.5)	<b>73</b> 16 (21.9)	<b>73</b> 10 (13.7)	<b>74</b> 22 (29.7)
Superficial spreading	<b>47</b> 13 (27.7)	<b>52</b> 6 (11.5)	<b>48</b> 3 (6.3)	<b>50</b> 11 (22.0)
<b>Anatomical site</b>		<b>0.038</b>	0.83	
	0.757		4	0.372
Limbs	<b>106</b> 34 (32.1)	<b>113</b> 12 (10.6)	<b>113</b> 11 (9.7)	<b>109</b> 30 (27.5)
Trunk	<b>49</b> 20 (40.8)	<b>52</b> 14 (26.9)	<b>51</b> 6 (11.8)	<b>53</b> 18 (34.0)
Head and neck	<b>48</b> 17 (35.4)	<b>50</b> 7 (14.0)	<b>50</b> 6 (12.0)	<b>48</b> 11 (22.9)

Mucosa	5	2 (40.0)	5	0 (0.0)	5	0 (0.0)	4	0 (0.0)
<b>Ulceration</b>					0.598		0.27	
		0.143					3	0.396
Absent	51	14 (27.5)	58	8 (13.8)	55	5 (9.1)	55	18 (32.7)
Present	88	35 (39.8)	88	15 (17.0)	91	14 (15.4)	88	23 (26.1)
<b>Peritumoral lymphocyte infiltration</b>							0.06	
		0.731			0.293		3	0.486
Absent	22	8 (36.4)	22	1 (4.5)	23	0 (0.0)	23	8 (34.8)
Present	74	24 (32.4)	79	13 (16.5)	76	11 (14.5)	77	21 (27.3)
<b>Intratatumoral lymphocyte infiltration</b>							0.18	
		0.138			0.054		5	0.448
Absent	38	17 (44.7)	41	2 (4.9)	42	2 (4.8)	38	11 (28.9)
Present	60	18 (30.0)	62	11 (17.7)	58	8 (13.8)	63	14 (22.2)
<b>Tumor regression</b>					0.417		1.00	
		0.709					0	1.000

No	<b>96</b> 35 (36.5)	<b>103</b> 13 (12.6)	<b>101</b> 12 (11.9)	<b>99</b> 23 (23.2)	
Yes	<b>8</b> 2 (25.0)	<b>9</b> 2 (22.2)	<b>8</b> 1 (12.5)	<b>9</b> 2 (22.2)	
<b>pT</b>		<b>0.016</b>	<b>0.002</b>	<b>0.03</b>	0.546
				<b>5</b>	
1 + 2	<b>57</b> 13 (22.8)	<b>63</b> 2 (3.2)	<b>61</b> 3 (4.9)	<b>60</b> 17 (28.3)	
3 + 4	<b>116</b> 48 (41.4)	<b>122</b> 24 (19.7)	<b>121</b> 19 (15.7)	<b>120</b> 29 (24.2)	
<b>pN</b>		<b>0.004</b>	<b>0.008</b>	0.64	0.481
				<b>1</b>	
0 + 1	<b>146</b> 43 (29.5)	<b>158</b> 17 (10.8)	<b>158</b> 16 (10.1)	<b>153</b> 39 (25.5)	
2 + 3	<b>48</b> 25 (52.1)	<b>50</b> 13 (26.0)	<b>48</b> 6 (12.5)	<b>49</b> 15 (30.6)	
<b>pM</b>		<b>0.012</b>	0.162	0.75	0.402
				<b>0</b>	
0	<b>169</b> 53 (31.4)	<b>183</b> 24 (13.1)	<b>181</b> 19 (10.5)	<b>177</b> 46 (26.0)	
1	<b>31</b> 17 (54.8)	<b>30</b> 7 (23.3)	<b>30</b> 4 (13.3)	<b>30</b> 10 (33.3)	
<b>Locoregional recurrence</b>		0.313	<b>0.002</b>	0.17	0.083
				<b>0</b>	
No	<b>155</b> 52 (33.5)	<b>164</b> 19 (11.6)	<b>164</b> 14 (8.5)	<b>158</b> 38 (24.1)	

Yes **43** 18 (41.9)      **46** 14 (30.4)      **45** 7 (15.6)      **46** 17 (37.0)

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WT: wild-type.

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**Table 4.** Association of MCT1, MCT4, GLUT1 and CAIX plasma membrane expression with the clinicopathological parameters (quantitative variables) in melanomas. Only expression in primary tumors was considered.

	<i>Breslow's thickness</i> (mm)			<i>Number of mitoses/mm<sup>2</sup></i>		
	n	Mean rank	<i>p</i>	n	Mean rank	<i>p</i>
<b>MCT1</b>			0.085			0.579
Negative	<b>117</b>	84.71		<b>69</b>	54.20	
Positive	<b>61</b>	98.70		<b>41</b>	57.68	
<b>MCT4</b>			<b>0.001</b>			<b>0.015</b>
Negative	<b>165</b>	90.95		<b>101</b>	55.99	
Positive	<b>26</b>	128.06		<b>16</b>	78.03	
<b>GLUT1</b>			<b>0.036</b>			<b>0.049</b>
Negative	<b>167</b>	91.97		<b>103</b>	56.33	
Positive	<b>22</b>	117.98		<b>13</b>	75.73	
<b>CAIX</b>			0.195			0.786

Negative	<b>135</b>	96.64		<b>84</b>	57.00	
Positive	<b>51</b>	85.18		<b>30</b>	58.90	

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**Table 5.** Prognostic factors for overall survival in melanomas. Only expression in primary tumors was considered for MCT1, MCT4, GLUT1 and CAIX variables.

Variable	Overall survival					
	Univariate analysis			Multivariate analysis		
	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>
Gender (male)	<b>1.601</b>	<b>1.199 – 2.137</b>	<b>0.001</b>	1.386	0.867 – 2.215	0.173
Age *	1.004	0.994 – 1.013	0.449			
Clinical stage (III-IV)	<b>3.654</b>	<b>2.655 – 5.029</b>	<b>&lt;0.001</b>	<b>4.559</b>	<b>2.716 – 7.754</b>	<b>&lt;0.001</b>
Breslow's thickness *	<b>1.098</b>	<b>1.067 – 1.129</b>	<b>&lt;0.001</b>	<b>1.053</b>	<b>1.009 – 1.098</b>	<b>0.018</b>
Clark (IV-V)	<b>1.805</b>	<b>1.231 – 2.646</b>	<b>0.002</b>	1.034	0.572 – 1.868	0.911
MCT1 (+)	<b>1.508</b>	<b>1.021 – 2.226</b>	<b>0.039</b>	0.864	0.530 – 1.407	0.557
MCT4 (+)	<b>2.049</b>	<b>1.308 – 3.210</b>	<b>0.002</b>	1.441	0.839 – 2.475	0.185
GLUT1 (+)	1.212	0.678 – 2.168	0.516			
CAIX (+)	1.178	0.768 – 1.909	0.269			

\*Quantitative variables.

**Table 6.** Main demographics, clinical and histological characteristics of melanoma patients.

<b>Variable</b>	<b>n</b>	<b>%</b>
<b><i>Gender</i></b>		
Female	<b>182</b>	51.1
Male	<b>174</b>	48.9
<b><i>Skin color</i></b>		
White	<b>313</b>	87.9
Other	<b>38</b>	10.7
N/A	<b>5</b>	1.4
<b><i>Anatomical site</i></b>		
Lower limbs	<b>120</b>	343.7
Trunk	<b>93</b>	26.1
Head and neck	<b>69</b>	19.4
Upper limbs	<b>40</b>	11.2
Mucosae	<b>5</b>	1.6
Unknown	<b>6</b>	1.7

N/A	<b>23</b>	6.3
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*Clinical stage*

0	<b>12</b>	3.4
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I	<b>62</b>	17.4
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II	<b>111</b>	31.2
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III	<b>98</b>	27.5
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IV	<b>58</b>	16.3
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X	<b>15</b>	4.2
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*Histological subtype*

Nodular	<b>113</b>	31.7
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Superficial spreading	<b>87</b>	24.4
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Acral lentiginous	<b>43</b>	12.1
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Mucosae	<b>9</b>	2.5
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Lentigo maligna	<b>7</b>	2.0
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Not classified	<b>15</b>	4.2
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N/A	<b>82</b>	23.1
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*Ulceration*

Absent	<b>85</b>	23.9
Present	<b>130</b>	36.5
N/A	<b>141</b>	39.6

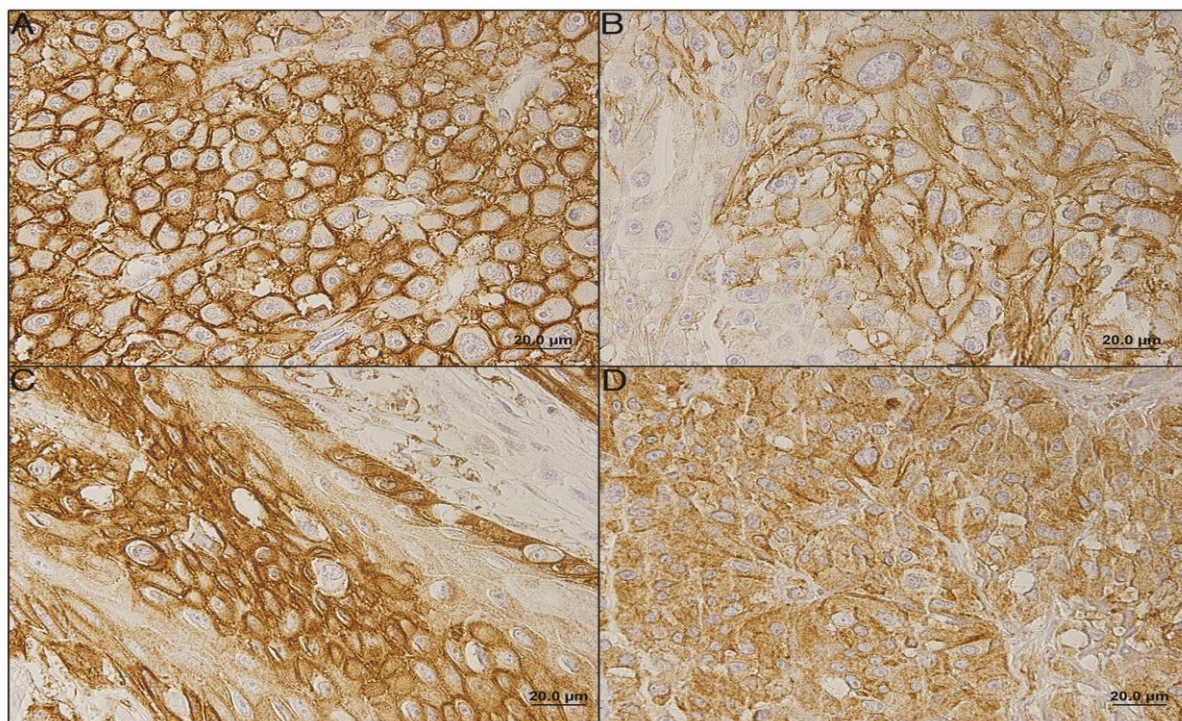
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*Mitoses/mm<sup>2</sup>*

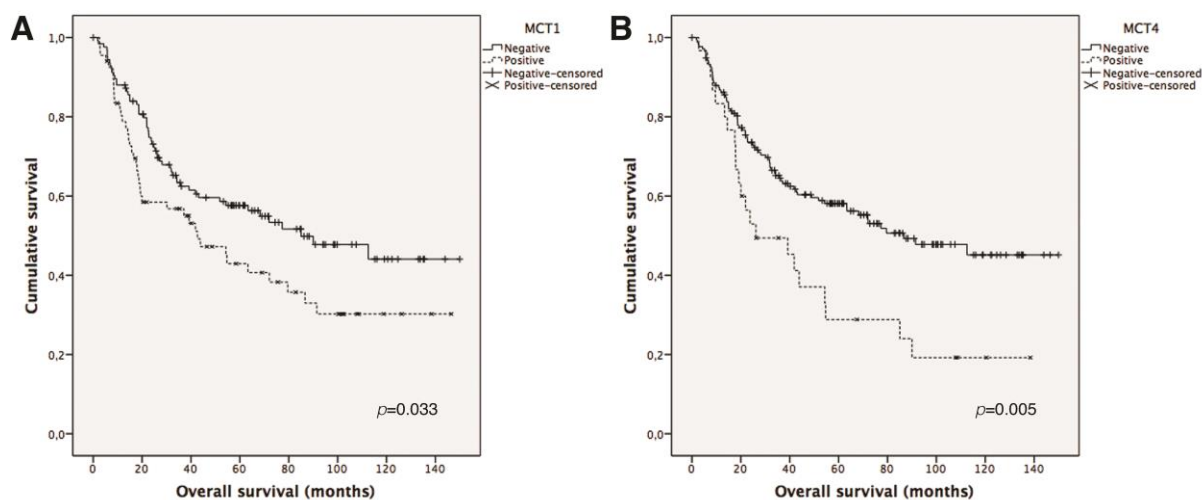
0	<b>9</b>	2.5
1	<b>36</b>	10.1
>1	<b>126</b>	35.4
N/A	<b>185</b>	52.0

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N/A – not available.

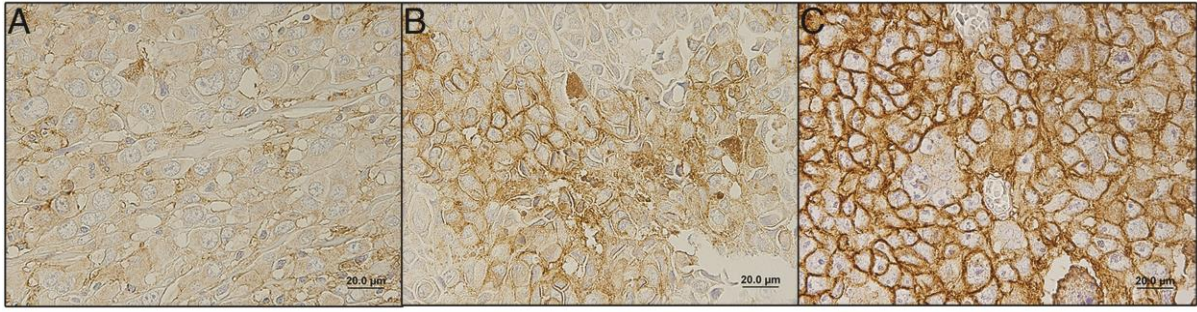


**Figure 1. Immunohistochemical expression of MCT1 (A), MCT4 (B), GLUT1 (C) and CAIX (D) in melanoma.** All the proteins were more importantly found in the plasma membrane of cells. (A) Stage IIC primary tumor; (B) Stage IIB primary tumor; (C) Distant metastasis; (D) Stage IV primary tumor.



**Figure 2. Overall survival curves of melanoma's patients.** The results are stratified according to protein immunohistochemical expression, using Kaplan Meier's method. Only significant results are shown. Continuous line refers to negative expression while interrupted line refers to positive expression. **(A)** Plasma membrane expression of MCT1; **(B)** Plasma membrane expression of MCT4.





**Figure 3. Photomicrographs representative of weak (A), moderate (B) and strong (C) scores. MCT1 immunohistochemical staining in melanoma samples is shown.**

Accepted Manuscript