La metilazione virale nel triage e nel follow-up

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Disegno studio

- Nested in braccio sperimentale NTCC
- Campioni stoccati per tutte le donne HPV positive (HC2) . Momento inizio cambia tra centri
- Tutte le donne HPV+ inviate in colposcopia
- Se nessuna CIN2+ trovata in colposcopia follow-up finché donna HPV+
- Campioni HPV+ genotipizzati. Solo campioni con infezione singola da tipi oncogeni (IARC1) inclusi.
- Campioni presi al reclutamento testati per metilazione virale e collegati ad individuazione CIN a reclutamento o follow-up
- Ulteriori campioni presi a tempo vicino ad individuazione CIN usati per analisi supplementari

Molecular Methods

- Methylation status analysis by PCR and Pyrosequencing of bisulfite modified DNA

- Hybrid Capture 2 targeted types hrHPV 16,18,31,33,35,39,45,51,52,56,58,59[68 excluded]

- Focus on L1 e L2 → more frequently highlighted as methylated across studies
  → higher sequence homology among types
  → potential generation of PCR consensus primers

Sequence alignment tool Clustal Omega

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HPV16 TAGATAGTGGTTTTATTAGGTAGTTAAAGAGATATTTGAAAAAAAATATGGTAAAYGTTT  1828
HPV35 TAGATAGTGGTTTYGTTAGATYGTTATAGAGATATTTGTAAAAAAATATGGGATAYGTTT  1772
HPV31 TAGATAGTAGTTTYGTTAGGTYGTTATAGAGATATTTGTAAAAAAATATGATATAYGTTT  2034
HPV58 TATATAGTGGTTTTATTAGGTYGTTATAYGGATATTTGTAAAAAAATATGGAAAYGTTT  1762
HPV33 TATATTGTGGTTTTATTAGGTYGTTATAYGGATATTTGTAAAAAAATATGGAAAAYGTTT  1932    PCR PRIMER 1 (FORWARD)
HPV52 TATATAGTGGTTTTATTAGGTYGTTATAYGGATATTTGTAAAAAAATATGGAAAYGTTT  1876
HPV56 TATATTTTATTTTTATTAGGTYGTTAYGTYGTTATTTGTAAAAAAATAGGGAATAYGTTT  1748
         ** **   *   ***   **** *  ****      *  ****** ***********  *   *  *    ***

HPV16 AYG-----TYGTTTTYGTAATATGTA----ATAA---TTAGGATGTAAATAAAAGTTATTTG--ATT  1882    PCR PRIMER 2 (REVERSE)
HPV35 AYG-----AYGTYGTTTTAATAAATA-- ATAA---TTAGGGTGTAAATAAAAGTTATTTT--TATT  1826
HPV31 AYG-----TYGAYGTTTTAATATATA- - ATAA---TTAGGGTGTAAATAAAAATTATTTT--TATT  2088
HPV58 AYG-----TTTGYGAYGTAAAATAAA-- ATAA---TTAGGGTGTAATATAAAATTAGTAT-TATT  1816
HPV33 AYG-----TTTGYGAYGTAAAATAAA- -ATAA---TTAGGATGTAAATAAAGTTAGTAT-YGTT  1986
HPV52 AYG-----TTTGYGAYGTAGTAAAAA-- ATAA---TTAGGATGTAAATAAAATTTGTAT-TATT  1930
HPV56 AYGGYGTTTAYGTTTAAAAAAAATATATAGGTTATAATGTAAAGGAGGATTTTTGTATATA  1808
         ***                 *   *    *  * ***   ***    ******    *    ** *    *
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HPV16 sequence as reference

Y = CpG

Molecular Methods

PCR: HPV Types grouped in two families by phylogenetic affinity

> HPV 16, 31, 33, 35, 52, 56, 58
> HPV 18, 39, 45, 51, 59

CpG 5601,5606,5609,5616 L1 I   **consensus** primers   HPV 16, 31, 33, 35, 52, 58
consensus primers   HPV 39, 45, 51, 59
dedicated primers   HPV 18, 56

CpG 6457   **L1 II**   **consensus** primers   HPV 16, 31, 33, 35, 52, 58
consensus primers   HPV 18,51**and**HPV 39,45,59
dedicated primers   HPV 31

CpG 4261   **L2**   **no consensus** primers
dedicated primers   HPV 16,18,31,33,35,39,45,51,52,56,58,59

Methylation quantification → Pyrosequencing
- For each region separately
- Overall methylation score = sum of the 3 values

Study flow-chart

Women tested for viral methylation

	# women tested	Valid test			
		L1I	L1II	L2	OVERALL
No CIN detected at baseline no follow-up	5	4	3	2	2
No CIN detected at baseline nor follow-up	807	655	607	651	539
No CIN2+ at baseline, CIN1 at follow-up	58	55	52	53	49
No CIN2+ at baseline CIN2 at follow-up	31	27	26	27	24
No CIN2+ at baseline CIN3+ at follow-up	26	24	25	25	24
CIN1 at baseline	146	122	118	129	105
CIN2 at baseline	62	60	57	60	56
CIN3+ at baseline	65	64	64	64	63
Total	1200	1011	952	1011	862

Flow-chart:

- HPV POSITIVE **2498**
  - SAMPLE FOR GENOTYPING NOT AVAILABLE 100
  - SAMPLE FOR GENOTYPING AVAILABLE **2398**
    - COLPOSCOPY NOT DONE 143
    - COLPOSCOPY DONE **2255**
      - No IARC group 1 type present 594
      - IARC group 1 type present **1661***
        - MULTIPLE HPV INFECTIONS 433
        - SINGLE HPV INFECTIONS **1228**
          - SAMPLE NOT AVAILABLE 28
          - TESTED FOR VIRAL METHYLATION **1200**

*includes only types detected by Digene Genotyping RH Kit (Qiagen)

Baseline: ≤1 year from recruitment Follow-up: >1 – 3 years from recruitment

All histology of women with CIN at baseline and/or follow-up reviewed (morphology only) and each woman classified according to worse in each period

Methylation significantly decreases with increasing ln(RLU Ratio)
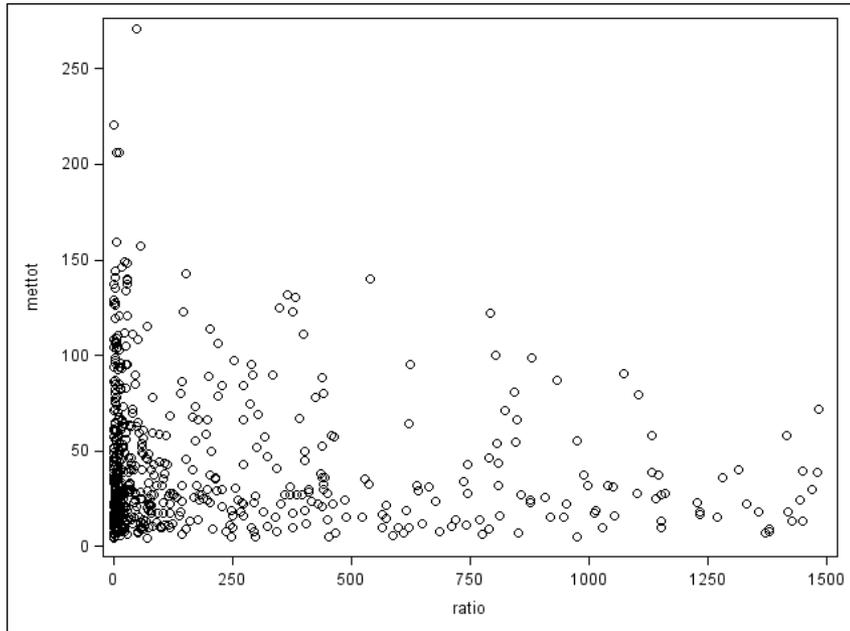
Methylation levels rapidly decrease with increasing viral load. Stable low levels above RLU ratio 200-250

Overall methylation in women with no CIN at baseline nor f.u.

- Beta for ln(RLU ratio) = -2.23 (p<0.0001)

  Type-adjusted

- Linear effect of viral load not significant:

  Beta for 100 RLU ratio = -0.022 (p=0.23)

  Type-adjusted

- "ratio normalized" methylation computed as methylation x ln(ratio)

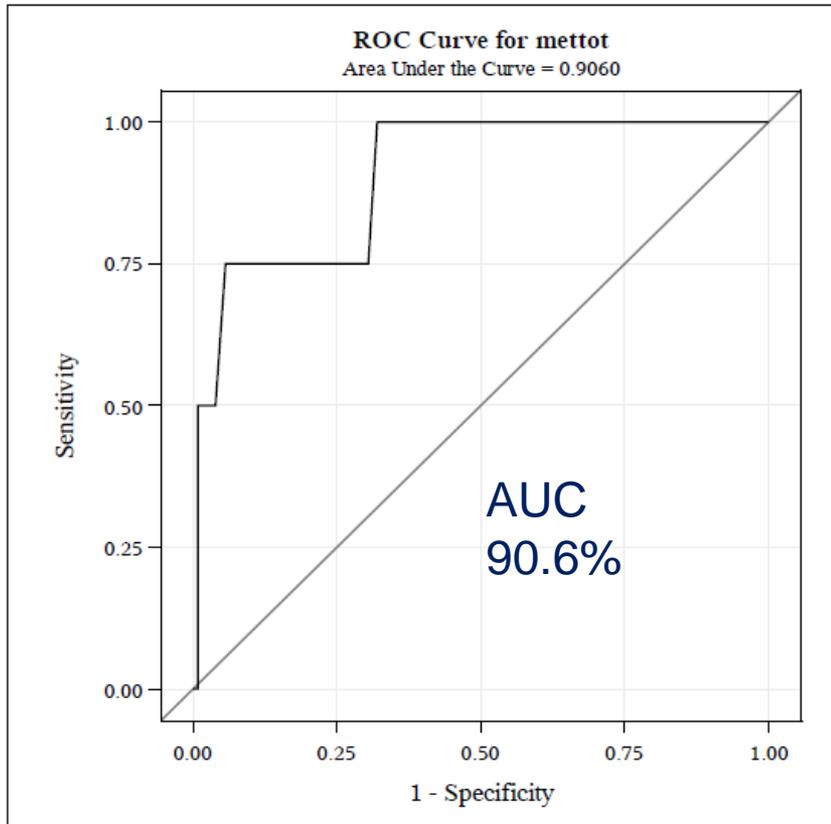# Methylation variability by HPV type among women with no CIN2+ detected at baseline nor at follow-up

genotype	Overall methylation			Ratio-normalised overall methylation		
	# women tested	Methylation score difference	p	# women tested	Methylation score difference	p
HPV16	166	reference		166	reference	
HPV31	130	5.8	0.0975	129	21.4	0.2062
HPV33	18	34.0	<.0001	18	59.1	0.0981
HPV35	20	-2.5	0.729	19	-10.8	0.7576
HPV52	39	22.5	<.0001	39	80.1	0.0018
HPV56	92	27.3	<.0001	91	116.2	<.0001
HPV58	53	26.4	<.0001	53	90.2	<.0001
HPV18	54	39.9	<.0001	54	146.9	<.0001
HPV45	53	62.9	<.0001	52	213.0	<.0001
HPV39	15	9.5	0.2378	15	90.5	0.0199
HPV51	39	1.3	0.806	37	27.3	0.2965
HPV59	17	0.4	0.9567	17	40.4	0.2703

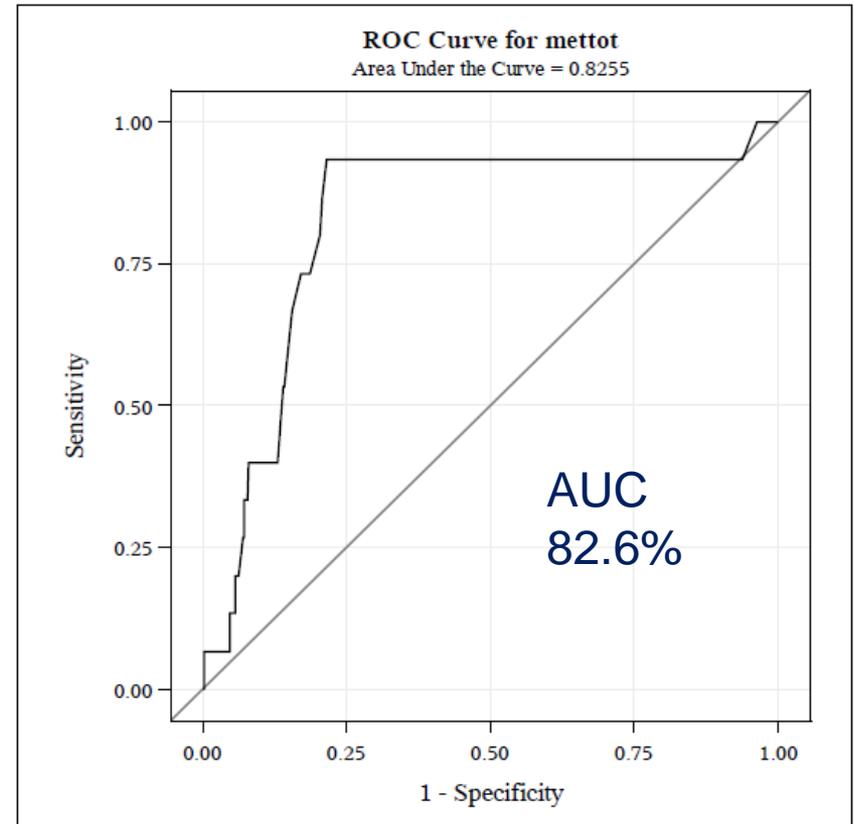# Cut-off values defined for groups of HPV types by ROC curves.
Overall methylation
## Endpoint: CIN3+ at follow-up vs. no CIN

HPV 33,18,45

ROC Curve for mettot
Area Under the Curve = 0.9060

AUC
90.6%

HPV 16,31,35,39,51,59

ROC Curve for mettot
Area Under the Curve = 0.8255

AUC
82.6%

HPV 52,56 and 58 excluded

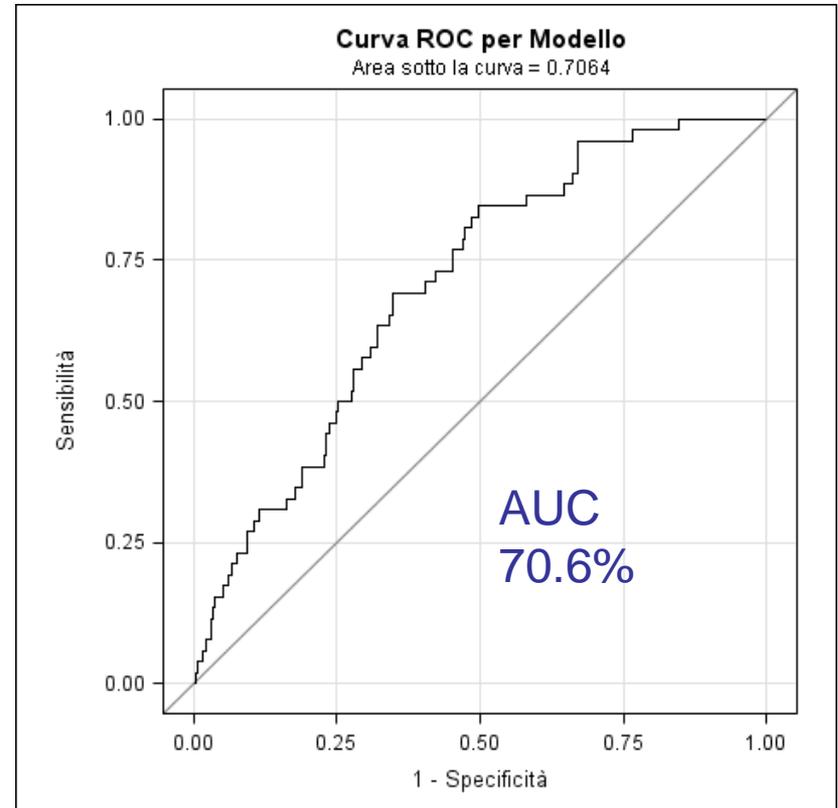| CUT-OFF	Overall	L1-I	L1-II	L2
HPV 33,18,45	90	30	30	30
HPV 16,31,35,39,51,59	30	10	10	10

Overall ratio-normalised methylation Endpoint: CIN3+ at baseline vs. no CIN
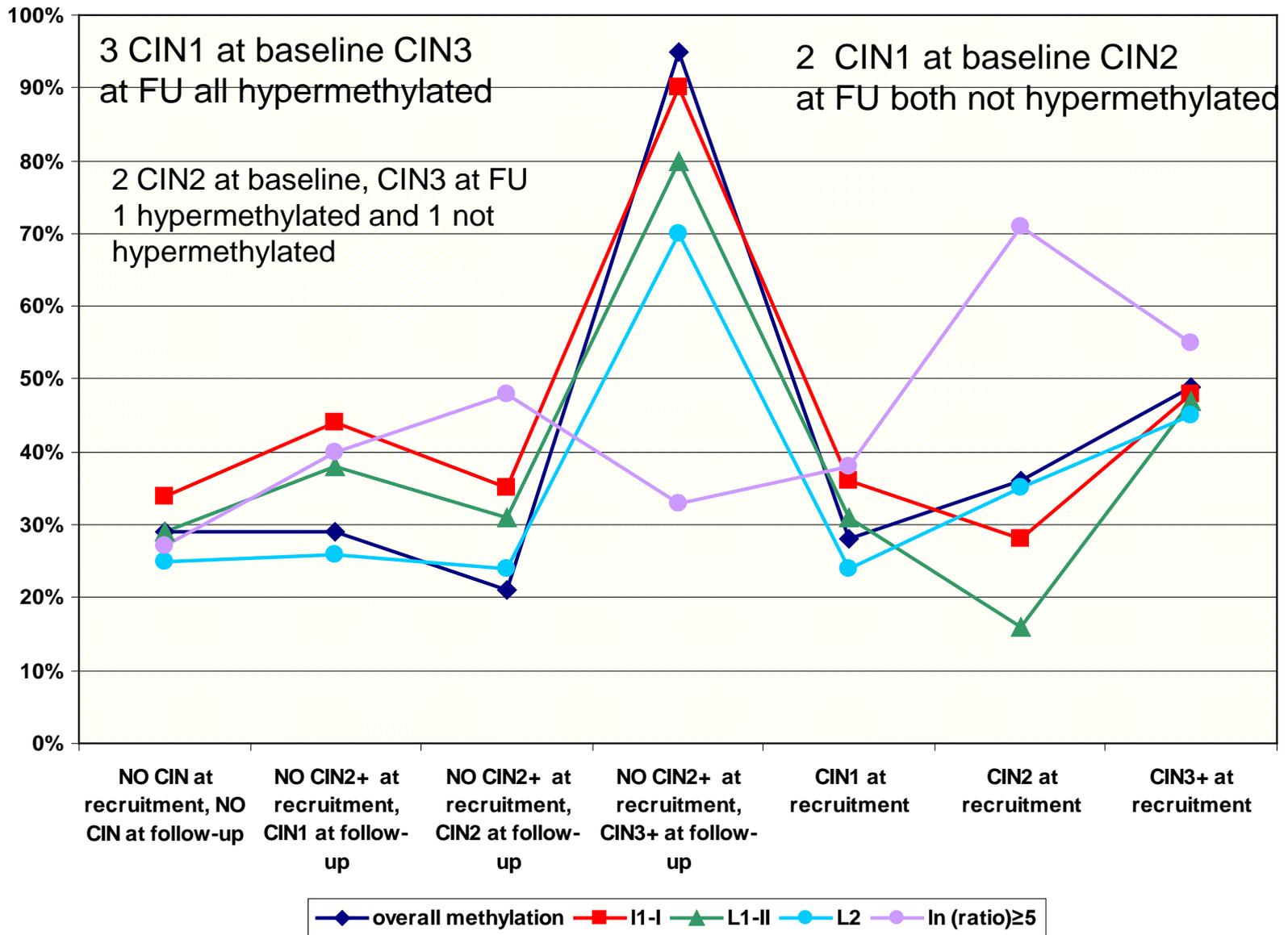
ROC curve for HPV 33,18,45
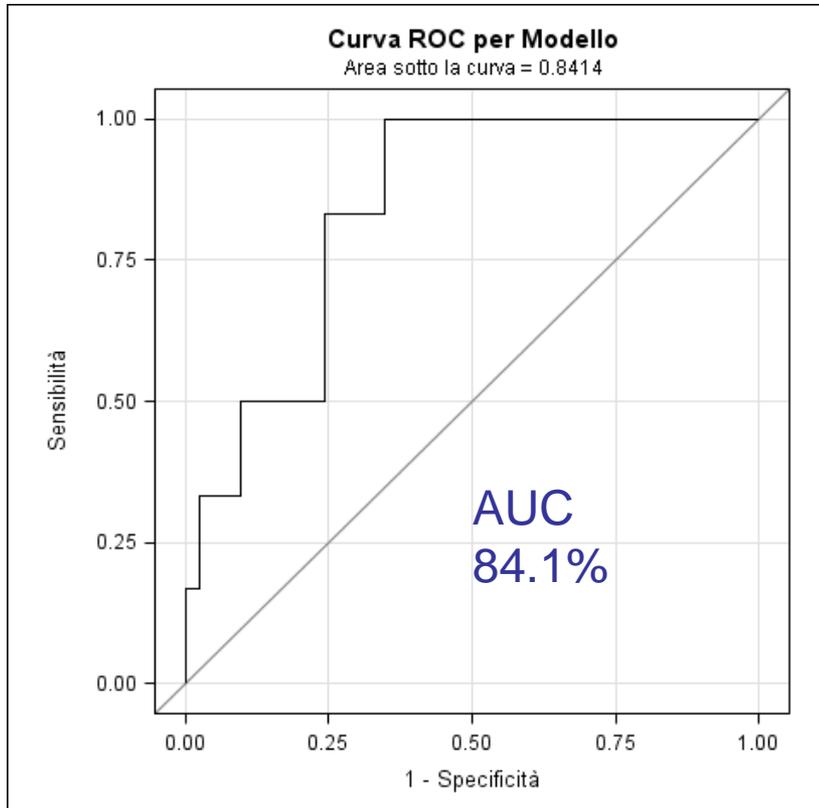
ROC curve for HPV 16,31,35,39,51,59

HPV 52,56 and 58 excluded

CUT-OFF	Overall	L1-I	L1-II	L2
HPV 33,18,45	280	93	93	93
HPV 16,31,35,39,51,59	50	17	17	17

% women positive for hypermethylation and high viral load at baseline by CIN subsequent or symultaneous detection and methylation region

3 CIN1 at baseline CIN3 at FU all hypermethylated

2 CIN1 at baseline CIN2 at FU both not hypermethylated

2 CIN2 at baseline, CIN3 at FU 1 hypermethylated and 1 not hypermethylated

Legend: overall methylation, l1-I, L1-II, L2, ln (ratio)≥5

X-axis categories: NO CIN at recruitment, NO CIN at follow-up; NO CIN2+ at recruitment, CIN1 at follow-up; NO CIN2+ at recruitment, CIN2 at follow-up; NO CIN2+ at recruitment, CIN3+ at follow-up; CIN1 at recruitment; CIN2 at recruitment; CIN3+ at recruitment

Odds Ratios for hypermethylation at baseline and CIN subsequent or symultaneous detection, by methylation region

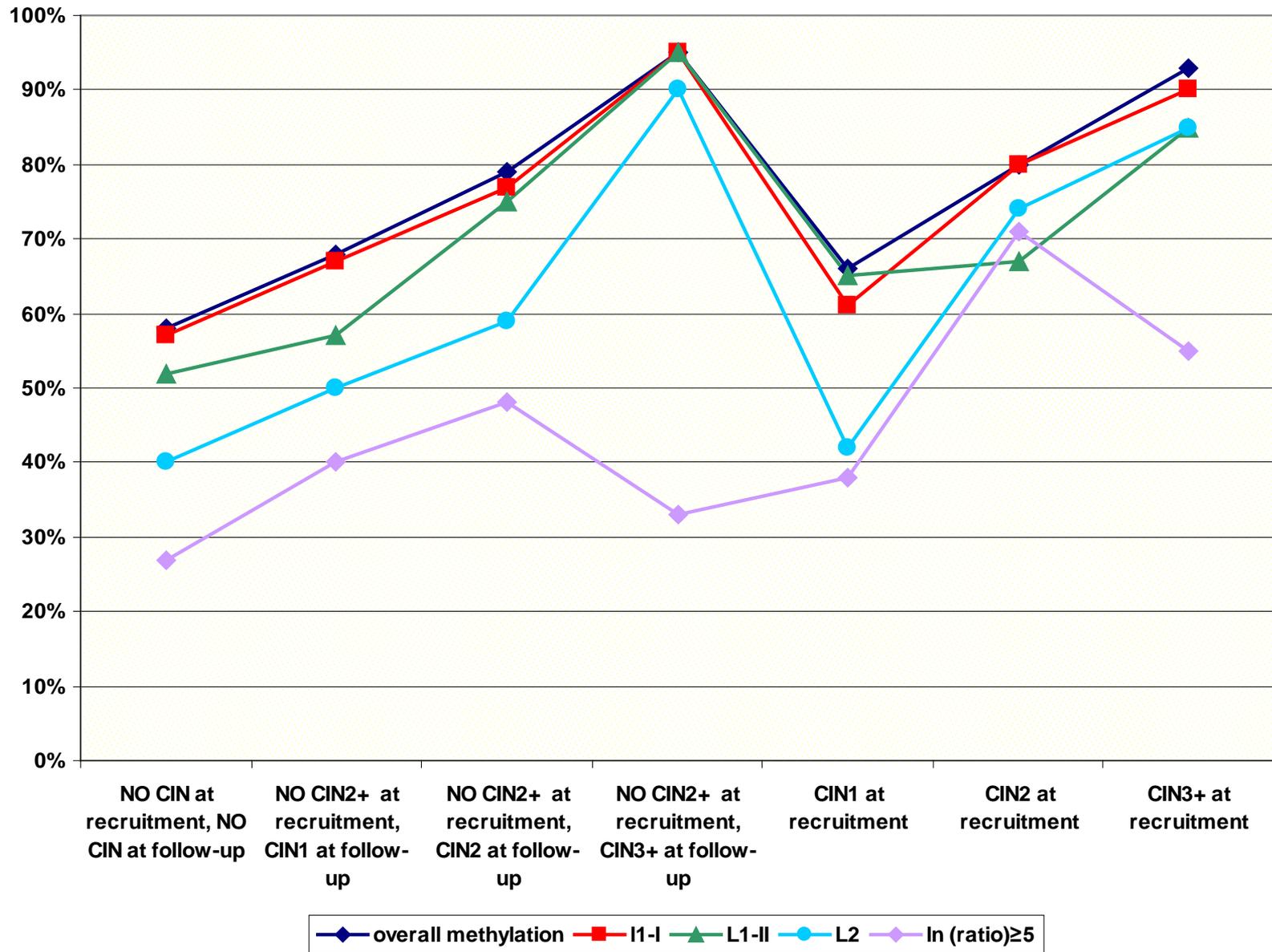|	CIN3+ F.U. VS. NO CIN	CIN3+ F.U. VS. CIN2 F.U.	CIN3+ BASELINE VS. NO CIN	CIN3+ BASELINE VS CIN2 BASELINE
Overall	**44.3** 5.9 - 335.0	**66.0** 6.1 - 716.2	**2.4** 1.4 - 4.1	**1.7** 0.8 - 3.7
L1-I	**16.2** 3.7 - 70.8	**15.6** 2.7 - 91.5	**1.8** 1.0 - 3.1	**2.4** 1.1 - 5.3
L1-II	**9.7** 3.2 - 29.6	**8.8** 1.9 - 40.3	**2.1** 1.2 - 3.7	**4.7** 1.9 - 11.7
L2	**6.9** 2.6 - 18.5	**7.6** 1.7 - 33.1	**2.4** 1.4 - 4.2	**1.5** 0.7 - 3.2
In (ratio) ≥5	**1.4** 0.5 - 3.4	**0.6** 0.2 - 1.9	**3.3** 1.9 - 5.7	**0.5** 0.2 - 1.1

% women positive for ratio-normalised hypermethylation and high viral load at baseline by CIN subsequent or symultaneous detection and methylation region

Legend: overall methylation, l1-I, L1-II, L2, ln (ratio)≥5

X-axis categories: NO CIN at recruitment, NO CIN at follow-up; NO CIN2+ at recruitment, CIN1 at follow-up; NO CIN2+ at recruitment, CIN2 at follow-up; NO CIN2+ at recruitment, CIN3+ at follow-up; CIN1 at recruitment; CIN2 at recruitment; CIN3+ at recruitment

OR for viral load normalised hypermethylation at baseline and CIN subsequent or symultaneous detection by methylation region

	CIN3+ F.U. VS. NO CIN	CIN3+ F.U. VS. CIN2 F.U.	CIN3+ BASELINE VS. NO CIN	CIN3+ BASELINE VS. CIN2 BASELINE
OVERALL	**13.3** 1.8 - 100.5	**4.9** 0.5 - 53.3	**10.0** 3.5 - 28.1	**3.4** 1.0 - 11.5
L1-I	**13.3** 1.8 - 100.7	**5.5** 0.6 - 55.5	**6.6** 2.8 - 15.5	**2.3** 0.8 - 6.6
L1-II	**17.4** 2.3 - 130.7	**6.3** 0.6 - 63.6	**5.1** 2.4 - 10.6	**2.8** 1.1 – 7.0
L2	**13.5** 3.1 - 58.9	**6.3** 1.1 - 36.6	**8.3** 4.0 - 17.4	**1.9** 0.8 – 5.0
ln (ratio) ≥5	**1.4** 0.5 - 3.4	**0.6** 0.2 - 1.9	**3.3** 1.9 - 5.7	**0.5** 0.2 - 1.1

Methylation in samples taken close to CIN detection at baseline or during follow-up

	CIN 2		CIN 3+	
	Hyper methylated	Not Hyper methylated	Hyper methylated	Not Hyper methylated
Overall Methylation				
CIN detected at baseline	24 **42.9%**	32 57.1%	33 **52.4%**	30 47.6%
CIN detected at follow up, sample taken close to CIN detection	4 **36.4%**	7 63.6%	10 **62.5%**	6 37.5%
Overall ratio-normalised methylation				
CIN detected at baseline	45 **80.4%**	11 19.6%	57 **91.9%**	5 8.1%
CIN detected at follow up, sample taken close to CIN detection	9 **81.8%**	2 18.2%	15 **93.8%**	1 6.3%

P16 and viral methylation at baseline in women who will have CIN2 or CIN3 detected during follow-up

CIN2+ at followup				
		hypermetilation at baseline		
		no	yes	tot
P16 baseline	no	3	3	6
	yes	5	2	7
tot		8	5	13

McNemar p=0.4795

McNemar p=0.0455

CIN3+ at followup				
		hypermetilation at baseline		
		no	yes	tot
P16 baseline	no	1	4	5
	yes	0	9	9
tot		1	13	14

Women recruited in phase 2 of NTCC

Discussione/Conclusioni

- Analizzate cellule desquamate in gran parte non provenienti da lesioni

- Metilazione virale funzione esponenziale negativa del carico virale. Coerente con incapacità cellule di metilare tutte le copie virali sopra soglia data.

- Quasi tutte le donne HPV+ con CIN3+ trovato nei 3aa successivi ipermetilate e con basso carico virale

- Tra le donne a cui verrà trovata CIN1 o CIN2 in futuro % iper-metlate simile a quella nelle donne HPV+ a cui non verranno trovate CIN (controlli).Più future CIN2 che controlli hanno alto carico virale.

- Ipermetilazione virale (e basso carico virale) parrebbero marker molto precoce di infezioni destinate a sviluppare CIN3+

- Tra le donne che già avevano una CIN3+ proporzione ipermetilate molto più bassa (e carico virale aumentato.). Metilazione ratio-normalised ha buona sensibilità trasversaòe per CIN3+.

- Coerente con carico virale che segue trend temporale esponenziale nelle infezioni trasformanti (*Depuydt, Cancer Med 2015*). Però sia carico virale che sovra-espressione p16 predicono le future CIN3+ peggio che metilazione virale (p16 non specifica per CIN3+ vs. CIN2).

- Ipermetilazione virale non è marker di CIN3+ recente. Campioni presi vicino a diagnosi CIN3+ trovate al f.u. (comparse di recente) non differenti da quelli presi vicino a CIN3+ trovate al reclutamento (possibilmente di lunga data)

- % di donne attualmente con CIN2 ipermetilate intermedia tra quelle con attualmente CIN3+ e CIN1. CIN2 future intermedie tra future CIN1 e CIN3+ se si considera "ratio-normalised" methylation (cioè tenendo conto della diluizione dovuta al carico virale). Perché trend temporale particolare carico virale per CIN2?

RR hypermethylation ratio ≥1,5 vs ratio <1,5	0,98	0,86	-	-	1,02	0,00	0,48
	97/335 vs 18/61	8/28 vs 2/6	3/14 vs 0/0	18/18 vs 0/1	19/68 vs 3/11	0/32 vs 18/18	27/56 vs 2/2

Legend: overall methylation — overall methylation weighted — RR