**Title and Subtitle**
Epithelial Interactions and the Angiogenic Phenotype of Breast Cancer

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**Abstract**
The objective of our work was to study the role of cell-ECM interactions in the increased survival, migratory and invasive phenotype found in the transition from DCIS to invasive carcinoma. We considered the possibility that loss of tissue architecture could elicit malignant transformation via integrin- growth factor receptor- regulated ERK effects on growth and survival as well as on angiogenesis, probably through a close interaction between tumor cells and macrophages. Ectopic expression of $\alpha_1\beta_1$ integrin enhanced invasion, migration and survival of the premalignant S3-C cells in vitro, as well as increasing their malignant phenotype in vivo in nude mice. We proposed that $\alpha_1\beta_1$ integrin could be affecting the cells phenotype via induction of the flt-1 VEGFR expression, which would participate in an autocrine survival loop. The enhanced malignant phenotype in vivo was characterized by an angiogenic switch, correlated with increased macrophage infiltration of the tumor. In vitro experiments suggest that this increase in angiogenesis potential might involve a paracrine loop between macrophages and MECs, in which macrophage- derived EGF induces an increase in IL-8 expression by MECs, which in turn increases their angiogenic ability.

**Subject Terms**
Breast cancer, angiogenesis, extracellular matrix, tissue architecture, alpha5beta1 integrin, fibronectin, VEGF, Interleukin-8, VEGF receptor 1, autocrine survival loop, uPA receptor, dormancy
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INTRODUCTION:

Since the exponential growth of new DCIS cases found during the last few years, which have recently reached epidemic proportions, understanding how a DCIS lesion evolves into an invasive tumor and finally gives rise to metastatic foci is fundamental for establishing a way to control the progression of breast cancer. To become a malignant tumor, cells from a DCIS lesion have to be able to invade the surrounding stroma. Consistently, breast cancer development from a DCIS is characterized by perturbations in stromal-epithelial interactions, and mammary tumors and cell lines characteristically exhibit alterations in their integrin expression when compared to cells from premalignant lesions (1-10). Moreover, during malignant transformation of the breast, there is an increase in FN and TN levels in the stroma, and breast tumors often show an increase in α5β1 and αvβ(x) integrin expression (10-15). This suggests that integrins might be playing an important role in malignant transformation of the breast.

The objective of our work was to study the role of cell-ECM interactions in the increased survival, migratory and invasive phenotype found in the transition from normal breast to pre-malignant lesion and from DCIS to invasive carcinoma. We considered the possibility that loss of tissue architecture could elicit malignant transformation via integrin- growth factor receptor- regulated ERK/ AKT effects on growth and survival as well as on angiogenesis, probably through a close interaction between tumor cells and macrophages.

BODY:

In the first two years of this grant we established that malignant transformation and breast cancer behavior is associated with α5β1 integrin over expression, and that inhibition of this receptor can induce a state of tumor dormancy that is tightly linked to tissue architecture. Inhibition of α5β1 integrin activity in tumor cells repressed tumor growth, and this was linked to inhibition of angiogenesis. Similarly, ectopic expression of α5β1 integrin by the nonmalignant S1 cells induced loss of tissue structure (hyperplasia) in vivo, and loss of polarity in 3D rBM+FN by these cells. This was also correlated with an increased angiogenic phenotype. Using the S3-C cells as a model of DCIS in vitro we sought to study how tumor progression occurs in vivo and to determine which molecules are functionally significant for transition from premalignancy to malignancy. In particular, we wanted to assess the role of α5β1 integrin in malignant transformation of the breast.

Ectopic expression of α5β1 integrin enhanced invasion, migration and survival of the premalignant S3-C cells in vitro, as well as increasing their malignant phenotype in vivo in nude mice. We proposed that α5β1 integrin could be affecting the cells phenotype via induction of the flt-1 VEGFR expression, which would participate in an autocrine survival loop. The enhanced malignant phenotype in vivo was characterized by an angiogenic switch, correlated with increased macrophage infiltration of the tumor. In vitro experiments suggest that this increase in angiogenesis potential might involve a paracrine loop between macrophages and MECs, in which macrophage- derived EGF
induces an increase in IL-8 expression by MECs, which in turn increases their angiogenic ability.

With these studies we can report significant progress on all three of the specific aims and the tasks detailed in my SOW:

1) To determine if there is a correlation between tumorigenicity, loss of acinar structure, $\alpha v$ and/or $\alpha 5$ integrin expression and angiogenesis.

2) To test if the reacquisition of a polarized acinar structure is related to the loss of the angiogenic phenotype and if this is linked to changes in $\alpha v$ and/or $\alpha 5$ integrin.

3) To determine if the up-regulation of $\alpha v$ and/or $\alpha 5$ integrins is a predictor of malignant behavior in HMT-3522 MECs, and functions by compromising tissue organization and inducing angiogenesis and invasion.

1. Our first aim was to determine if there is a correlation between tumorigenicity, loss of acinar architecture and angiogenesis.

We previously showed that when the cells in our model progress towards malignancy, they present altered tissue architecture including cell-cell interactions, integrin expression and organization, ECM expression and deposition and expression of proangiogenic molecules such as VEGF and IL-8 (Fig 1a, b and 2c). Using our 3D multicellular MEC rBM/collagen I EC model we observed an increased angiogenic ability correlated with tumor progression in vitro (Fig 2b). When the cells in our model were injected into nude mice for in vivo angiogenesis and tumor studies, we were able to further confirm their angiogenic phenotype in vivo (Fig 2a).

Due to the consistency of the data obtained in our in vitro as well as in vivo angiogenesis studies, we decided not to do the acute angiogenesis assays to avoid the unnecessary use of animals.

2. Our second aim was to test if tumor reversion and reformation of a “differentiated” tissue-like structure is associated with repression of angiogenic behavior and to determine if this is linked to altered expression and/or activity of $\alpha 5$ and $\alpha v$ integrin.

We found that phenotypic reversion of the tumors using EGFR inhibitor and $\beta 1$-integrin function blocking mAb in the 3D rBM assay induced normalization of the integrin expression profile (Fig 3b), on ECM protein expression (inhibiting FN deposition; Fig 3a) and inhibiting the expression of VEGF and IL-8 in the tumor cells (Fig 5e, f). To specifically test the importance of altering $\alpha v$ and $\alpha 5$ integrins in the reverted tumor phenotype we inhibited $\alpha v$ and $\alpha 5$ integrins using function-blocking mAbs. We found that by blocking $\alpha 5$ integrin activity we could induce a state of tumor dormancy similar to the one achieved by blocking $\beta 1$ integrin function (Fig 3c, d). We determined that $\alpha 5\beta 1$ integrin-NF interaction regulates ECM deposition, angiogenic molecule expression (Fig 5 e, f) and tissue organization in 3D rBM cultures, as well as the tumor cells angiogenic behavior in vitro (in the 3D multi-cellular MEC assay; Fig 5c). We showed that $\alpha 5\beta 1$ integrin sustains tumorigenicity and angiogenesis in vivo (Fig 5a, b).

By inducing $\alpha 5$ integrin over-expression in the T4-2 cells using an exogenous retroviral promoter we determined that exogenous over-expression of $\alpha 5$ integrin interferes with EGFR inhibitor-tumor reversion potential, increasing 10 times the amount
of EGFR inhibitor necessary to induce their phenotypic reversion (not shown). While the inhibition of α5 integrin function in T4-2 cells induced their reversion to a polarized state (basal deposition of β4 integrin and ECM proteins and apicolateral deposition of β-catenin) over-expression of α5 integrin in these cells did not affect their phenotype in 3D rBM (not shown). Since over expression of α5 integrin by T4-2 cells also had no effect on their proliferation, motility, or invasiveness in the transwell assay, we decided not to continue with the following specific aims (f-n) and focused instead in other important modulators of tumor growth which had been shown to be linked to α5β1 integrin such as uPAR and EGFR that had not been included in the original SOW.

Tumor dormancy has been tightly linked to urokinase-type plasminogen activator receptor (uPAR) deficit, and uPAR levels increase in cancer (16). Moreover, it has been shown that α5β1 integrin-uPAR interaction through EGFR is required for tumor growth in vivo, and that interfering with any of these receptors can induce tumor dormancy in hepatocarcinoma cells (17). Based on these data, we decided to evaluate the molecules involved in the regulation of malignant progression and tumor dormancy. Specifically, we studied the uPAR status in the HMT-3522 cell series and found that α5β1 integrin expression induces the expression of uPAR, and determined via function blocking studies that this receptor is involved in tissue architecture-mediated tumor dormancy through regulation of angiogenesis (Fig 6).

3. Our third aim was to determine if over expression of αv- and/or α5-integrins would be sufficient to drive invasive and angiogenic behavior in either pre-malignant or non-malignant MECs and whether this is associated with disruption of cell-cell interactions, perturbations in tissue polarity and is mediated through effects on angiogenic regulator molecules.

We achieved this goal by directly up-regulating the expression of αv, α2 and α5 integrins in the nonmalignant S1 and pre-malignant S3 cells using inducible retroviral expression constructs and assayed for effects on tissue architecture, invasion and expression of angiogenic molecules using the 3D rBM assay.

In the S1 non-malignant cells, we found that α5β1 integrin expression and ligation was enough to induce loss of tissue polarity promoting an angiogenic switch, although not enough to induce invasion (Fig 4 and 5). We used the multi-cellular 3D model to assess the cells angiogenic and invasive behavior ex vivo and tumor injections in nude mice to study in vivo tumorigenicity and angiogenesis and confirmed again that α5β1 integrin expression and ligation induces an angiogenic phenotype both in vitro as well as in vivo, and induces loss of the normal phenotype in the nonmalignant S1 cells, which give rise to hyperplastic lesions (Fig 5).

Finally, ectopic expression of α5β1 integrin enhanced invasion, migration and survival of the premalignant S3-C cells in vitro, as well as increasing their malignant phenotype in vivo in nude mice (Fig 7). There is evidence showing that increased survival of breast cancer cells might involve an autocrine loop in which VEGF-activated VEGFR can repress apoptosis and enhance growth (18). We found expression of α5β1 integrin to induce increased survival of the pre-malignant cells by inducing the expression of VEGFR-1 (Fig 7f). This receptor was linked to increased survival, since its inhibition inhibited the cells ability to grow anchorage independently in soft agar (Fig
7g). Expression of $\alpha_5\beta_1$ integrin also increased the S3 cells’ angiogenic ability in vivo (Fig 8a). This correlated with a higher macrophage infiltration of the S3 $\alpha_5$- derived tumors. Premalignant S3 cells already express high levels of VEGF, thus, $\alpha_5$ integrin did not affect the expression of this molecule. Surprisingly, EGF induced the expression of IL-8 only in the context of $\alpha_5\beta_1$ integrin- expressing cells (Fig 8c). These data, together with the finding that S3 $\alpha_5$ cells- derived tumors had a higher macrophage infiltration, confirmed that $\alpha_5\beta_1$ integrin- EGFR interaction is important for malignant progression and suggested that a paracrine loop involving macrophages and MECs could be taking place, in which macrophage- derived EGF induces an increase in IL-8 expression by MECs, which in turn increases their angiogenic ability.

**KEY RESEARCH ACCOMPLISHMENTS:**

- *In vivo* characterization of S3 cells regarding their malignant and their angiogenic phenotype.
- Expression characterization of protein by FACs analysis in S-3 cells expressing $\alpha_5$, $\alpha_v$ and $\alpha_2$ integrins. In 2D monolayer.
- Immunological characterization of S-3 cells expressing $\alpha_5$, $\alpha_v$ and $\alpha_2$ integrins in 3D rBM.
- Invasion characterization of S-3 cells expressing $\alpha_5$ integrin.
- Angiogenesis characterization of S-3 cells expressing $\alpha_5$ integrin in co-cultures.

**REPORTABLE OUTCOMES for the last period:**

**Products**

*Cell lines, tissue or serum repositories developed*

- Prepared and characterized pooled populations of HMT-3522-derived T4-2 (malignant) cells expressing $\alpha_5$ integrin and S3 (premalignant) cells expressing $\alpha_2$ integrin.
- With the research done with the support of this grant, I completed my Thesis work and obtained my PhD degree from the University of Buenos Aires (December, 2005).
- With the experience obtained from my research I was able to obtain a post-doctoral position at the University of North Carolina (starting in June, 2006).

**CONCLUSIONS:**

In conclusion, the data presented here suggest that $\alpha_5\beta_1$ integrin is involved in the conversion from a normal to a premalignant phenotype and that inhibition of $\alpha_5\beta_1$ integrin function can render a tumor dormant. This, together with our finding that $\alpha_5\beta_1$ integrin also plays a fundamental role in the transition from pre-malignancy to malignancy, suggests that $\alpha_5\beta_1$ integrin has a very prominent role in malignant transformation.

This work also demonstrated a putative link between uPA-uPAR and $\alpha_5\beta_1$ integrin expression that could be involved in the loss of tissue architecture and development of metastasis. In fact, $\alpha_5\beta_1$ integrin is found in metastatic breast disease as well as other tumors that metastasize, and it has been postulated that it may be promoting
angiogenesis through regulation of VEGF - and via loss of tissue integrity -. This could be also true for the link that we found between α5β1 integrin expression, tumor progression and IL-8 expression. Surprisingly, EGF induced the expression of IL-8 only in the context of α5β1 integrin- expressing cells. These data, together with the finding that S3 α5 cells- derived tumors had a higher macrophage infiltration, confirmed that α5β1 integrin- EGFR interaction is important for malignant progression and suggested that a paracrine loop involving macrophage- derived EGF and MECs- derived IL-8 could be taking place.

Furthermore, our results indicate that tissue differentiation - architecture mediated dormancy- may be functionally linked to metastatic tumor dormancy mechanisms, suggesting that if we could delineate the molecular mechanisms by which cells acquire dormancy in a tissue this may reveal novel insights into how metastatic tissue dormancy may arise.

Put together, these data imply that there is not just one factor that controls the state of a tumor, but that there are interconnected factors that regulate each other. This are promising results, since they imply that tumor dormancy could be achieved and maintained by various means, giving the cancer patient more chances to win over the tumor.

REFERENCES:

Figure 1. Malignant transformation is associated with loss of growth control and polarity, and increased expression of α5 and αv integrins and their ligands FN and TN
A. Top lane: Phase contrast micrographs showing differences in colony size and architecture of S1 non-malignant, S3-C premalignant and T4-2 tumor cells after 12 days of culture in 3D rBM. Rest of the panel: Immunostaining for polarity markers: β-catenin, β4 integrin and for reactive stromal proteins: FN and TN. B. FACS analysis of surface expression of the α1, α2, α3, α5, α6, αv, β1, and β6 integrins by S1, S3-A, S3-B, S3-C or T4-2 cells. Expression values were normalized to S1 for each integrin in order to allow comparison of experiments using different antibodies. Only α5, α6, αv, β4 and β1 integrin expression is significantly higher in T4-2 than S3 and S1 cells (p<0.05).

Figure 2. Tumor progression is associated with an angiogenic switch.
A. Photographs of S1, S3 and T4-2-derived lesions showing the presence of blood vessels both in the S3-C and the T4-2 tumors. B. Micrographs showing ECs invasion of collagen layer of co-cultures of HDMVECs and S1 cells, S3 cells or T4-2 cells (same results were obtained either with iHUVEC2A or HDMVECs). Note that the only cells capable of generating an angiogenic response are the S3s and the T4-2s. C. VEGF, Il-8 and bFGF levels in conditioned media from 3DrBM cultures of S1, S3-C and T4-2 cells, obtained by ELISA.

Figure 3. Tumor dormancy/ reversion is associated with normalized tissue architecture and repression of expression of α5 and αv integrins and their ligands FN and TN. Inhibition of α5β1 integrin induces tumor dormancy and phenotypic reversion in vitro.
A. Top scheme: representation of S1, T4-2 and T4-2 reverted (T4 Rvt) morphology. Top lane: Phase contrast micrographs showing differences in colony size and architecture of S1 non-malignant, T4-2 tumor and T4 Rvt cells (by β1 integrin blocking; same results were obtained by inhibiting EGFR, PI3K or MEK). Rest of the panel: Immunostaining for FN and TN, using 488 AlexaFluor secondary Ab (green). B. Western blot of protein lysates from 3D rBM cultures of S1, T4-2 and T4 Rvt cells, showing how phenotypic reversion by blocking β1 integrin in the tumor cells down-regulates α5 and αv integrin protein levels together with β1 down-modulation. C. Immunofluorescence showing restoration of polarization (α6 integrin and β-catenin expression) by treatment of T4-2 tumor cells with α5 integrin blocking, but not α2 or αv integrin blocking Ab (T4-2 cells were grown in 3D rBM for 12 days in the presence or absence of β1, α5, α2, αv integrin blocking antibodies. S1 cells were used as control). D. Histogram showing % of positive
colonies (>40 \mu m) that grew after 21 days of culture in soft agar in the presence or absence of \( \beta 1, \alpha 5, \alpha 2, \alpha v \) integrin blocking Abs. S1 cells were used as control. E. Immunostaining for polarity markers: \( \beta 4 \) integrin and \( \beta \)-catenin of 12 days 3D rBM +FN cultures, with or without treatment with the inhibitors Tyrphostin AG 1478 (200 nM), PD98059 or LY 294002 (40 \mu m) (inhibition of either EGFR, PI3K or MEK results in restoration of the normal phenotype).

Figure 4. Ectopic expression of \( \alpha 5 \beta 1 \) integrin in nonmalignant S1 cells induces loss of polarity and growth control only in the presence of its ligand FN. \( \alpha 5 \beta 1 \) integrin regulates AKT and ERK activity in MECs, resulting in a transforming phenotype.

A. Top lane: Phase contrast micrographs showing differences in colony size and morphology of S1 \( \alpha 5 \) cells versus S1 \( \alpha 2 \) and S1 eGFP cells, only in the presence of FN (cells were grown in 3D rBM or 3D rBM +FN for 12 days). Rest of the panel: Immunostaining for polarity markers: \( \beta 4 \) integrin, \( \beta \)-catenin and collagen IV, using AlexaFluor secondary Ab (red). Green fluorescence is due to eGFP. Only S1 \( \alpha 5 \) cells grown in the presence of FN were able to overcome their natural growth control, still proliferating by day 12. B. Immunostaining for polarity markers: \( \beta 4 \) integrin and \( \beta \)-catenin of 12 days 3D rBM +FN cultures, with or without treatment with the inhibitors Tyrphostin AG 1478 (200 nM), PD98059 or LY 294002 (40 \mu m) (inhibition of either EGFR, PI3K or MEK results in restoration of the normal phenotype). C. Time-course of EGF-induced AKT and ERK activation. Representative immunoblot of total and phosphorylated AKT and ERK in S1 \( \alpha 5 \) cells with repressed (+tet) or induced (-tet) \( \alpha 5 \) integrin expression.

Figure 5. Blocking of \( \alpha 5 \beta 1 \) function induces tumor dormancy in vivo in association with modulation of angiogenesis. \( \alpha 5 \beta 1 \) integrin induces an angiogenic switch

A. Columns 1 and 2: H&E staining of paraffin sections of samples from S1 eGFP, S1 \( \alpha 5 \), T4-2 IgG-treated and T4-2 \( \alpha 5 \) integrin blocking Ab-treated-derived tumors. Observe the important necrosis in the latter. Columns 3 and 4: Immunohistochemistry showing PCNA and activated caspase 3 staining of the same samples, in red, and nuclei in blue (DAPI). Columns 5 and 6: Photographs of the excised tumors showing important angiogenesis only in the T4-2 IgG-treated tumors and some angiogenesis in the S1 \( \alpha 5 \) lesions, and immunohistochemistry for CD34 (red) as an endothelial marker to show angiogenesis in paraffin sections (nuclei were stained with DAPI). B. Table describing the characteristics of the different lesions: number of cell masses per group (between brackets is the average +/- SD weight of the lesions) and histological features. C. Micrographs showing ECs invasion of collagen layer of co-cultures of HDMVECs and S1 eGFP, S1 \( \alpha 2 \), S1 \( \alpha 5 \), T4-2 and T4 Rvt cells. D. Quantification of average network formation by HDMVECs co-cultured with T4-2 cells in the presence of IgG control, EGFR inhibitor (Rvt) or \( \beta 1, \alpha 5, \alpha v \) or \( \alpha 2 \) integrin blocking Abs. The same results were obtained in all cases with either iHUVEC2A or HDMVECs. Note that the only cells capable of generating an angiogenic response are the S1 \( \alpha 5 \) and the control T4-2s, and that reverting the tumor phenotype impairs the angiogenic ability of the tumor cells. E. ELISA quantification of VEGF (pg/ml) released to the conditioned media by S1 eGFP and S1 \( \alpha 5 \) cells (left) and T4-2 and T4 Rvt cells (right). F. ELISA quantification of IL-8
(pg/ml) released to the conditioned media by S1 eGFP and S1 α5 cells (left) and T4-2 and T4 Rvt cells (right).

**Figure 6.** α5β1 integrin ligation of FN regulates tumor dormancy through cooperative activation of uPAR and induction of angiogenesis, and this is reciprocally linked to disrupted tissue architecture

A. ELISA quantification of uPA (pg/ml) released to the conditioned media by S1 eGFP, S1 α5, T4-2 and T4 Rvt cells. B. uPAR levels on the surface of S1 eGFP, S1 α5 and T4-2 cells assessed by FACS. C. Quantification of average network formation by ECs invasion of collagen layer in co-cultures of HDMVECs with S1 eGFP, S1 α5 and T4-2 cells, co-incubated without or with uPAR blocking antibody. D. Immunostaining for some polarity markers (β4 integrin, β-catenin and LM 5) of 3D rBM cultures of these MECs co-incubated with uPAR function-blocking Ab. E. % of T4-2 cells colonies that grew in soft agar +/- uPAR blocking Ab. F. Cartoon depicting the central role of α5β1 integrin-FN/uPAR/EGFR interaction in tumor progression and dormancy.

**Figure 7.** Pre-malignant S3-C cell expressing α5β1 integrin acquire characteristics of malignancy showing enhanced migration, invasion and survival, and increased expression of flt-1

A. Quantification of adhesion to FN by S3-C eGFP or S3-C α5 cells. B. Graph quantifying migration of these cells in the wound healing assay, after 8 hr incubation. C. Quantification of the % of cells that invaded through the rBM-covered Boyden chambers, in the presence of EGF. D. Quantification of the % of colonies which were able to grow in soft agar. E. Top: H&E staining of paraffin sections of S3-C eGFP and S3-C α5-derived tumors. Lanes 2 and 3: Immunohistochemistry showing DAPI (nuclei), PCNA (proliferating cells) and activated caspase 3 (cells undergoing apoptosis) staining. F. Immunostaining for flt-1 in S3-C eGFP and S3-C α5 cells plated on 2D. G. Graph quantifying the % of colonies that were able to grow in soft agar in the presence of different concentrations of recombinant flt-1 (rflt-1) used to inhibit flt-1-VEGF interaction.

**Figure 8.** S3-C cells expressing α5β1 integrin induce enhanced macrophage infiltration of the tumor, which enhance the angiogenic ability of MECs

A. Top: Photographs of S3-C eGFP and S3-C α5- derived tumors showing the presence of blood vessels only in the latter. Middle: Immunohistochemistry showing CD34 staining of blood vessels in these same samples. B. Immunohistochemistry showing F4/80 staining of macrophages in these tumors. C. Quantification of ECs invasion of the collagen layer by HDMVECs co-cultured with S3-C eGFP and S3-C α5 cells in the absence or presence of EGF; same results were obtained either with iHUVEC2A or HDMVECs. D. II-8 concentration in conditioned media from 12 days 3D rBM cultures of these same cells lines, measured by ELISA (VEGF and bFGF levels were unchanged).
FIGURES:

Figure 1:

A

B

Phase

β-catenin

β4 Integrin

Fibronectin

Tenascin

Figure 2:

A

Photo image

S1

S3-C

T4-2

B

HDMVECs co-cultured with

S1

S3-C

T4-2

C

VEGF production (ng/ml)

IL-8 production (ng/ml)

P4G2 production (ng/ml)
Figure 3:
Figure 4:

A

<table>
<thead>
<tr>
<th>rBM 3D + FN</th>
<th>S1 eGFP</th>
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B

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C

EGFR Inhib: - - +

EGF (t, min): 0 30 60 90 0 30 60 90

pAKT
AKT
pERK
ERK

(no α5 integrin expression) (α5 integrin expression)
Figure 6:
Figure 7:
Figure 8: