Circulating Cell-free DNA in Melanoma Patients

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1 Introduction

Molecular features of solid tumours become central in tailoring targeted therapies, but the accessibility to tumour tissue may be sometime limited due to the size of bioptic samples or the unavailability of biological material, particularly in patient follow up after tumour removal. In this context cancer-derived cell-free DNA (cfDNA) in blood represents a promising biomarker for cancer diagnosis and an useful surrogate material for molecular characterization (Hodgson et al., 2010).

The two classes of alterations detectable in cfDNA from cancer patients include quantitative and qualitative abnormalities. Concerning the former aspect, it is now evident that cancer patients have a higher concentration of cfDNA than healthy individuals (see Fleischhacker and Schmidt, 2007 for a review). The concentration of cfDNA is influenced by tumour stage, size, location, and other factors (Jung et al., 2010). On the other hand, increased plasma cfDNA level is not a specific marker of carcinogenesis, as it is observed also in patients with premalignant states, inflammation or trauma (Fleischhacker and Schmidt, 2007). Total cfDNA concentration has been proposed as a marker for early cancer detection, but the studies conducted so far showed a scarce discriminatory power between patients and controls as well as limited sensitivity and specificity, not allowing to reach any final conclusion on the diagnostic impact of this parameter. Several studies report a prognostic value of total cfDNA, while conflicting results have been obtained in testing this marker for therapy monitoring (Jung et al., 2010).

A higher specificity in cancer diagnosis can be achieved by detecting tumor specific alterations in cfDNA, such as DNA integrity, genetic and epigenetic modifications (Jung et al., 2010). Blood cfDNA in cancer patients originates from apoptotic or necrotic cells. In solid cancers, necrosis generates a spectrum of DNA fragments with variable size, due to random digestion by DNases. In contrast, cell death in normal blood nucleated cells occurs mostly via apoptosis that generates small and uniform DNA fragments. It has generally been observed that in patients affected by several neoplastic diseases plasma DNA contains longer fragments than healthy subjects (Wang et al., 2003; Umetani et al., 2006; Hanley et al., 2006; Hauser et al., 2010 Jiang et al., 2006; Tomita et al., 2007).

The above mentioned parameters can obviously be considered as non-specific biomarkers, since the increase of cfDNA concentration and integrity is common to the large majority of human solid cancers. When cfDNA is used to detect genetic and epigenetic modifications in a particular neoplasia, it is necessary to select specific molecular targets, expected to be altered in affected patients.

*BRAF* is a serine–threonine protein kinase involved in the RAS–RAF–MEK–ERK pathway (Dhomen and Marais, 2007) which regulates cell growth, survival, differentiation and senescence (Huang et al., 2009). The oncogene *BRAF* is frequently mutated in many human cancers constitutively activating the MAPK pathway. The most common *BRAF* mutation, which accounts for more than 90% of cases of cancer involving this gene, is the T1799A transversion, converting valine to glutamic acid at position 600 (V600E) (Davies et al., 2002). *BRAF* somatic mutations have been reported in 66% of malignant melanomas and at a lower frequency in a wide range of human cancers (Davies et al., 2002). *BRAF* mutations are likely to be a crucial step in the initiation of melanocytic neoplasia, as they are found also in nevi (Pollock et al., 2003). *BRAF* mutations are an attractive target for therapeutic interventions, as they represent an early event in melanoma pathogenesis and are preserved throughout tumor progression (Omholt et al., 2003). Specific inhibitors of mutant *BRAF*, such as PLX4032, were developed (Flaherty et al., 2009). *BRAF*<sup>V600E</sup> mutation has been investigated as a marker in cfDNA from melanoma patients by Daniotti et al., 2007 and Yancovitz et al., 2007.
Finally, it is widely demonstrated that a limited number of genes is epigenetically disregulated in cutaneous melanoma. RASSF1A (Ras association domain family 1 isoform A) is a tumor suppressor gene, which regulates mitosis, the cell cycle and apoptosis. It is inactivated mostly by inappropriate promoter methylation in many types of cancers (Donninger et al., 2007). RASSF1A promoter is methylated in 55% of cutaneous melanomas (Spugnardi et al., 2003). Methylation of RASSF1A increases significantly with advancing clinical stage, suggesting that the inactivation of this gene is associated with tumor progression (Tanemura et al., 2009). RASSF1A promoter hypermethylation has been detected in cfDNA from melanoma patients (Hoon et al., 2000; Marini et al., 2006) in association to a worse response to therapy and reduced overall survival (Mori et al., 2005; Koyanagi et al., 2006).

The aim of the present study was to identify a sequential multi-marker panel in cfDNA able to increase the predictive capability in the diagnosis of cutaneous melanoma in comparison with each single marker alone. To this purpose we tested total cfDNA concentration, cfDNA integrity, \textit{BRAF}^{V600E} mutation and RASSF1A promoter methylation associated to cfDNA in a series of 76 melanoma patients and 63 healthy controls.

2 Materials and Methods

2.1 Patients

Seventy six patients (32 females and 44 males, median age 63, range 23-94 years) affected by cutaneous melanoma (12 patients with in situ melanoma, 49 with local disease, 5 with regional metastatic disease and 10 with distant metastatic disease) were enrolled at the Department of Dermatological Sciences of the University of Florence. As a control group 63 healthy subjects (median age 62, range 25-79 years) were enrolled in the study upon a dermatological examination to exclude the presence of melanoma and to provide the number of nevi. Blood samples (5 ml) were collected in EDTA tubes during the dermatologic examination and before surgery. The research protocol was approved by the review board of the University of Florence and all the patients signed an informed consent.

2.2 DNA Extraction

Plasma was separated from blood in EDTA tubes, within three hours from blood draw by two centrifugation steps at 4 °C for 10 min: at 1600 rcf and 14000 rcf, respectively. Plasma aliquots (505 µl) were stored at −80 °C. DNA was extracted from 500 µl of plasma, by the QIAamp DSP Virus Kit (Qiagen, Italy) according to the manufacturer’s instructions. RNAse digestion was included in the procedure to prevent RNA interference during the subsequent qPCR reactions.

2.3 Molecular Biomarkers in cfDNA

All the cfDNA samples from melanoma patients and healthy controls were submitted in duplicate to six qPCR assays targeting the chosen biomarkers, for a total of about 1500 determinations. All the qPCR reactions were performed using the 7900HT instrument (Applied Biosystems).

2.3.1 qPCR Assays for Total Free Circulating DNA and Integrity Indexes

Absolute quantification of the single copy gene \textit{APP} (Amyloid Precursor protein, chr.4q11-q13) was performed on DNA from plasma to accurately measure the total amount of free circulating DNA per ml
plasma, using primers and probe targeting a 67 bp sequence (Pinzani et al., 2011).

Quantification of DNA concentration was obtained using an external reference curve ranging from 10 to $10^{5}$ pg/tube of genomic DNA extracted from a blood pool of healthy donors and measured spectrophotometrically (Nanodrop ND1000, Nanodrop, USA). Results were expressed as ng circulating DNA/ml of plasma.

Three additional qPCR assays were determined by targeting longer target sequences (respectively a 180 bp or 306 bp or 476 bp) on the single copy gene APP, as already reported (Pinzani et al., 2011). The ratio between the absolute concentration of each of the longer amplicons and the shortest one (67 bp used to quantify total cfDNA) defined the integrity indexes 180/67, 306/67 and 476/67 which were used to assess the fragmentation of cfDNA. The primers and the hydrolysis probes for the different amplicons were previously described (Lehmann et al., 2000; Pinzani et al., 2011) and are reported in Table 1. The reactions were carried out separately for each different-length template in a 12.5 µl mix containing 1x Quantitect® Probe PCR Master Mix (QIAgen), 300 nm primers, 200 nm probe and 1 µl sample. The thermal profile of the amplification was 50 cycles of PCR as follows: i) 15 s at 95 °C and 60 s at 60 °C for shorter amplicons (67 and 180 bps), ii) 15 s at 95 °C, 60 s at 56 °C and 60 s at 72 °C for the longer amplicons (306 and 476 bps). All the measurements were performed in triplicate on 1 µl of DNA. For cfDNA quantification targeting the 180, 306 and 476 amplicons, we used an external reference curve as reported for the 67 bp amplicon.

| Forward Primer | 5'-TCAGGTTGACGCCGCTGT-3' |
| Hydrolysis Probe | 5'-FAM-ACCCCAGAGGAGGCCACCTG-TAMRA-3' |
| Reverse Primer - 67 bp | 5'-TTCGTAGGCCTCTGCTGC-3' |
| Reverse Primer - 180 bp | 5'-TCTATAAAATGGACACCCAGATGGGTAGT-3' |
| Reverse Primer - 306 bp | 5'-GAGAGATAGAATAACCTACTGATGTTGGAT-3' |
| Reverse Primer - 476 bp | 5'-TAAAGTACCTAATGGTCACAAAC-3' |

Table 1 : Sequence of primers and probe.

*2.3.2 Double Allele Specific qPCR to Detect BRAF$^{V600E}$ Mutation*

Circulating cfDNA bearing the mutation $BRAF^{V600E}$ was quantified by an allele-specific qPCR assay, as already reported (Pinzani et al., 2010). The specificity for the mutated allele was conferred by the forward primer and the LNA probe. cfDNA was amplified in a reaction mixture containing 1× Quantitect® Probe PCR Master Mix (QIAgen), 200 nm primers and 200 nm probe in a final volume of 20 µl. The thermal profile of the reaction included a denaturation step at 95 °C for 10 min and 50 cycles of PCR (95 °C for 15 s, 64 °C for 1 min). $BRAF^{V600E}$ percentage was calculated by referring to a standard curve obtained by mixing DNA from mutant (SKMEL28) and wild type (MCF7) cell lines in the following proportions: 100%, 50%, 20%, 10%, and 1% mutated alleles. Results were expressed as percentage of mutated DNA (% $BRAF^{V600E}$) and in terms of absolute concentration of $BRAF^{V600E}$ mutated DNA (ng/ml of plasma) using the following formula:

\[
BRAF^{V600E} \text{ mutated DNA (ng/ml plasma)} = (\% \, BRAF^{V600E} \times \text{total DNA})/100
\]

*2.3.3 qPCR Assay for RASSF1A Methylated form Quantification*

The methylated form of RASSF1A promoter was quantified in plasma from melanoma patients and con-
trols after digesting unmethylated DNA by a methylation-sensitive enzyme: 5 µl of plasma DNA were treated with 10 units of Bsh1236I (Fermentas, Canada) in a reaction volume of 25 µl at 37 °C for 16 hours. The enzyme recognizes the restriction site CGCG in the promoter of the gene RASSF1A and digests DNA only if the cytosines are unmethylated. The methylated DNA is then amplified by a qPCR assay targeting the promoter region of the gene comprising the restriction site of Bsh1236 I. If the digestion is complete the qPCR reaction will quantify only methylated DNA. In particular 5 µl of enzyme-treated DNA underwent a qPCR assay for RASSF1A promoter, in a final volume of 25 µl, according to the protocol already described by Chan et al., 2006. A reference curve obtained by serial dilutions of genomic DNA was used to quantify the methylated alleles. Results were expressed as genomic equivalents (GEQ, each corresponding to 6.6 pg DNA) per ml plasma.

2.4 Statistical Analysis

All the considered biomarkers were analysed as continuous variables in their original scale or after an appropriate transformation. Comparison of biomarkers distribution in melanoma patients (cases) and healthy subjects (controls) was performed by using the Kolmogorov-Smirnov test (KST) (Hollander & Wolfe, 1999). The relationship between each biomarker and the disease status was investigated by resorting to a logistic regression model in both univariate and multivariate fashion (Hosmer & Lemeshow, 1989). The biomarker that was statistically significant in the univariate analysis was considered in the initial model of multivariate analysis. A final more parsimonious model was then obtained using a backward selection procedure in which only the variables reaching the conventional significance level of 0.05 were retained (final model).

The predictive capability (i.e. diagnostic performance) of each biomarker was investigated by means of the area under the ROC curve (AUC) (Hanley & McNeil, 1982). This curve measures the accuracy of biomarkers when their expression is detected on a continuous scale, displaying the relationship between sensitivity (true-positive rate, y-axes) and 1-specificity (false-positive rate, x-axes) across all possible threshold values of the considered biomarker. An useful way to summarize the overall diagnostic accuracy of the biomarker is the area under the ROC curve (AUC) the value of which is expected to be 0.5 in absence of predictive capability, whereas it tends to be 1.00 in the case of high predictive capacity (Hanley & McNeil, 1982). To aid the reader to interpret the value of this statistic, we suggest that values between 0.6 and 0.7 can be considered as indicating a weak predictive capacity, values between 0.71 and 0.8 a satisfactory predictive capacity and values greater than 0.8 a good predictive capacity (Gasparini et al., 1996).

Finally the contribution of each variables to the predictive capability of the final model was investigated by comparing the AUC value in the model with that of the same model without the variable itself. All statistical analyses were carried out using SAS software (Version 9.2.; SAS Institute Inc. Cary, NC).

3 Results

All the subjects enrolled in the study were tested for all the biomarkers in cfDNA.

3.1 Total cell-free DNA Concentration in Plasma

When submitted to qPCR for total cfDNA concentration in plasma, all samples from patients and controls showed positive amplification plots.
Figure 1 displays through a specific box-plot the distribution of circulating plasma cfDNA in patients with melanoma (cases) and in healthy subjects (controls) and in the table below are reported some descriptive statistics of these distributions. As shown in Figure 1, melanomas patients had an increase of about 3-fold of the amount of plasma cfDNA, since in control subjects plasma DNA level (median: 5.260 ng/ml, IQR: 6.210) was significantly lower than in melanomas patients (median:15.641 ng/ml, IQR: 19.687).

**Figure 1: Total circulating plasma cfDNA distribution.** Boxplot reflecting the free-circulating plasma DNA concentration distribution in cases and controls. The two horizontal sides of the box identify the 25th and 75th centile, the horizontal line inside the box indicates the median and the limits of the two whiskers indicates the extreme measured values. The inner box represents a zoom window to aid the visualization of the of the distribution.

<table>
<thead>
<tr>
<th>cfDNA (ng/ml plasma)</th>
<th>min</th>
<th>25th centile</th>
<th>median</th>
<th>75th centile</th>
<th>max</th>
<th>IQR</th>
<th>p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>cases</td>
<td>0.894</td>
<td>11.098</td>
<td>15.641</td>
<td>30.785</td>
<td>208.560</td>
<td>19.687</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>controls</td>
<td>0.990</td>
<td>2.530</td>
<td>5.260</td>
<td>8.740</td>
<td>47.490</td>
<td>6.210</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: IQR, Interquartile range (75th centile – 25th centile).
† p-value of the Kolmogorov-Smirnov test by comparing the distribution of cases and controls.
3.2 Integrity Indexes 180/67, 306/67 and 473/67 of Cell-free DNA in Plasma

cfDNA integrity indexes were evaluated in plasma of our cohort of subjects. The results show a decrease of cfDNA concentration as the amplicon dimensions increase in both healthy and melanoma subjects. Moreover for each single amplicon a statistically significant difference was detected between healthy subjects and patients (Student t-Test, p-value <0.05) with constantly higher values in the melanoma group (data not shown).

Figure 2 depicts the distribution of the three integrity indexes in patients with melanoma (cases) and in healthy subjects (controls). The distribution of the integrity index 180/67 was significantly different in melanoma patients with respect to that of control subject (p-value < 0.0001). Conversely, these findings were not observed when we analysed the integrity index 306/67 (p-value = 0.981) and the integrity index 476/67 (p-value = 0.363).

![Boxplot](Figure 2: Integrity index distributions. Boxplot reflecting the distribution of the integrity index 180/67 (Panel A), 306/67 (Panel B) and 476/67 (Panel C) in cases and controls. The two horizontal sides of the box identify the 25th and 75th centile, the horizontal line inside the box indicates the median and the limits of the two whiskers indicate the extreme measured values.)

<table>
<thead>
<tr>
<th>Integrity Index</th>
<th>Min</th>
<th>25th centile</th>
<th>Median</th>
<th>75th centile</th>
<th>Max</th>
<th>IQR</th>
<th>p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>180/67 cases</td>
<td>0.070</td>
<td>0.560</td>
<td>0.750</td>
<td>0.950</td>
<td>2.568</td>
<td>0.390</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>180/67 controls</td>
<td>0.090</td>
<td>0.290</td>
<td>0.460</td>
<td>0.670</td>
<td>1.810</td>
<td>0.380</td>
<td></td>
</tr>
<tr>
<td>306/67 cases</td>
<td>0.050</td>
<td>0.150</td>
<td>0.260</td>
<td>0.440</td>
<td>1.100</td>
<td>0.290</td>
<td>0.981</td>
</tr>
<tr>
<td>306/67 controls</td>
<td>0.070</td>
<td>0.150</td>
<td>0.290</td>
<td>0.400</td>
<td>1.260</td>
<td>0.250</td>
<td></td>
</tr>
<tr>
<td>476/67 cases</td>
<td>0.010</td>
<td>0.050</td>
<td>0.135</td>
<td>0.240</td>
<td>1.010</td>
<td>0.190</td>
<td>0.363</td>
</tr>
<tr>
<td>476/67 controls</td>
<td>0.000</td>
<td>0.080</td>
<td>0.130</td>
<td>0.290</td>
<td>1.530</td>
<td>0.210</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: IQR, Interquartile range (75th centile – 25th centile).
† p-value of the Kolmogorov-Smirnov test by comparing the distribution of cases and controls.

3.3 \(BRAF^{V600E}\) Percentage and Concentrations in Plasma

The median plasma percentage of \(BRAF^{V600E}\) DNA was higher in cases (median: 1.585, IQR: 4.655) than in control subjects (median: 1.440, IQR: 2.840). Similar results were found when evaluating the \(BRAF^{V600E}\) results expressed as ng/ml of plasma. In particular, a statistically difference (p-value: 0.001) in the distribution of \(BRAF^{V600E}\) DNA concentration (ng/ml of plasma) was observed between cases (median: 0.200 ng/ml plasma, IQR: 0.603) and controls (median: 0.080 ng/ml plasma, IQR: 0.153).
Figure 3: \(BRAF^{V600E}\) distributions. Boxplot reflecting the distribution of the plasma percentage of \(BRAF^{V600E}\) DNA (Panel A) and \(BRAF^{V600E}\) quantification in ng/ml of plasma (Panel B) in cases and controls. The two horizontal sides of the box identify the 25th and 75th centile, the horizontal line inside the box indicates the median and the limit of the two whiskers indicates the extreme measured values. The inner box represents a zoom window to aid the visualization of the distribution.

### 3.4 Methylated RASSF1A Concentration in Plasma

Plasma concentration of the methylated form of \(RASSF1A\) gene promoter were evaluated in control subjects as well as in melanoma patients. The distribution of methylated \(RASSF1A\) resulted significantly different in melanoma patients with respect to the control subjects (p-value: 0.0003).

### 3.5 Multimarker Approach

As shown in the previous paragraphs, circulating plasma total cfDNA concentration, integrity index 180/67, \(BRAF^{V600E}\) and methylated \(RASSF1A\) showed a significant difference in the distribution of melanoma patients in comparison to that of controls, confirming the potential clinical relevance of these biomarkers for the melanoma diagnosis. For these biomarkers considered in the logistic regression model we found that a linear relationship between the log odds and their values on the original (methylated \(RASSF1A\)) or logarithm (total cfDNA, integrity index 180/67 and \(BRAF^{V600E}\)) scale was appropriate. As reported in Table 2 disease status was significantly associated with all these biomarkers in the logistic univariate analysis. Consequently all the considered biomarkers were included in the initial model of the logistic multivariate regression analysis.
Figure 4: Methylated RASSF1A distribution. Boxplot reflecting the distribution of the methylated RASSF1A in cases and controls. The two horizontal sides of the box identify the 25\textsuperscript{th} and 75\textsuperscript{th} centile, the horizontal line inside the box indicates the median and the limit of the two whiskers indicates the extreme measured values.

<table>
<thead>
<tr>
<th></th>
<th>cases</th>
<th>25\textsuperscript{th} centile</th>
<th>median</th>
<th>75\textsuperscript{th} centile</th>
<th>max</th>
<th>IQR</th>
<th>p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>methylated RASSF1A (GE/ml plasma) cases</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>11.040</td>
<td>208.680</td>
<td>11.040</td>
<td>0.0003</td>
</tr>
<tr>
<td>methylated RASSF1A (GE/ml plasma) controls</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>4.010</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

*Abbreviations: IQR, Interquartile range (75\textsuperscript{th} centile – 25\textsuperscript{th} centile).† p-value of the Kolmogorov-Smirnov test by comparing the distribution of cases and controls.*

The AUC values (Table 2 and Figure 5) computed for each biomarker (univariate logistic model) indicated a weak/satisfactory level of predictive capability by ranging between 0.64 (BRAF\textsuperscript{V600E}) to 0.85 (cfDNA). Of note for all the considered biomarkers the 95\% Confidence Interval (95\%CI) of the AUC fails to include the 0.5 value (i.e. absence of predictive capability).

As reported in Table 3, cfDNA, integrity index 180/67 and methylated RASSF1A retained a statistically significant (p-value < 0.05) association with disease status also in the multivariate final logistic model. A good predictive capability was observed for the final logistic model with an AUC of 0.95 (95\% CI: 0.91-0.98) (Table 3 and Figure 6).

Finally, the contribution of each variable to the predictive capability of the final model (AUC: 0.95, 95\%CI: 0.91-0.98) was investigated by comparing the AUC value in the model with that of the same model without the variable itself: the highest predictive capability was given by cfDNA (AUC:0.86, 95\%CI: 0.80-0.92) followed by integrity index 180/67 (AUC:0.90, 95\%CI: 0.85-0.95) and methylated RASSF1A (AUC:0.89, 95\%CI: 0.84-0.95).
<table>
<thead>
<tr>
<th>Biomarker</th>
<th>OR(^a)</th>
<th>OR 95%CI</th>
<th>p value(^b)</th>
<th>AUC</th>
<th>AUC 95%CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>cfDNA (ng/ml plasma)</td>
<td>5.621</td>
<td>3.102-10.185</td>
<td>&lt;0.0001</td>
<td>0.853</td>
<td>0.788-0.918</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Integrity Index 180/67</td>
<td>4.790</td>
<td>2.356-9.740</td>
<td>&lt;0.0001</td>
<td>0.759</td>
<td>0.677-0.840</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Methylated RASSF1A (GE/ml plasma)</td>
<td>1.413</td>
<td>1.112-1.795</td>
<td>0.005</td>
<td>0.688</td>
<td>0.621-0.754</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(BRAF^{V600E}) (ng/ml plasma)</td>
<td>6.061</td>
<td>1.650-22.263</td>
<td>0.007</td>
<td>0.635</td>
<td>0.540-0.730</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Abbreviations: OR, Odds Ratio; CI, Confidence Interval; AUC, area under the ROC curve.

\(^a\)Odds Ratio for any increase of one unit.

\(^b\)p-value of the Wald statistic.

**Table 2:** Univariate logistic analysis.

**Figure 5:** ROC Curves deriving from the univariate logistic analysis. ROC curves derived from the univariate logistic analysis corresponding to cfDNA (AUC = 0.85), integrity index 180/67 (AUC = 0.76), methylated RASSF1A (AUC = 0.69) and \(BRAF^{V600E}\) (AUC = 0.64).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>OR(^a)</th>
<th>OR 95%CI</th>
<th>p value(^b)</th>
<th>AUC</th>
<th>AUC 95%CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cfDNA (ng/ml plasma)</td>
<td>6.592</td>
<td>3.084-14.088</td>
<td>&lt;0.0001</td>
<td>0.945</td>
<td>0.910-0.980</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Integrity Index 180/67</td>
<td>7.783</td>
<td>2.944-20.579</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated RASSF1A (GE/ml plasma)</td>
<td>1.450</td>
<td>1.100-1.910</td>
<td>0.008</td>
<td></td>
<td></td>
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</table>

Abbreviations: OR, Odds Ratio; CI, Confidence Interval; AUC, area under the ROC curve.

\(^a\)Odds Ratio for any increase of one unit.

\(^b\)p-value of the Wald statistic.

**Table 3:** Final multivariate logistic model.
Figure 6: ROC Curve deriving from the multivariate final logistic model. ROC curve derived from the final multivariate logistic model (AUC = 0.95).

4 Discussion

The analysis of cfDNA may have the potential to complement or replace the existing cancer tissue and blood biomarkers in the future (Schwarzenbach et al., 2011). In order to reach this goal, specific and sensitive analytical procedures must be developed and optimized to compute proper circulating target molecules showing differences between patients and healthy subjects. It is now widely accepted that a single biomarker cannot fully discriminate between controls and patients and consequently an approach based on different markers is preferable in order to achieve a stronger predictive ability (Pinsky and Zhu, 2011). It has been demonstrated that in prenatal screening a combination of multiple markers, each of which by itself has limited sensitivity and/or specificity, can lead to a powerful screening test (Malone et al., 2005). Analogously, Schneider and Mizejewski (2007) suggest to develop a multi-marker screening approach for cancer. The authors observed that so far the approach for cancer testing has been limited to the research of single biomarkers. Unfortunately this strategy has been proven unsuccessful, notwithstanding the high number of new biomarkers reported in the literature. There are already some examples on prostate and ovarian cancer clearly showing that multi-marker screening can have its place in early cancer detection (Schneider & Mizejewski 2007).

In this preliminary study the principal aim was to investigate the diagnostic performance of four markers associated to cfDNA in identifying melanoma patients. Particular efforts were dedicated to the technical aspects of the methods adopted for each single parameter allowing to reach accurate and reproducible measurements. Following the standard approach (Verderio et al 2010) for the clinical validation of biomarkers for early detection we are planning new studies focused on the assessment of the impact of these biomarkers on clinical practice including the identification of the most suitable thresholds to use for the early detection of melanoma by clinicians.

Here we considered total cfDNA concentration by a qPCR assay for the single copy gene APP, as well as its fragmentation expressed by the integrity index 180/67. On the other hand, tumour contribution to cfDNA was assessed by quantifying BRAF<sup>V600E</sup> mutated alleles and RASSF1A promoter methylation. These markers have been used in a panel in all patients, thus representing a simple model potentially adoptable by any laboratory. Our preliminary results show that by jointly considering the panel of bi-
markers here investigated the highest predictive capability is given by cfDNA followed by integrity index 180/67 and methylated RASSF1A. According to these results, an approach based on the simultaneous determination of the three biomarkers (cfDNA, integrity index 180/67 and methylated RASSF1A) could be suggested to improve the diagnostic performance in melanoma (Salvianti et al 2012).

Even though each biomarker investigated in the present work is not exclusively associated with melanoma, their combination reveals a high specificity for melanoma detection.

**Acknowledgement**

This paper is dedicated to Prof. Claudio Orlando who initiated the research on cell-free DNA in our Laboratory.

**References**


