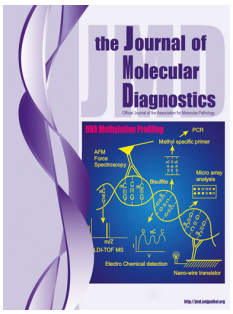

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Pyrosequencing assay for *BRCA1* methylation analysis: results from a cross-validation study

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Abstract

Epithelial Ovarian Cancers (EOCs) harboring germline or somatic pathogenic variants in *BRCA1* and *BRCA2* genes show sensitivity to poly(ADP-ribose) polymerase (PARP) inhibition. It has been suggested that *BRCA1* promoter methylation is perhaps a better determinant of therapy response, due to its intrinsic dynamic feature, with respect to genomic scars or gene mutation. Conflicting evidence was reported so far and the lack of a validated assay to measure promoter methylation was considered a main confounding factor in data interpretation. To contribute to the validation process of a pyrosequencing assay for *BRCA1* promoter methylation, 109 EOCs from two Italian centers were reciprocally blindly investigated. By comparing two different pyrosequencing assays, addressing a partially overlapping region of *BRCA1* promoter, an almost complete concordance of results was obtained. Moreover, the clinical relevance of this approach was also supported by the finding of *BRCA1* transcript downregulation in *BRCA1* methylated EOCs.

These findings could lead to the development of a simple and cheap pyrosequencing assay for diagnostics, easily applicable to FFPE tissues. This technique may be implemented in routine clinical practice in the near future to identify EOCs sensitive to PARPi therapy, thus increasing the subset of women affected by EOCs that could benefit from such treatment.

Introduction

The inhibition of poly(ADP-ribose) polymerase (PARP) in cancer cells, which causes the inactivation of the homologous recombination-mediated repair (HR) pathway, is a current strategy used for therapy in Epithelial Ovarian Cancers (EOCs) harboring germline or somatic pathogenic variants in the *BRCA1* and *BRCA2* genes [1, 2, 3, 4]. There is now clear evidence that HR defects can arise not only through inactivating germline and/or somatic mutations but also, in a mutually exclusive manner, when gene silencing is due to promoter methylation of *BRCA* genes and also of HR related genes [5, 6, 7, 8, 9]. However, clinical studies revealed that *BRCA1* gene methylation is involved in a consistent subset of EOCs but provided conflicting evidence, and therefore its accuracy and reliability as a biomarker for predicting PARP inhibitor responses in EOC patients cannot currently be established. On the contrary, *BRCA2* promoter methylation plays a marginal role in EOCs[10, 11, 12, 13, 14].

The lack of consistency of *BRCA1*-methylation clinical studies was primarily attributed to technical issues, given the significant differences in the methods used to evaluate *BRCA* promoter methylation [15]. There is evidence that clinical studies using *BRCA1* promoter methylation were confounded by technical factors associated with the measurement of tumour DNA methylation and with the zygosity of *BRCA1* methylation alleles [15].

The main reported methods for *BRCA1* tumour methylation analysis are pyrosequencing, methylation-specific PCR (MSP) with gel electrophoresis (MSP-GE) or quantitative (q-MSP), methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), and more recently genome wide methylation arrays (GWMA) [16]. These methods differ for many analytical parameters (e.g., discrimination of bisulfite induced C to T transition; cleavage of genomic DNA by methylation-sensitive restriction enzymes; number of CpG sites investigated; determination of methylation cutoffs) and, therefore, their results are difficult to compare.

In this context, pyrosequencing analysis is the most used among methods to obtain quantitative data with a good analytical sensitivity [17], which is crucial for the correct identification of *BRCA1* methylation levels. Other methods (e.g., MS-MLPA, MSP) give qualitative results independently from zygosity because they are based on the evaluation of the methylation difference between each sample and normal reference DNAs.

Noteworthy, it has been shown that the response to PARPi depends on the complete impairment of *BRCA1* function[15] and recent findings from an exploratory biomarker analysis of ARIEL2 trial samples[18] demonstrated that a high level of *BRCA1* methylation is a strong predictor of a response to Rucaparib. These findings were validated using highly quantitative methylation specific droplet digital PCR to measure *BRCA1* methylation. The authors also demonstrated that *BRCA1* promoter methylation seemed to be a more accurate biomarker respect to genomic scars to identify HRD. In fact, genomic scarring, once established, persists and does not provide a real-time predictor of sensitivity after multiple treatment lines; on the contrary, methylation status is a dynamic mechanism and could be investigated by methylation quantitative assessments.

Although the pyrosequencing approach has been described as a robust, quantitative and sensitive assay applicable to FFPE samples in several studies [19, 20, 21], no data focused on obtaining formal technical validations of this approach were retrieved from the literature.

In order to contribute to the validation process of a pyrosequencing assay for *BRCA1* promoter methylation, in the present study 109 EOCs from two Italian centers [21, 19] were investigated comparing two similar pyrosequencing assays. To gain evidences for their application in routine diagnostics, blinded analyses on the same sample set between different laboratories were performed and the robustness of the pyrosequencing assay was also evaluated using orthogonal assays.

Materials and Methods

Samples

A retrospective series of 109 EOCs was investigated for *BRCA1* promoter methylation including 50 formalin-fixed paraffin-embedded (FFPE) samples from Varese Center (ASST Settelaghi, Ospedale di Circolo – University of Insubria) and 59 FFPE samples from Genoa Center (IRCCS San Martino Hospital). All investigated EOCs were selected from routine clinical practice because they were negative for Homologous Recombination (HR) somatic and germline variants. The clinico-pathological characteristics of Varese and Genoa EOCs were previously described respectively by Sahnane et al. [19] and Rivera et al[21].

All EOC samples were obtained from primary debulking surgery or needle biopsy and the histopathological diagnosis was performed using standard pre-analytical procedures with a fixation time less than 72 hours and according to the criteria of WHO Classification [22]. The majority of analyzed samples showed a high percentage of tumour cells (Supplementary Table S1).

All analyses were performed in agreement with the Declaration of Helsinki and the study was approved by the Ligurian Ethical Committee (472REG2015) and the Research Ethics Committee of ATS Insubria (ID 238/2018).

BRCA1 methylation assays by pyrosequencing

Varese assay

The assay used in ASST Settelaghi (Varese) has been extensively described elsewhere by Sahnane et al. [19]. Briefly, DNA was extracted from FFPE sections using automatic procedures (Maxwell RSC FFPE Plus DNA kit, Madison, Promega Corporation, Wisconsin, USA). About 200ng of tumour DNA underwent bisulfite-conversion by using EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA). A total of 8 CpG sites are tested by analyzing two PCR amplicons, addressing the non-coding exon 1 of *BRCA1* (chr17: 41,277,595–41,277,289). To set-up the methylation tests, artificial control samples at different percentages of DNA methylation (0, 10, 50, and 100%) were analyzed by appropriately mixing commercial fully methylated DNA and fully unmethylated DNA (Human WGA Methylated and Non-methylated DNA Set, Zymo Research, Irvine, CA, USA). These experiments demonstrated that the quantitative measurements of methylated cytosines performed well and there's no preferential amplification of either methylated sequences nor unmethylated ones. The LoB (limit of blank) for *BRCA1* methylation tests was set at a value of 10%, corresponding to the mean value plus three standard deviations of 10 independent measures. Subsequently, to set the limit of detection (LoD), data from three independent pyrosequencing analyses of the 10%-methylated control were analyzed for each primer set. The obtained values ranged from 6.94 to 14% of methylation, thus the LoD was set at a cut-off of 15%.

Genova assay

DNA samples have been extracted using an automated device (QIASymphony, Qiagen, Hilden, Germany) and applying GeneRead DNA FFPE Treatment Kit. For samples with low starting material, crude DNA was manually isolated using overnight proteinase K digestion. Bisulfite conversion was performed with the Epitect Bisulfite Kit (Qiagen, Hilden, Germany) using up to 300ng of DNA for each reaction. The assay developed at the IRCCS San Martino Hospital (Genoa) was designed with the Pyrosequencing Assay Design Software (Qiagen, Hilden, Germany) to cover 14 CpG sites in the promoter region of the *BRCA1* gene. The genomic coordinates of CpG sites were derived from the literature [23 24 25]. The pyrosequencing analysis was conducted by amplifying two promoter regions, and using three sequencing primers (Table 1) with the Pyro Q-CpG software (version 1.0.9) that provides an internal control for the completeness of the bisulfite conversion. The primer pairs were tested in order to not match with genomic DNA and to avoid any preferential amplification towards a methylated or unmethylated template (Supplementary Table S2). To set the methylation threshold, data from three independent pyrosequencing analyses of the unmethylated controls were analyzed for each primer set. The obtained values ranged from 2% to 9% of methylation (Supplementary Table S2), and the mean value plus two standard deviation corresponds to the value of 11%. Thus, the cut-off to call methylation was set at 15%, which was in agreement with other assays [19, 26].

Design of the study

The selected 109 EOCs from Varese and from Genoa were used for interlaboratory comparison of *BRCA1* methylation results using both pyrosequencing approaches: DNA samples from 59 EOC analyzed in Genoa were investigated in Varese lab using Varese methylation assay and *vice versa* DNA samples from 50 EOC analyzed in Varese were investigated in Genoa lab using Genoa methylation assay**.** Methylation status of samples was assessed by each laboratory using the in-house developed pyrosequencing assay and a methylation cut-off of 15% was used by both laboratories, according to intra-laboratory set-up [19]. The same DNA preparations used in the in-house assay were exchanged between the two laboratories and the external

150 samples were blindly analyzed by each laboratory using the in-house assay. Methylation results were
151 expressed as the mean value of all the analyzed cytosines. Figure 1 shows a schematic illustration of CpG
152 dinucleotides *BRCA1* promoter regions analyzed with Varese (blue) and with Genoa (grey) assays. Black
153 arrows indicate amplification primers: light-colored arrows (blue for Varese and Gray for Genoa) indicate
154 sequencing primers. The two assays test two different regions of the *BRCA1* promoter (NM_007294.4,
155 https://www.ncbi.nlm.nih.gov/nuccore/NM_007294.4, last access January 11, 2023), but they shared two
156 overlapping regions from chr17:41,277,581 to 41,277,547 and from chr17:41,277,445 to 41,277,427
157 positions on chromosome 17, GChr37/Hg19 assembly. Table 2 reported a comparison of the technical
158 characteristics of Varese and Genoa assays.

159

160 **MS-MLPA analysis**

161 A total of 100 ng of tumour DNA from a subset of 33 samples was used for Methylation-Specific Multiplex
162 Ligation-dependent Probe Amplification (MS-MLPA) analysis using the ME053 *BRCA1-BRCA2* X1-0914
163 methylation assay, according to manufacturer's instructions (MRC-Holland, the Netherlands). As described
164 elsewhere [27], the methylation assay included two digestion (methylation) control probes. Normal ovarian
165 tissue from three patients were included as template controls. For methylated samples, another tumour area
166 was used as positive control and healthy tissue or peripheral blood were used as negative controls. Data
167 analysis was performed with Coffalyser software (DB v.140701.0000, Client v.210604.1451). The methylation
168 status of the samples was determined by comparing the percentages of the methylation-specific probes
169 (containing an HhaI site) of digested sample to its undigested counterpart.

170

171 ***BRCA*1 transcript analysis**

172 *BRCA1* expression analysis was performed on a subset of 14 FFPE EOCs from the Genoa's series, including 8
173 methylated and 6 unmethylated EOCs. Total RNA was extracted using RNAeasy Micro Kit (Qiagen, Valencia,
174 CA) according to the manufacturer's instructions and subsequently quantified by Nanodrop 2000 instrument

(Invitrogen). An amount of 1.5µg of RNA was used for reverse transcription by using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) with random hexamers in a total volume of 20µL, as previously described [26]. Real-time PCR reactions were performed in triplicate on StepOnePlus Real Time System (Applied Biosystem) according to manufacturer's instructions, with a cDNA input of 75 ng. Taqman Probes (Thermo Fisher Scientifics) were used to detect *BRCA1* (assay ID: Hs01556193_m1) and two housekeeping genes: *RPLP0* (assay ID: Hs99999902_m1) and *β-Actin* (assay ID: Hs99999903_m1). As Ct values for *β-Actin* were greater than those observed for *RPLP0*, this latter gene was selected as the internal control. All measurements were performed in triplicate, so the ΔCT calculation was performed on the average of the three values. Data of *BRCA1* expression levels assessed in EOC samples were expressed as the fold change with respect to a pool of 10 histologically normal ovarian tissues, using the ΔΔCT method.

Results

Figure 2 summarizes the results of EOCs methylation analysis from each Center. The complete *BRCA1* methylation data are enlisted in Supplementary Table S1. Concordance of methylation status, using a cut-off value of 15% to score methylated versus unmethylated EOCs, was observed for all but one EOC (99%, 108/109 cases): case GE25 (Supplementary Table S1, Figure 3A and 3B pink dot) showed 8.52% methylation value (scored as unmethylated) with the Varese assay and 18.8% methylation value (scored as methylated) with the Genoa test.

In order to better correlate the results, quantitative data from the two laboratories were plotted by a regression test and by Bland-Altman analysis. A high level of correlation was observed between the two assays (R^2=0.84, Figure 3A), except for VA27 and VA17 samples. Both samples were outliers (see Figure 3B, green dots) as showed the greater differences between the two measurements: VA27 case resulting 78% by Genoa assay versus 31% by Varese assay and VA17 case showing 15% by Genoa test and 42% by Varese test. When the methylation levels were grouped in different level classes, the comparison of the data from two centers showed a high concordance of methylation assessment (Figure 3C).

A subset of 33 samples including 10 methylated and 23 unmethylated EOCs were analyzed also by Methylation Sensitive-Multiple Ligation Probe Amplification (MS-MLPA) by using ME053 BRCA1-BRCA2 X1-0914 kit (MRC-Holland, the Netherlands). MS-MLPA analysis confirmed *BRCA1* methylation status in 32 out of 33 analyzed EOCs. Interestingly, the discordant pyrosequencing sample (case GE25, Supplementary Table S1) revealed methylation at only one out of three analyzed probes at MS-MLPA analysis (Figure 4).

In a subset of 14 EOCs including 8 methylated and 6 unmethylated EOCs, transcript analysis was also performed to verify methylation driven *BRCA1* downregulation. *BRCA1* expression analysis by Real-Time PCR showed that transcript levels were significantly lower in *BRCA1*-methylated versus *BRCA1*-unmethylated EOCs (p=0.0032, Figure 5B). On the whole, in each case we observed a correlation between high degree of promoter methylation and transcript downregulation, although in one case a decreased expression was not supported by promoter methylation (Figure 5A, GE2). Unfortunately, transcript analysis of the discordant case (case GE25 Genoa) was not possible because additional tumour tissue was not available.

Discussion

The results of this cross-validation study suggest that pyrosequencing is a robust, reproducible and feasible method for *BRCA1* promoter methylation analysis in EOC FFPE samples. The concordance of methylation results obtained by the two laboratories using similar in-house developed assays was 99% and the only discordance was detected on a poorly differentiated EOC obtained from a primary debulking surgery. From a technical point of view, this discordant case was analyzed using a sample with FFPE storage time of ≤3 years old and an adequate pre-analytical phase complied with optimal fixation/storage, high representativeness of the entire neoplasia, good tumour cellularity (40%) and low presence of necrosis. Hence, the observed discrepancy probably reflects methylation heterogeneity of the sample, in agreement with the result of MS-MLPA methylation analysis that revealed methylation with only one out of three *BRCA1*-probes (Figure 4). Interestingly, other two cases, despite a clear classification as “methylated cases”, displayed different methylation values comparing the two assays. As both laboratories analyzed the same DNA preparation, such

differences might be due to post-extraction processes, like bisulfite-conversion, or the preferential allele amplification of methylated or unmethylated templates in PCR reaction. However, conversion efficacy was good in these cases (criteria in Table 2) and a preferential allele amplification might be excluded based on the set-up experiments performed in both laboratories. Thus, we speculate that the differences of methylation levels from the two assays could be due to methylation heterogeneity within these two samples (similarly to the discordant case).

The correct quantification of methylated alleles is very relevant from a clinical point of view, as recently reported by Menghi and colleagues [28]. The authors studied a series of primary EOCs using droplet digital PCR and demonstrated that the degree of *BRCA1* methylation had a strong and significant negative correlation with *BRCA1* expression. Moreover, using xenografts model, it was demonstrated that *BRCA1* methylation showed a functionally plastic behavior and can be lost upon chemotherapy regimens. On this ground, the quantification of *BRCA1* methylation is clinically relevant to check methylation status over the course of therapeutic cycles in order to readily change the therapy management. This aspect is particularly important in light of the availability of new therapeutic approach with PARPi, stressing the need of a strong predictive marker, able to longitudinally identify promoter methylation/demethylation and to have the real-time picture of the sensitivity profile. Of note, the correct assessment of the silenced alleles versus the functional ones is further complicated by chromosomal instability in EOC (https://mitelmandatabase.isb-cgc.org/ last access January 11, 2023).

The high concordance observed in this inter-laboratory comparison suggests that these pyrosequencing assays could be used in clinical practice to investigate *BRCA1* methylation. Noteworthy, the analysis of this *BRCA1* promoter region is indicative of gene silencing as it encompassed the promoter sequence between chr17:41,277,443 and chr17:41,277,717 which was identified by in-vitro studies as the promoter regulatory element that, when methylated, could silence gene expression [20]. In fact, in eight *BRCA1*-methylated cases a significantly downregulation of gene transcript was observed with respect to six *BRCA1*-unmethylated cases, confirming that the pyrosequencing assays are able to provide clinically relevant data to identify cases with BRCA1 function reduction. Remarkably, the same results were obtained when *BRCA*1 methylation results

obtained using all CpGs data were compared with results from only four CpGs (from chr17:41,277,445 to chr17:41,277,427) common between Varese and Genoa assays (Figure 6A and 6B).

The comparison of the two pyrosequencing assays encourages new considerations about this method. First, both methylation assays exploited the ability of pyrosequencing to investigate small amplicons and this characteristic enables the analysis of FFPE samples even when the pre-analytical steps are difficult to standardize [29]. This typically happens in routine practice when a single laboratory collects samples from many surgery/pathology units. Second, these data demonstrated that two different DNA conversion methods performed equally well. In order to exclude the overestimation of methylation levels, it is however important to maintain the presence of an intra-assay control of bisulfite-conversion efficiency. Third, this method performs in similar way of MS-MLPA technique, a broadly used method, considering costs and turnaround time, but has the advantage to be able to virtually analyze all genomic loci, while MS-MLPA, based on a restriction enzyme-based strategy, could target only regions containing the "recognition sites", moreover providing a semi-quantitative data.

In conclusion, these results confirmed that *BRCA1* promoter methylation of the investigated regions caused *BRCA1* downregulation and suggest that a high discrimination between methylated and unmethylated samples may be obtained by sequencing a single PCR product that includes only four shared CpG doublets in order to develop simple and cheap pyrosequencing assay for diagnostics.

Pyrosequencing analysis is a feasible and robust method to quantitatively detect *BRCA1* promoter methylation and, in the near future, this test may be implemented in routine clinical practice as a real-time predictor of PARPi therapy sensitivity, thus increasing the subset of women affected by EOCs that could benefit from this therapy during different treatment lines.

276 **Author Contributions:** LL, SF, DR, BB performed the methylation experiments; NS, LL, DR, BB analyzed the
277 data; GC and DR performed gene expression analysis; VG, MP, VGV, LV secured funding; NS, IC, DR, BB
278 designed the experiment; MGT, LV planned the research project; NS, DR wrote the manuscript; MGT, LV
279 revised the manuscript; all authors read and approved the manuscript. N.S. is the guarantor of this work and,
280 as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and
281 the accuracy of the data analysis.

282

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Figure Legends:

Figure 1: *BRCA1* methylation assays design. Schematic illustration of *BRCA1* promoter region showing CpG dinucleotides analysed with Varese (in blue) and Genoa (in grey) assays. *Black arrows are for amplification primers and light-coloured arrows (blue for Varese and grey for Genoa) represent sequencing primers.*

Figure 2: Summary of *BRCA1* methylation results obtained from blinded pyrosequencing assays in both Varese and Genoa Centers. E*OCs: Epithelial Ovarian Cancers; Met: BRCA1 methylated cases; Unmet: BRCA1 unmethylated cases*

Figure 3: Correlation between Varese and Genoa *BRCA1* methylation levels and classes. (**A**) Scatter plot of the regression analysis between Varese and Genoa *BRCA1* methylation assays. Each dot identifies single samples analyzed with both assays (Varese methylation levels on the x-axis and Genoa methylation levels on y-axis). Red dotted lines indicate 15% methylation level for each assay (cut-off level). (B) Bland-Altman plot between Varese and Genoa *BRCA1* methylation assays: for each sample are reported the average methylation values between Varese and Genoa measurements on the x-axis and on the y-axis is reported the difference between Varese and Genoa measurements. Red dotted lines indicate upper and lower 95% confident interval whereas solid blue line indicate no difference between observations. (**C**) Histogram which compares Varese (blue bars) and Genoa (grey bars) *BRCA1* methylation classes. *The discordant case (GE25) is highlighted as a pink dot. Green dots identify two cases that show discrepant methylation levels using Varese's or Genoa's assays (VA27: 78% Genoa vs 31% Varese, VA17: 15% Genoa vs. 42% Varese).*

Figure 4: **ME053 *BRCA1-BRCA2* MS-MLPA assay result of the discordant sample (GE25).** The upper panel shows the copy number analysis output of genes regions enlisted at the bottom of the figure. The lower panel shows the methylation analysis output of the methylation sensible gene regions ([HHA] probes). The red lines and the blue-indigo-purple lines are the lower and upper borders, respectively. They are placed at -/+ 0.3 from the average probe value of each probe over the normal reference samples. Because the average value of the probes over the reference samples is different for every probe, the borders are not straight lines. In the black box area, the three probes for *BRCA1* promoter methylation are highlighted , which shows methylation of only one out of three probes, at the limit of the upper border.

426 **Figure 5**: ***BRCA1* expression versus methylation status.** (A) Comparison between *BRCA1* expression (fold-
427 change, orange histograms) and *BRCA1* methylation status (percentage of methylation, blue dots/lines) for
428 each analysed sample. (B) The box-plot shows a significant downregulation of *BRCA1* transcript in 7 *BRCA1*
429 methylated EOCs in comparison to 6 unmethylated cases.

430

431 **Figure 6: Correlation of *BRCA1* methylation levels evaluated using "all CpGs" versus "four CpGs" shared by**
432 **both Varese and Genoa assays. (A)** Scatter plot of the regression analysis between "all CpG" versus "four
433 CpGs" of Varese (left) and Genoa (right) *BRCA1* methylation assays. Each dot identifies a single sample ("All
434 CpGs" methylation levels on the y-axis and "four CpGs" methylation levels on x-axis). Red dotted lines
435 indicate 15% methylation level for each assay (cut-off level). (B) Bland-Altman plot between "all CpG" versus
436 "four CpGs" of Varese (left) and Genoa (right) *BRCA1* methylation assays: for each sample are reported the
437 average methylation values between "all CpG" and "four CpG" measurements (x-axis) and the difference
438 between "all CpG" and "four CpG" measurements (y-axis). Red dotted lines indicate upper and lower 95%
439 Confident Interval (CI) whereas solid blue line indicate no difference between observations. *Yellow dots*
440 *indicate those samples which are outside of the 95% CI.*

441

442 **Table 1. Genoa assay - Primers and PCR conditions of pyrosequencing analysis**

Primer name	Primer sequences	AT (°C)	Amplicon length (bp)
B1PHM 1-2F	5'-GATGGGAGGGATAGAAAGAGTTAA-3'	62	97
B1PHM 1-2R^{biotin}	5'-TCCTCTTCCRTCTCTTTCCTTTT-3'		
B1PHM 1-2S	5'-GGGAGGGATAGAAAGAGT-3'		
B1PHM 3-14F	5'-AGAGTAGAGGGTGAAGGTTTTTT-3'	56.7	228
B1PHM 3-14R^{biotin}	5'-TCTATCCCTCCCATCCTCTAATTA-3'		
B1PHM 3-14S1	5'-GAGTAGAGGGTGAAGGTT-3'		
B1PHM 3-14S2	5'-TTTGTTTTTRGTTTAGGAAG-3'		

443

444

445 **Table 2. Comparison of technical characteristics of the Genoa and Varese assays.**

	Genoa assay (IRCCS San Martino Hospital)	**Varese assay (ASST Settelaghi)**
CpG sites	14	8
DNA input	up to 300ng	100-200ng
Bisulfite conversion	Epitect bisulfite kit (Qiagen)	EZ DNA Methylation kit (Zymo Research)
Amplicons	2	2
Sequencing primers	3	2
Pyrosequencer	Pyrosequencing PSQMA96 (Qiagen)	PyroMark Q96 ID (Qiagen)
Design software	Pyrosequencing Assay Design Software (v1.0.9)	Pyrosequencing Assay Design Software (v.2.0)
Methylation controls	0 - 25 - 50 - 75 - 100% hemimethylated DNA	0 - 10 - 50 - 100%
Limit of Detection	≤ 15% unmethylated (U) ≥ 15% methylated (M)	≤ 15% unmethylated (U) ≥ 15% methylated (M)
Partial methylation status	Heterogeneity (UM/M) of CpG methylation sites	not defined
Bisulfite score	10% passed, 15% check	12% passed

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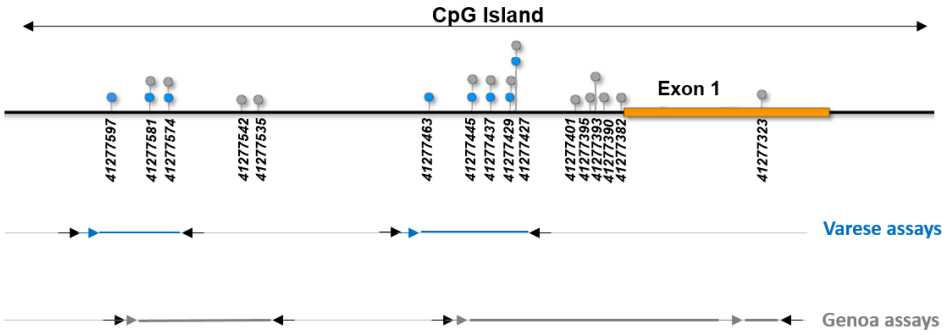

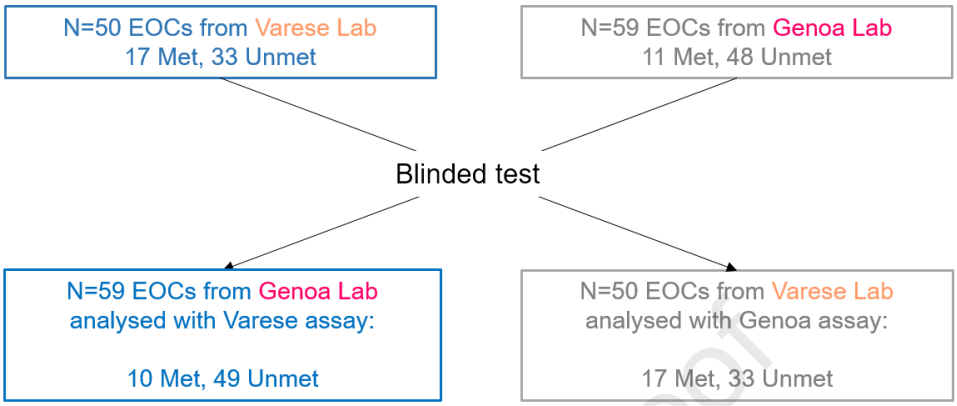

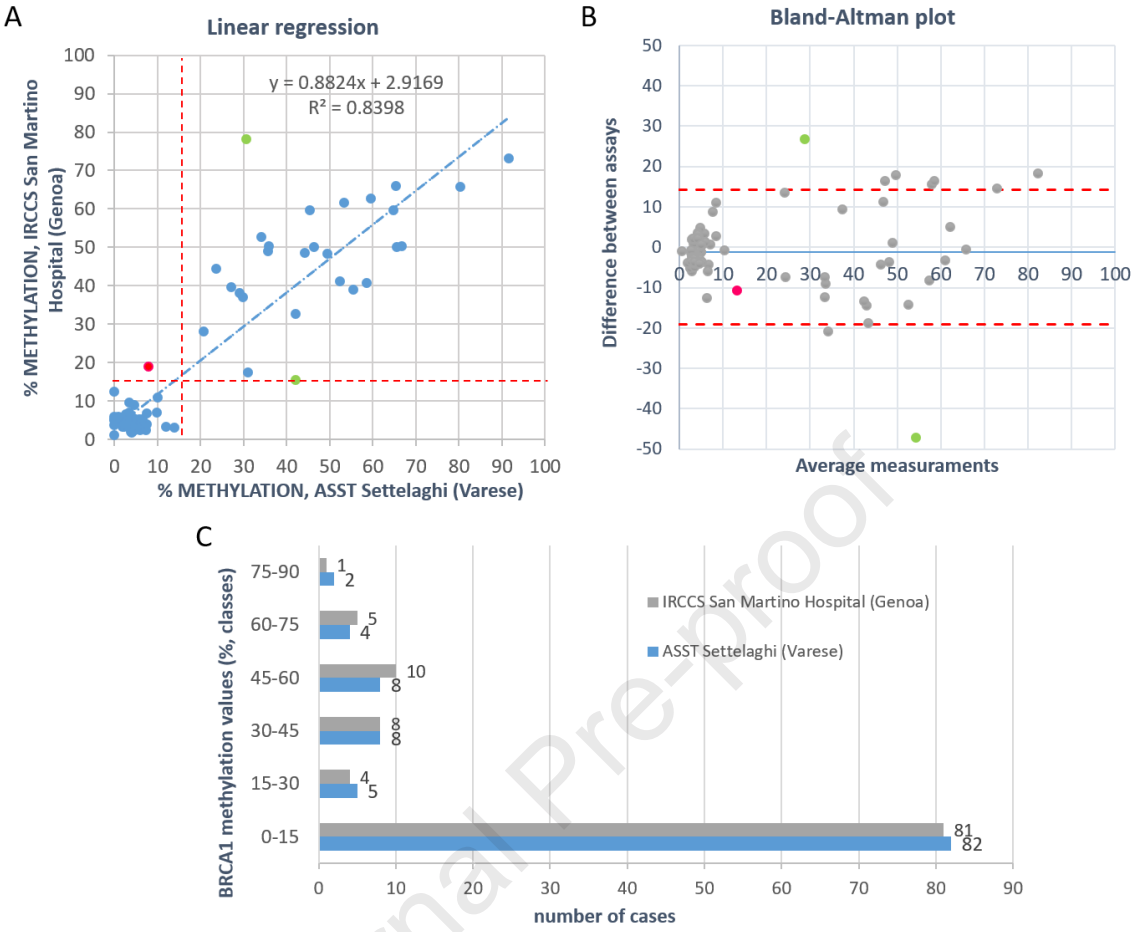

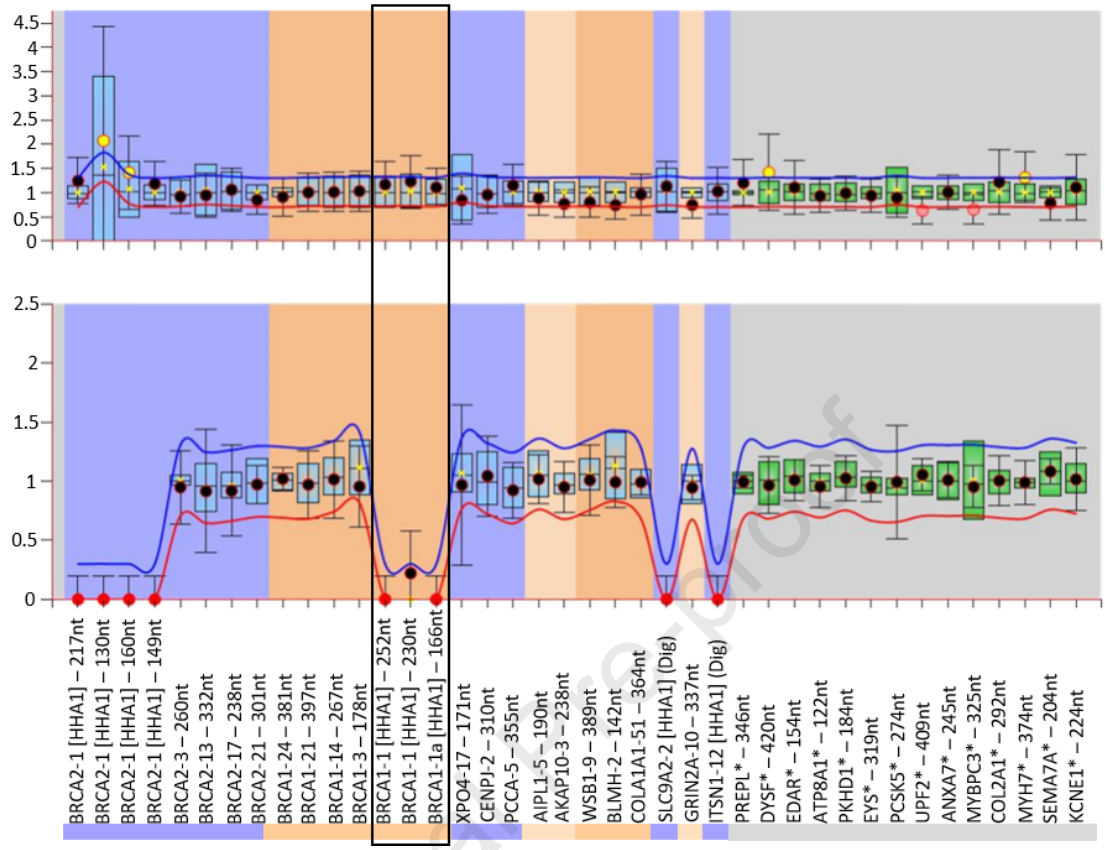

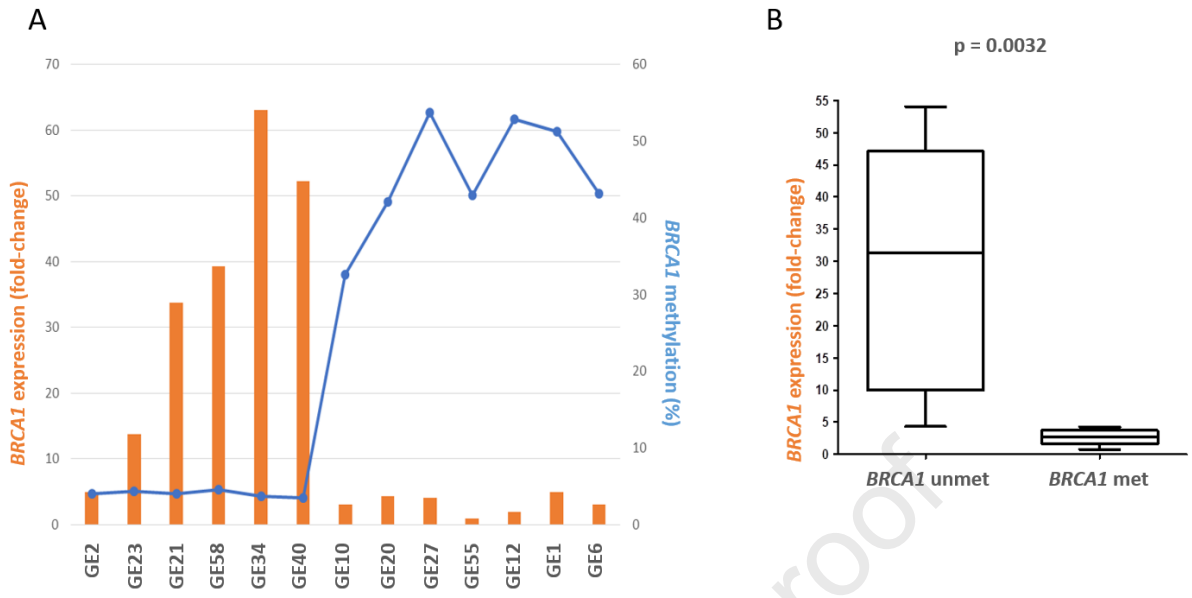

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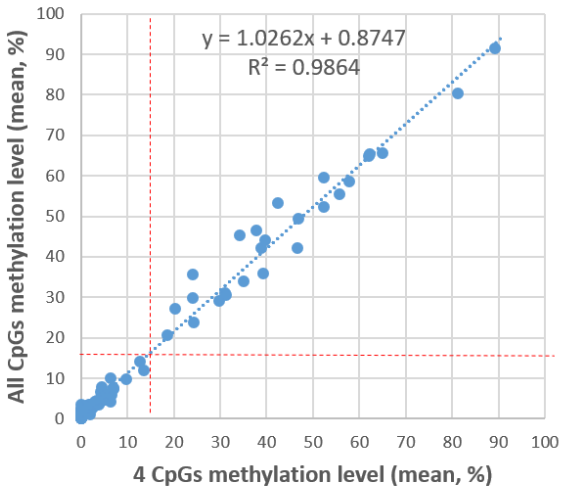

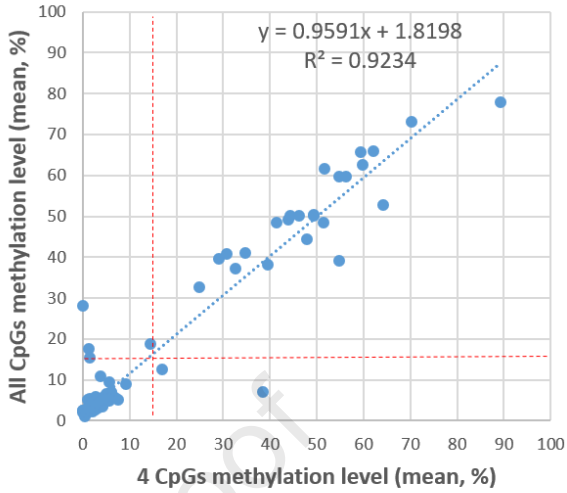

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