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Case Report

Preliminary Findings on Multi-Targeted Epigenetic Therapy in Modifying Telomerase Activity

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Abstract

Researcher's recent intention to inhibit telomerase as a therapeutic measure has failed in general, as the cancer cells have a secondary adaptive cellular mechanism of resistance to such approach, using an "Alterative Lengthening Telomeres" (ALT) pathways, involving mitochondria, (by increased expression of PGC-1beta), to [1-7]. The highly aggressive and metabolically active cancer cells are able to survive regardless of the telomere's activity [8]. This has important clinical implications, as clinically we see cases that are treated with different therapeutic interventions and we can show that surprisingly, the telomerase activity *increases* with an effective therapy. This can be shown in the samples of the circulatory tumor cells that have no telomerase activity, but very highly aggressive features, such as G250 in renal cell carcinoma, which is suggestive of activated ALT [9,10]. After a successful therapy they lose their aggressiveness by a lack of G250, and they start to show positive telomerase activity in their signature in liquid biopsy. To date no clinical study or theory explains this. Here we present a case of renal cell carcinoma, which supports the concept and offers a possible explanation for presence of telomeres as a "sign of response" to therapy. We also present several case studies that show response to Multi-targeted Epigenetic Therapy, as evident by the eradication of telomerase positive cells in liquid biopsy.

Keywords: Cancer prevention, Telomeres, Epigenetic therapy

Case Studies

Sample Case 1: Renal cell carcinoma

67 year old male with a 10 cm renal mass incidentally found in an ultrasound, which was suspected for malignancy. He was scheduled for laparotomy, the biopsy was deemed to be too dangerous to perform. He was referred for evaluation and treatment on 8/11/14. Labs were drawn which showed increased VEGF at 248 (pg/ml), serum interleukin-8 of 448.4. His D-Dimer was also elevated at 0.61. He started the daily IV epigenetic therapies, consisting of Quercetin and Sodium phenyl butyrate as part of the patented multitargeted epigenetic therapy (MTET) protocol. He did not experience any negative side effects, and did not change his diet or his supplements.

His labs were repeated after 10 treatments (two weeks), which showed substantial improvements. His VEGF dropped from 248 down to 58 and his Interleukin-8 dropped from 448.4 down to 16.6. His CRP dropped from 160 to 133. He was scheduled for surgery, as his risk of angiogenesis has decreased significantly. His initial thrombocytosis as a sign of angiogenesis resolved, as his platelets dropped from 388 to 201. He had CTC analysis through Biofocus laboratory before and after the treatments. The results indicated significant reduction of the tumor burden in CTC as well as CTC markers, including CD20, telomerase, and cytokeratin markers. The results may indicate response in the micrometastatic environment. The markers for angiogenesis are suggested in our research to correlate with survival.

The first sample showed positive G250 and second has a negative G250. Telomerase overexpression was observed in the second sample, which suggests our concept with positive response to therapy, as explained above (Figures 1 and 2).

Sample Case 2: Metastatic malignant melanoma

29 year old female with history of melanoma, diagnosed in April 2012 status post

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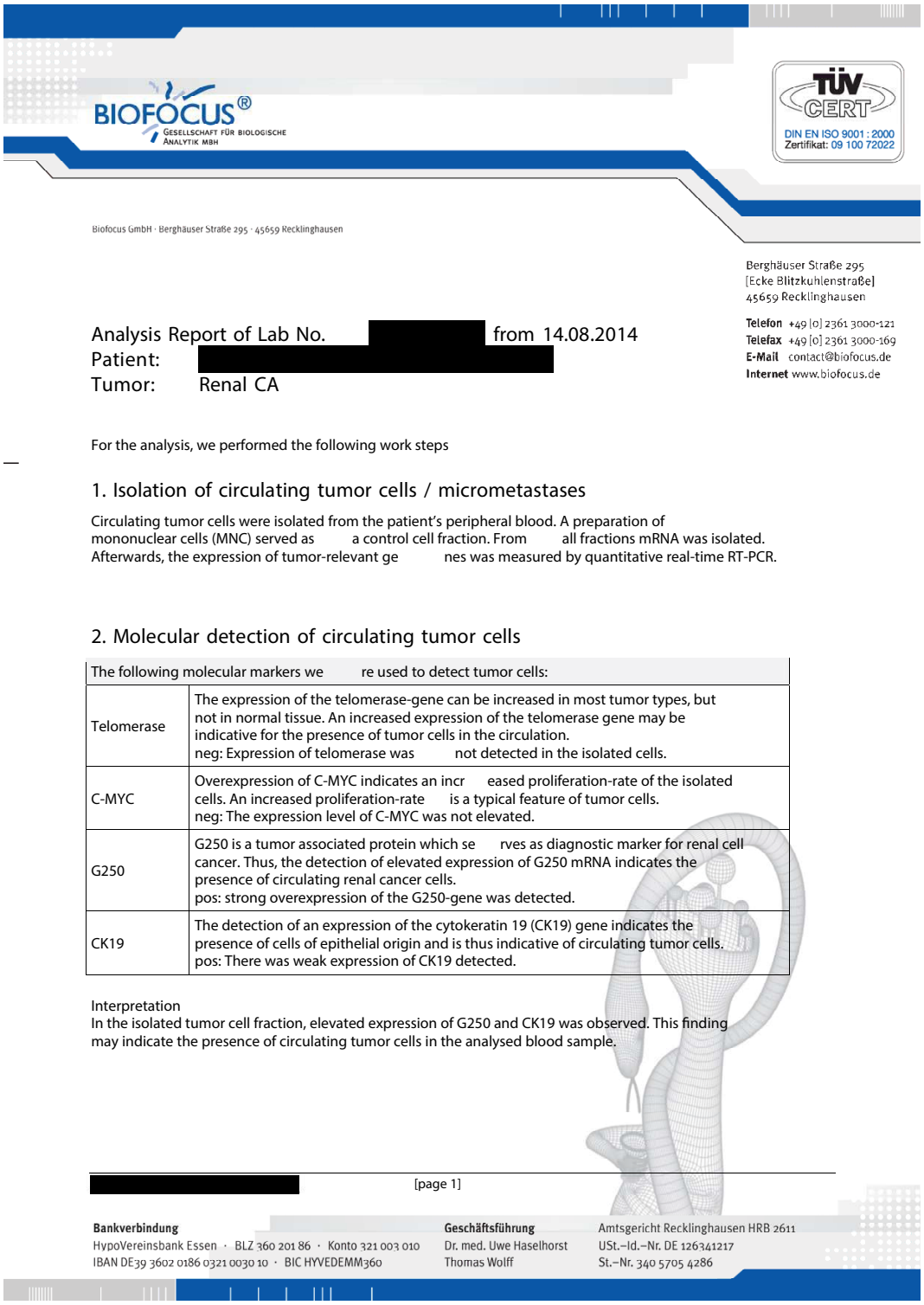

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Analysis Report of Lab No. [redacted] from 14.08.2014
Patient: [redacted]
Tumor: Renal CA

For the analysis, we performed the following work steps

1. Isolation of circulating tumor cells / micrometastases

Circulating tumor cells were isolated from the patient's peripheral blood. A preparation of mononuclear cells (MNC) served as a control cell fraction. From all fractions mRNA was isolated. Afterwards, the expression of tumor-relevant ge nes was measured by quantitative real-time RT-PCR.

2. Molecular detection of circulating tumor cells

The following molecular markers we re used to detect tumor cells:	
Telomerase	The expression of the telomerase-gene can be increased in most tumor types, but not in normal tissue. An increased expression of the telomerase gene may be indicative for the presence of tumor cells in the circulation. neg: Expression of telomerase was not detected in the isolated cells.
C-MYC	Overexpression of C-MYC indicates an incr eased proliferation-rate of the isolated cells. An increased proliferation-rate is a typical feature of tumor cells. neg: The expression level of C-MYC was not elevated.
G250	G250 is a tumor associated protein which se rves as diagnostic marker for renal cell cancer. Thus, the detection of elevated expression of G250 mRNA indicates the presence of circulating renal cancer cells. pos: strong overexpression of the G250-gene was detected.
CK19	The detection of an expression of the cytokeratin 19 (CK19) gene indicates the presence of cells of epithelial origin and is thus indicative of circulating tumor cells. pos: There was weak expression of CK19 detected.

Interpretation
In the isolated tumor cell fraction, elevated expression of G250 and CK19 was observed. This finding may indicate the presence of circulating tumor cells in the analysed blood sample.

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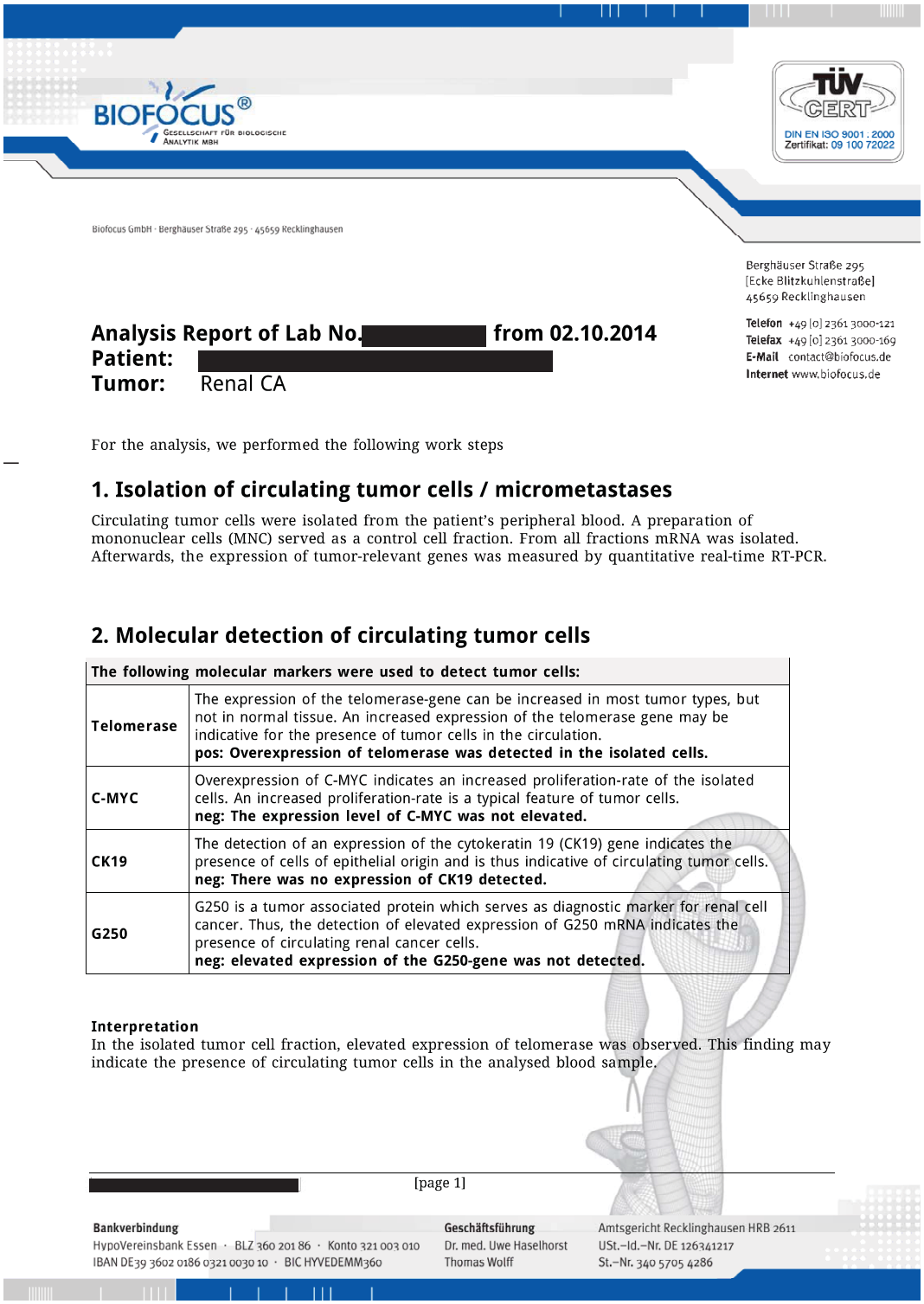
Figure 1

biopsy of her right forearm nevus, side margin excision, and graft and LN biopsy. MRI showed disease free status, status post a recurrence in 9 months, with vision problem in January 2013, with multiple brain mets, including Chiasm of Optic nerve, status post 5 cycles of Immunophresis in Germany, Interferon, and Yervoy (5 mg/kg for two rounds), and subsequent Craniotomy by in Australia, and cyberknife of remaining lesion in brain. In February 2013, she had received Sutent.

Her molecular profiling of her tumor showed positive NRAS mutations.



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Analysis Report of Lab No. [redacted] from 02.10.2014
Patient: [redacted]
Tumor: Renal CA

For the analysis, we performed the following work steps

1. Isolation of circulating tumor cells / micrometastases

Circulating tumor cells were isolated from the patient's peripheral blood. A preparation of mononuclear cells (MNC) served as a control cell fraction. From all fractions mRNA was isolated. Afterwards, the expression of tumor-relevant genes was measured by quantitative real-time RT-PCR.

2. Molecular detection of circulating tumor cells

The following molecular markers were used to detect tumor cells:	
Telomerase	The expression of the telomerase-gene can be increased in most tumor types, but not in normal tissue. An increased expression of the telomerase gene may be indicative for the presence of tumor cells in the circulation. **pos: Overexpression of telomerase was detected in the isolated cells.**
C-MYC	Overexpression of C-MYC indicates an increased proliferation-rate of the isolated cells. An increased proliferation-rate is a typical feature of tumor cells. **neg: The expression level of C-MYC was not elevated.**
CK19	The detection of an expression of the cytokeratin 19 (CK19) gene indicates the presence of cells of epithelial origin and is thus indicative of circulating tumor cells. **neg: There was no expression of CK19 detected.**
G250	G250 is a tumor associated protein which serves as diagnostic marker for renal cell cancer. Thus, the detection of elevated expression of G250 mRNA indicates the presence of circulating renal cancer cells. **neg: elevated expression of the G250-gene was not detected.**

Interpretation
In the isolated tumor cell fraction, elevated expression of telomerase was observed. This finding may indicate the presence of circulating tumor cells in the analysed blood sample.

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Figure 2

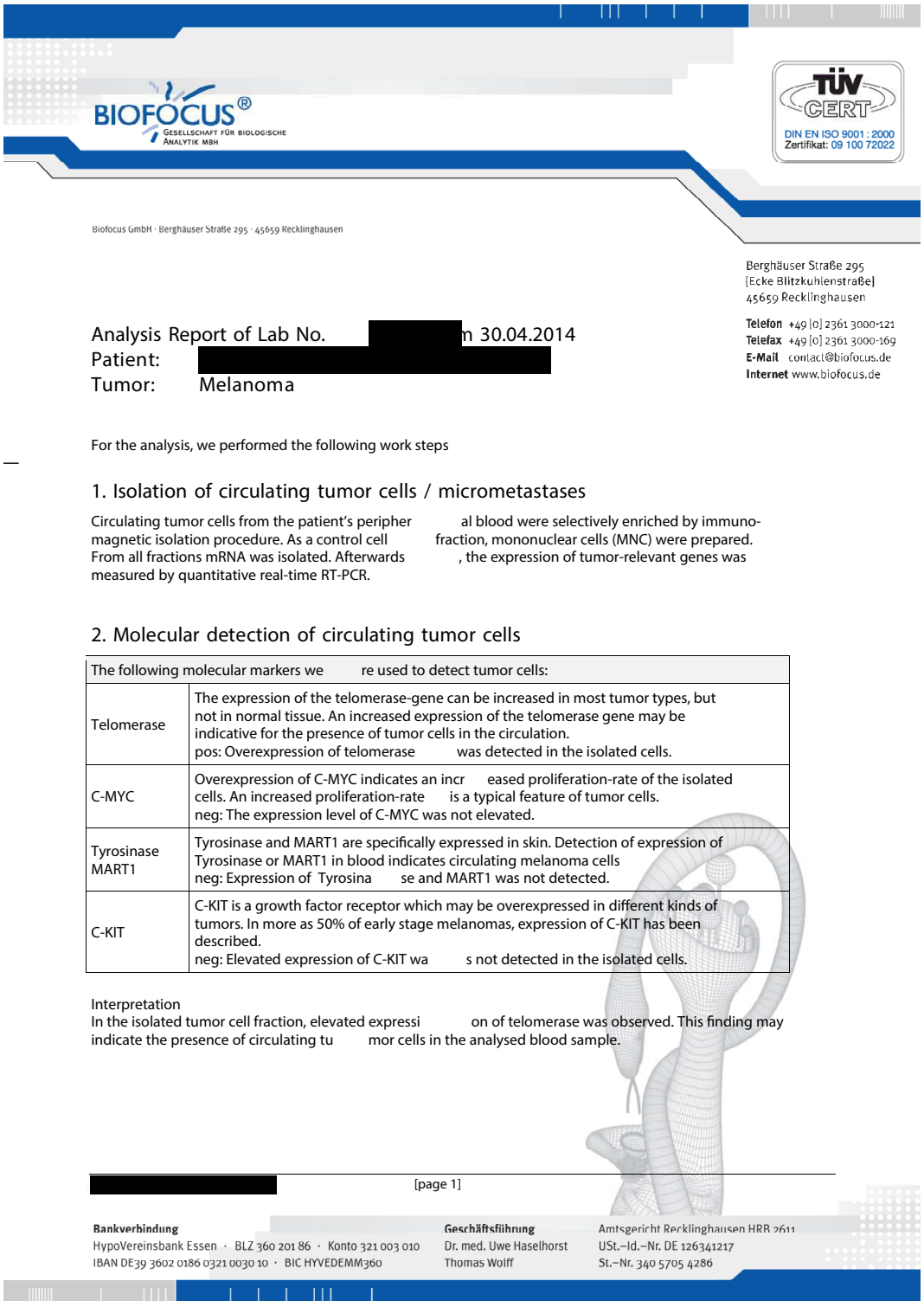
After initial evaluation, immediately she was started on IV epigenetic therapies which she received on daily basis. After ten treatments she expressed improvement in her function and vision. Her ECOG score improved as was her vision. Her labs were repeated which showed following results:

Neuron Specific Enolase (NSE) dropped from 45 to 9.2 and her interleukin 8 has dropped from 76 to 53, in two weeks, repeated on 4/28/14. LASA dropped from 64 to 27 (measured on 5/15/14 and 5/5/14 respectively).

We used Biofocus lab for her circulatory tumor analysis. The

test was done PRE and POST epigenetic therapies. She did not receive any chemotherapies, or immune therapies or cytotoxic or targeted therapies during this time. Her CTC showed complete resolution post therapy, with disappearance of telomerase positive cells. Her CT scan was repeated on 6/2/14, which showed mixed response with many lesions in her chest and neck improved in size and activity.

This was a rapid response in an advanced case of RAS positive refractory to immune therapy in melanoma (Figures 3 and 4).



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Analysis Report of Lab No. [redacted] m 30.04.2014
Patient: [redacted]
Tumor: Melanoma

For the analysis, we performed the following work steps

1. Isolation of circulating tumor cells / micrometastases

Circulating tumor cells from the patient's peripher al blood were selectively enriched by immuno-magnetic isolation procedure. As a control cell fraction, mononuclear cells (MNC) were prepared. From all fractions mRNA was isolated. Afterwards , the expression of tumor-relevant genes was measured by quantitative real-time RT-PCR.

2. Molecular detection of circulating tumor cells

The following molecular markers we re used to detect tumor cells:	
Telomerase	The expression of the telomerase-gene can be increased in most tumor types, but not in normal tissue. An increased expression of the telomerase gene may be indicative for the presence of tumor cells in the circulation. pos: Overexpression of telomerase was detected in the isolated cells.
C-MYC	Overexpression of C-MYC indicates an incr eased proliferation-rate of the isolated cells. An increased proliferation-rate is a typical feature of tumor cells. neg: The expression level of C-MYC was not elevated.
Tyrosinase MART1	Tyrosinase and MART1 are specifically expressed in skin. Detection of expression of Tyrosinase or MART1 in blood indicates circulating melanoma cells neg: Expression of Tyrosina se and MART1 was not detected.
C-KIT	C-KIT is a growth factor receptor which may be overexpressed in different kinds of tumors. In more as 50% of early stage melanomas, expression of C-KIT has been described. neg: Elevated expression of C-KIT wa s not detected in the isolated cells.

Interpretation
In the isolated tumor cell fraction, elevated expressi on of telomerase was observed. This finding may indicate the presence of circulating tu mor cells in the analysed blood sample.

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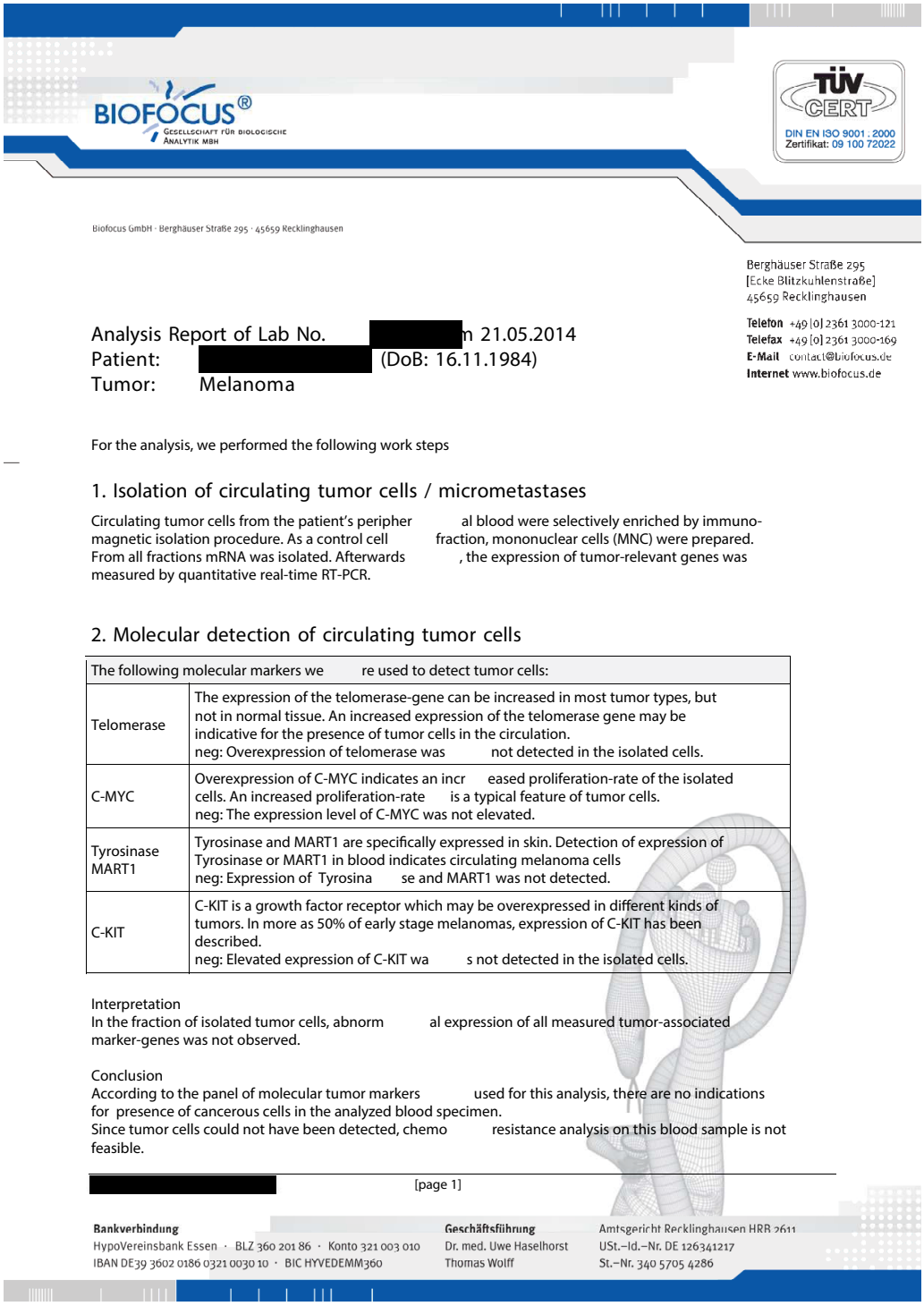
Figure 3



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Analysis Report of Lab No. [redacted]n 21.05.2014
Patient: [redacted] (DoB: 16.11.1984)
Tumor: Melanoma

For the analysis, we performed the following work steps

1. Isolation of circulating tumor cells / micrometastases

Circulating tumor cells from the patient's peripher al blood were selectively enriched by immuno-magnetic isolation procedure. As a control cell fraction, mononuclear cells (MNC) were prepared. From all fractions mRNA was isolated. Afterwards , the expression of tumor-relevant genes was measured by quantitative real-time RT-PCR.

2. Molecular detection of circulating tumor cells

The following molecular markers we re used to detect tumor cells:	
Telomerase	The expression of the telomerase-gene can be increased in most tumor types, but not in normal tissue. An increased expression of the telomerase gene may be indicative for the presence of tumor cells in the circulation. neg: Overexpression of telomerase was not detected in the isolated cells.
C-MYC	Overexpression of C-MYC indicates an incr eased proliferation-rate of the isolated cells. An increased proliferation-rate is a typical feature of tumor cells. neg: The expression level of C-MYC was not elevated.
Tyrosinase MART1	Tyrosinase and MART1 are specifically expressed in skin. Detection of expression of Tyrosinase or MART1 in blood indicates circulating melanoma cells neg: Expression of Tyrosina se and MART1 was not detected.
C-KIT	C-KIT is a growth factor receptor which may be overexpressed in different kinds of tumors. In more as 50% of early stage melanomas, expression of C-KIT has been described. neg: Elevated expression of C-KIT wa s not detected in the isolated cells.

Interpretation
In the fraction of isolated tumor cells, abnorm al expression of all measured tumor-associated marker-genes was not observed.

Conclusion
According to the panel of molecular tumor markers used for this analysis, there are no indications for presence of cancerous cells in the analyzed blood specimen.
Since tumor cells could not have been detected, chemo resistance analysis on this blood sample is not feasible.

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Figure 4

Sample Case 3: Squamous cell carcinoma of the lung

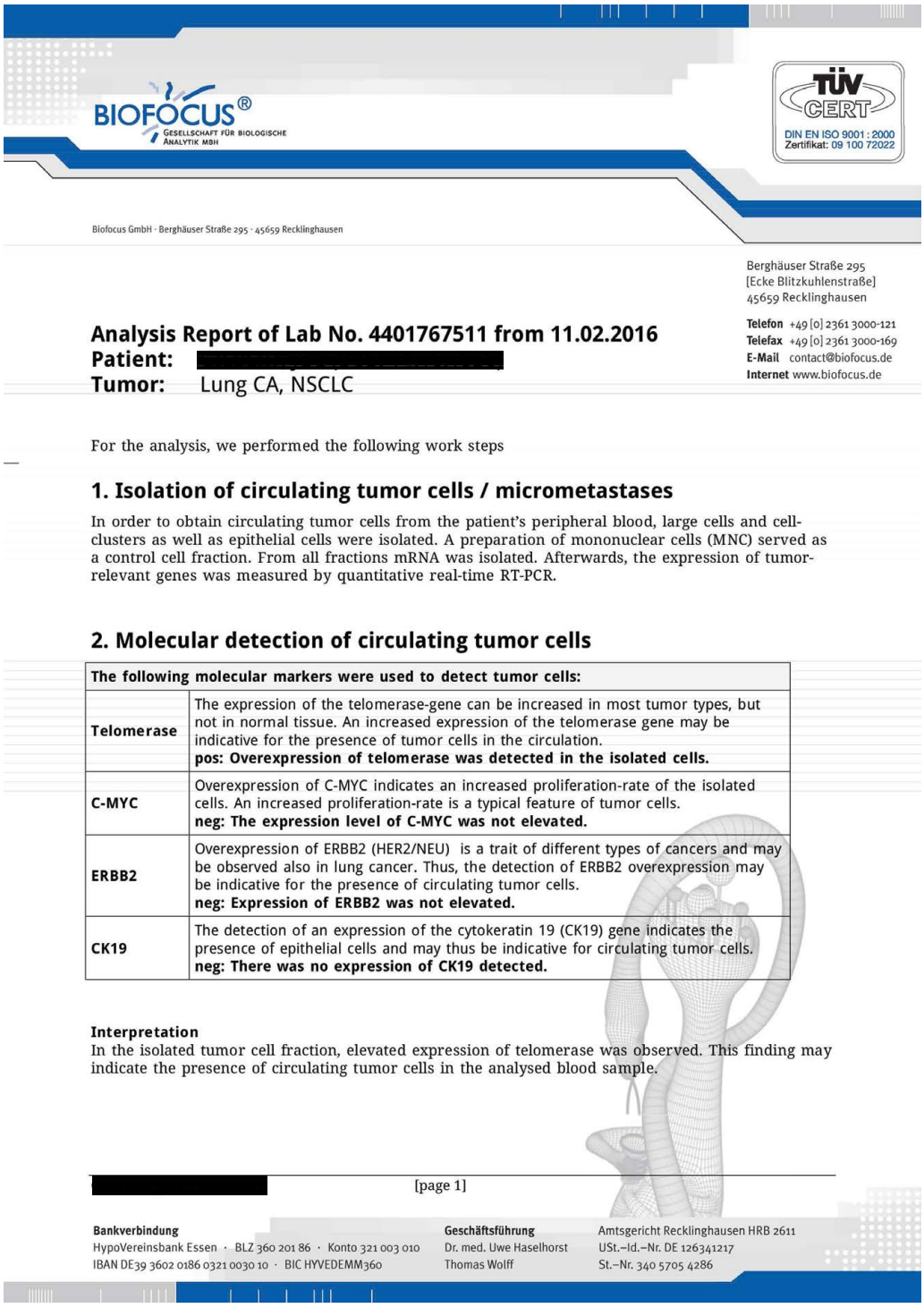
79 year old male with history of Stage IV squamous cell lung cancer, with metastasis to the shoulder, ribs, muscles, with some possible lung and peritoneal involvement. He was first diagnosed in April 2015, when he developed shoulder pain. An MRI done by orthopedic surgeon reveal lesion, and he was then referred to multiple doctors, resulting in a PET scan and referral to an oncologist. He initially received Carboplatin and Abraxane with radiation in June 15, which failed. He was then switched to Nivolumab, which was given until his most recent scan in Jan 16 showed mixed results. Some of the radiated bone lesions responded, however metastatsis were still widespread. He lost over 25 pounds and had decreased quality of life after the therapies. He had not been given any other options other than palliative care.

His CTC done by Biofocus on 2/11/16 on showed positive EGFR overexpression and showed positive Telomerase.

He was started on IV epigenetic therapies immediately. Following 10 treatments, his CTC was repeated on 2/25/16, and showed eradication of the Telomerase positive cells, with no indications for remaining CTCs. His quality of life has also improved, as he has gained weight and his ECOG score improved from 2 to 1 (Figures 5 and 6).

Conclusion and Discussion

The literature has shown that in a majority of cancer cells, telomerase activity is present. As evident by the above cases



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Analysis Report of Lab No. 4401767511 from 11.02.2016
Patient: [redacted]
Tumor: Lung CA, NSCLC

For the analysis, we performed the following work steps

1. Isolation of circulating tumor cells / micrometastases

In order to obtain circulating tumor cells from the patient's peripheral blood, large cells and cell-clusters as well as epithelial cells were isolated. A preparation of mononuclear cells (MNC) served as a control cell fraction. From all fractions mRNA was isolated. Afterwards, the expression of tumor-relevant genes was measured by quantitative real-time RT-PCR.

2. Molecular detection of circulating tumor cells

The following molecular markers were used to detect tumor cells:	
Telomerase	The expression of the telomerase-gene can be increased in most tumor types, but not in normal tissue. An increased expression of the telomerase gene may be indicative for the presence of tumor cells in the circulation. **pos: Overexpression of telomerase was detected in the isolated cells.**
C-MYC	Overexpression of C-MYC indicates an increased proliferation-rate of the isolated cells. An increased proliferation-rate is a typical feature of tumor cells. **neg: The expression level of C-MYC was not elevated.**
ERBB2	Overexpression of ERBB2 (HER2/NEU) is a trait of different types of cancers and may be observed also in lung cancer. Thus, the detection of ERBB2 overexpression may be indicative for the presence of circulating tumor cells. **neg: Expression of ERBB2 was not elevated.**
CK19	The detection of an expression of the cytokeratin 19 (CK19) gene indicates the presence of epithelial cells and may thus be indicative for circulating tumor cells. **neg: There was no expression of CK19 detected.**

Interpretation
In the isolated tumor cell fraction, elevated expression of telomerase was observed. This finding may indicate the presence of circulating tumor cells in the analysed blood sample.

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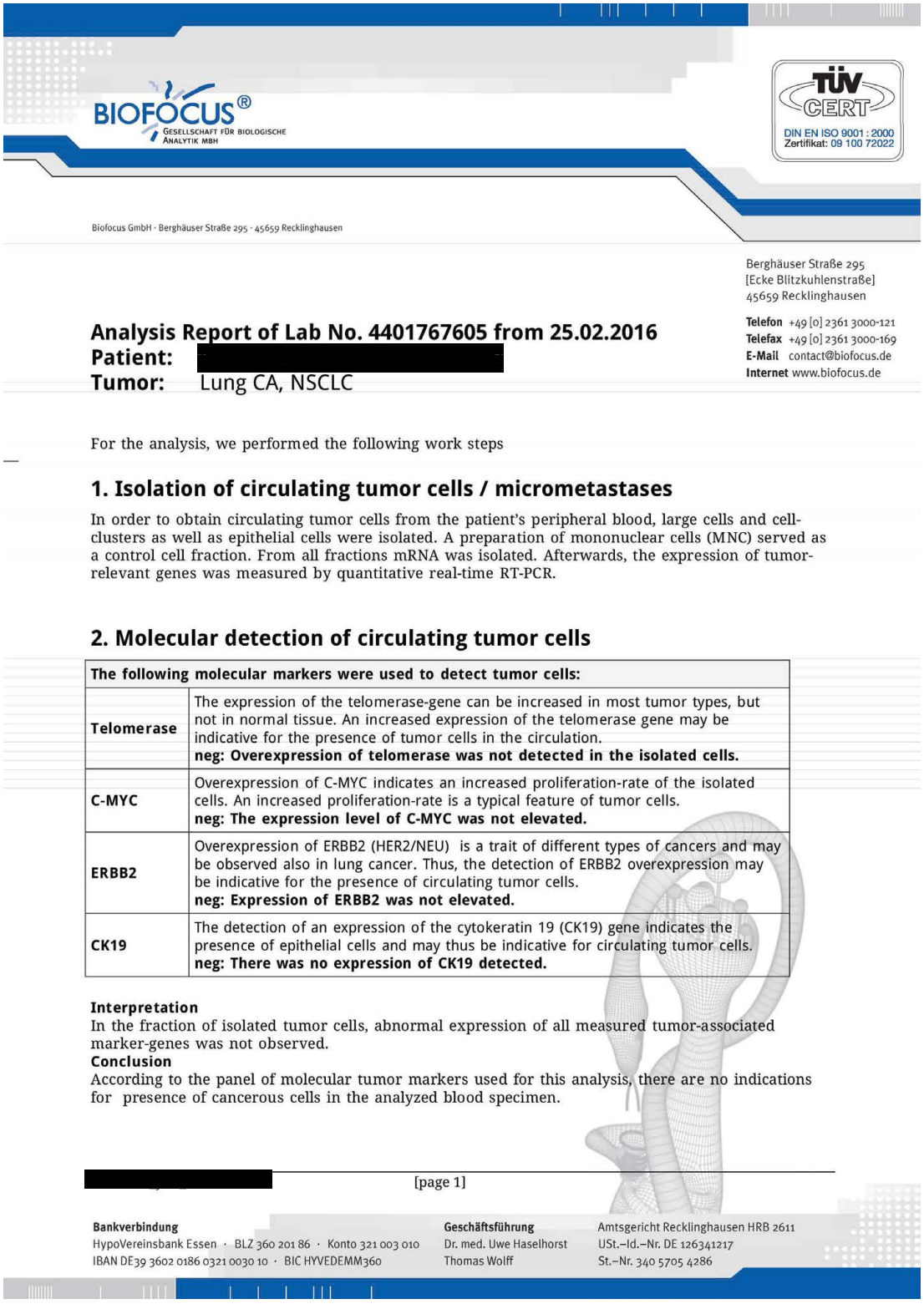
Figure 5



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Analysis Report of Lab No. 4401767605 from 25.02.2016
Patient: [redacted]
Tumor: Lung CA, NSCLC

For the analysis, we performed the following work steps

1. Isolation of circulating tumor cells / micrometastases

In order to obtain circulating tumor cells from the patient's peripheral blood, large cells and cell-clusters as well as epithelial cells were isolated. A preparation of mononuclear cells (MNC) served as a control cell fraction. From all fractions mRNA was isolated. Afterwards, the expression of tumor-relevant genes was measured by quantitative real-time RT-PCR.

2. Molecular detection of circulating tumor cells

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C-MYC	Overexpression of C-MYC indicates an increased proliferation-rate of the isolated cells. An increased proliferation-rate is a typical feature of tumor cells. **neg: The expression level of C-MYC was not elevated.**
ERBB2	Overexpression of ERBB2 (HER2/NEU) is a trait of different types of cancers and may be observed also in lung cancer. Thus, the detection of ERBB2 overexpression may be indicative for the presence of circulating tumor cells. **neg: Expression of ERBB2 was not elevated.**
CK19	The detection of an expression of the cytokeratin 19 (CK19) gene indicates the presence of epithelial cells and may thus be indicative for circulating tumor cells. **neg: There was no expression of CK19 detected.**

Interpretation
In the fraction of isolated tumor cells, abnormal expression of all measured tumor-associated marker-genes was not observed.
Conclusion
According to the panel of molecular tumor markers used for this analysis, there are no indications for presence of cancerous cells in the analyzed blood specimen.

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Figure 6

studies, MTET therapy was able to suppress the telomerase activity. In the case renal cell carcinoma presented above, there was a paradoxical finding of increased telomerase as a marker of less aggressive cancer phenotype, due to accessory shifts. The clinical responses presented here, across various types of tumors, represents a potentially effective and novel method of treating cancers with metastatic disease, and needs further attention in large trials. Clinical investigations are required to evaluate

the correlation of clinical response to presence of telomerase overexpression in the circulatory tumor cells.

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