THE LANCET Oncology

Supplementary appendix

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Sankaranarayanan R, Prabhu PR, Pawlita M, et al, for the Indian HPV vaccine study group. Immunogenicity and HPV infection after one, two, and three doses of quadrivalent HPV vaccine in girls in India: a multicentre prospective cohort study. *Lancet Oncol* 2015; published online Dec 1. http://dx.doi.org/10.1016/S1470-2045(15)00414-3.

Appendix

Supplementary table 1. Mean MFI values of HPV 16, 18, 6 and 11 L1 antibodies on day 1 and at months 7, 18, 36 and 48 after the first dose among girls who received vaccination per protocol, and at months 12, 18 and 36 after the first dose among girls who did not have their eir complete vaccine schedules by default

Vaccine doses received	No. samples	Geometric mean MFI (95% CI)	MFI ratio (95% CI)* (alternate dose/standard 3-dose at days 1, 60, 180)	No. (%) with MFI ≥ sero-conversion levels**	No. (%) with MFI ≥ than the lowest in standard 3-dose at that time point[$]
		Day 1			
HPV 16 L1					
3-dose group	1000	11 (10 - 12)	1·00	46 (4·6)	
2-dose group	937	9 (8 - 10)	0·86 (0·74 - 0·99)	52 (5·5)	
HPV 18 L1					
3-dose group	1000	6 (5 - 7)	1·00	41 (4·1)	
2-dose group	937	5 (4 - 5)	0·81 (0·72 - 0·92)	63 (6·7)	
HPV 6 L1					
3-dose group	1000	24 (22 - 26)	1·00	51 (5·1)	
2-dose group	937	26 (24 - 29)	1·10 (0·97 - 1·25)	44 (4·7)	
HPV 11 L1					
3-dose group	1000	6 (6 - 7)	1·00	56 (5·6)	
2-dose group	937	7 (6 - 7)	1·03 (0·91 - 1·15)	43 (4·6)	
		Month 7			
HPV 16 L1					

3-dose (days 1, 60, 180)	308	5460	(5195 | - | 5738)	1·00			308	(100·0)	308	(100·0)
2-dose (days 1, 180)	317	6125	(5785	-	6485)	1·12	(1·02	- 1·23)	316	(99·7)	315	(99·4)
HPV 18 L1												
3-dose (days 1, 60, 180)	308	2942	(2733 | - | 3167)	1·00			308	(100·0)	308	(100·0)
2-dose (days 1, 180)	317	3068	(2812	-	3347)	1·04	(0·92	- 1·19)	317	(100·0)	313	(98·7)
HPV 6 L1												
3-dose (days 1, 60, 180)	308	4715	(4484 | - | 4957)	1·00			308	(100·0)	308	(100·0)
2-dose (days 1, 180)	317	4922	(4675	-	5182)	1·04	(0·97	- 1·13)	317	(100·0)	316	(99·7)
HPV 11 L1												
3-dose (days 1, 60, 180)	308	6163	(5909 | - | 6427)	1·00			308	(100·0)	308	(100·0)
2-dose (days 1, 180)	317	6905	(6622	-	7200)	1·12	(1·06	- 1·19)	317	(100·0)	317	(100·0)
Month 12												
HPV 16 L1												
2 doses (days 1, 60)	471	437	(398 | - | 480)	1·00			430	(91·3)		
A single dose	528	106	(96	-	116)	0·24	(0·21	- 0·28)	260	(49·2)		
HPV 18 L1												
2 doses (days 1, 60)	471	233	(214 | - | 254)	1·00			449	(95·3)		
A single dose	528	50	(45	-	55)	0·21	(0·19	- 0·24)	304	(57·6)		
HPV 6 L1												
2 doses (days 1, 60)	471	509	(469 | - | 552)	1·00			373	(79·2)		
A single dose	528	167	(153	-	183)	0·33	(0·29	- 0·37)	192	(36·4)		
HPV 11 L1												
2 doses (days 1, 60)	471	610	(562 | - | 662)	1·00			469	(99·6)		
A single dose	528	163	(149	-	179)	0·27	(0·24	- 0·30)	466	(88·3)		

Month 18											
HPV 16 L1											
3-dose (days 1, 60, 180)	313	1209	(1105	-	1323)	1·00			311	(99·4)	313	(100·0)
2-dose (days 1, 180)	314	1222	(1116	-	1338)	1·01	(0·87	- 1·18)	312	(99·4)	312	(99·4)
2 doses (days 1, 60)	449	401	(366	-	440)	0·33	(0·29	- 0·38)	411	(91·5)	417	(92·9)
A single dose	476	113	(102	-	126)	0·09	(0·08	- 0·11)	255	(53·6)	273	(57·4)
HPV 18 L1											
3-dose (days 1, 60, 180)	313	377	(337	-	422)	1·00			307	(98·1)	313	(100·0)
2-dose (days 1, 180)	314	269	(241	-	299)	0·71	(0·60	- 0·84)	305	(97·1)	313	(99·7)
2 doses (days 1, 60)	449	192	(176	-	209)	0·51	(0·43	- 0·59)	426	(94·9)	443	(98·7)
A single dose	476	46	(40	-	51)	0·12	(0·10	- 0·14)	259	(54·4)	347	(72·9)
HPV 6 L1											
3-dose (days 1, 60, 180)	313	986	(900	-	1080)	1·00			296	(94·6)	313	(100·0)
2-dose (days 1, 180)	314	830	(756	-	911)	0·84	(0·73	- 0·97)	292	(93·0)	313	(99·7)
2 doses (days 1, 60)	449	476	(440	-	514)	0·48	(0·42	- 0·55)	357	(79·5)	431	(96·0)
A single dose	476	169	(154	-	186)	0·17	(0·15	- 0·20)	182	(38·2)	332	(69·7)
HPV 11 L1											
3-dose (days 1, 60, 180)	313	1327	(1216	-	1449)	1·00			313	(100·0)	313	(100·0)
2-dose (days 1, 180)	314	1328	(1223	-	1443)	1·00	(0·87	- 1·15)	314	(100·0)	314	(100·0)
2 doses (days 1, 60)	449	530	(489	-	574)	0·40	(0·35	- 0·45)	449	(100·0)	449	(100·0)
A single dose	476	164	(148	-	180)	0·12	(0·11	- 0·14)	427	(89·7)	388	(81·5)
Month 36											
HPV 16 L1											
3-dose (days 1, 60, 180)	271	221	(197	-	247)	1·00			225	(83·0)	271	(100·0)

2-dose (days 1, 180)	278	163	(147 - 181)	0·74	(0·63 - 0·87)	197	(70·9)	278	(100·0)
2 doses (days 1, 60)	513	136	(126 - 147)	0·62	(0·54 - 0·71)	324	(63·2)	511	(99·6)
A single dose	510	72	(66 - 78)	0·33	(0·28 - 0·37)	166	(32·5)	500	(98·0)
HPV 18 L1									
3-dose (days 1, 60, 180)	271	184	(162 - 208)	1·00		249	(91·9)	271	(100·0)
2-dose (days 1, 180)	278	117	(104 - 132)	0·64	(0·53 - 0·76)	238	(85·6)	277	(99·6)
2 doses (days 1, 60)	513	101	(93 - 109)	0·55	(0·47 - 0·64)	423	(82·5)	510	(99·4)
A single dose	510	45	(41 - 49)	0·25	(0·21 - 0·29)	271	(53·1)	485	(95·1)
HPV 6 L1									
3-dose (days 1, 60, 180)	271	623	(556 - 699)	1·00		227	(83·8)	271	(100·0)
2-dose (days 1, 180)	278	472	(420 - 530)	0·76	(0·64 - 0·89)	210	(75·5)	278	(100·0)
2 doses (days 1, 60)	513	287	(265 - 312)	0·46	(0·40 - 0·53)	306	(59·6)	511	(99·6)
A single dose	510	131	(120 - 143)	0·21	(0·18 - 0·24)	138	(27·1)	490	(96·1)
HPV 11 L1									
3-dose (days 1, 60, 180)	271	683	(609 - 765)	1·00		268	(98·9)	271	(100·0)
2-dose (days 1, 180)	278	653	(585 - 729)	0·96	(0·82 - 1·12)	277	(99·6)	278	(100·0)
2 doses (days 1, 60)	513	265	(243 - 288)	0·39	(0·34 - 0·45)	490	(95·5)	507	(98·8)
A single dose	510	122	(111 - 135)	0·18	(0·16 - 0·21)	407	(79·8)	462	(90·6)
Month 48									
HPV 16 L1									
3-dose (days 1, 60, 180)	89	218	(181 - 262)	1·00		69	(77·5)	89	(100·0)
2-dose (days 1, 180)	127	183	(160 - 209)	0·84	(0·67 - 1·04)	101	(79·5)	124	(97·6)
HPV 18 L1									
3-dose (days 1, 60, 180)	89	206	(165 - 257)	1·00		82	(92·1)	89	(100·0)

2-dose (days 1, 180)	127	129	(111	-	151)	0·63	(0·49	-	0·82)	118	(92·9)	124	(97·6)
HPV 6 L1													
3-dose (days 1, 60, 180)	89	659	(551	-	789)	1·00				77	(86·5)	89	(100·0)
2-dose (days 1, 180)	127	512	(439	-	596)	0·78	(0·61	-	0·98)	100	(78·7)	127	(100·0)
HPV 11 L1													
3-dose (days 1, 60, 180)	89	762	(637	-	911)	1·00				89	(100·0)	89	(100·0)
2-dose (days 1, 180)	127	690	(594	-	801)	0·91	(0·72	-	1·14)	127	(100·0)	125	(98·4)

MFI: median fluorescence intensities; CI: confidence interval; HPV: human papilloma virus; * Other dose schedules were non-inferior to the 3-dose schedule for month 7, 18, 36 and 48 or to the incomplete 2 doses (Day 1, 60) for month 12 if the lower bound of the 95% CI for the MFI ratio was above 0·5 (2 times difference); ** The MFI sero-conversion levels for HPV 16, 18, 6 and 11 L1antibodies were 240, 48, 100 and 41 respectively; [$] The respective lowest MFI levels for HPV 16, 18, 6 and 11 L1 antibodies among the girls who received the standard 3 doses (Day 1, 60, 180) were 1011, 271, 521 and 1244 at 7 months, 90, 25, 100 and 61 at 18 months, 12, 11, 24 and 30 at 36, and 43, 20, 58 and 92 at 48 months after first dose.

Supplementary table 2. Geometric mean avidity index of MFI for HPV 16, 18, 6 and 11 L1 antibodies at 7 and 18 months after the first dose among girls who received vaccination per protocol, and those who did not have their complete vaccine schedules

HPV type and dose Received	No. samples	Geometric mean 0M MFI	Geometric mean 5M MFI	Geometric mean avidity index* (%, 95% CI)			Avidity index ratio (95% CI) Alternate dose/3-dose[$]	
Month 7								
HPV 16 L1								
3-dose (days 1, 60, 180)	97	1063	749	70	(68	- 73)	1·00	
2-dose (days 1, 180)	99	1758	1147	65	(62	- 68)	0·93	(0·83 - 1·04)
HPV 18 L1								
3-dose (days 1, 60, 180)	97	660	540	82	(79	- 85)	1·00	
2-dose (days 1, 180)	99	907	724	80	(75	- 85)	0·97	(0·87 - 1·09)
HPV 6 L1								
3-dose (days 1, 60, 180)	97	1590	1230	77	(74	- 81)	1·00	
2-dose (days 1, 180)	99	2216	1629	74	(70	- 78)	0·95	(0·84 - 1·07)
HPV 11 L1								
3-dose (days 1, 60, 180)	97	1975	1694	86	(82	- 90)	1·00	
2-dose (days 1, 180)	99	2867	2306	80	(76	- 85)	0·94	(0·83 - 1·06)
Month 18								
HPV 16 L1								
3-dose (days 1, 60, 180)	136	287	193	67	(64	- 71)	1·00	
2-dose (days 1, 180)	139	335	221	66	(63	- 70)	0·98	(0·90 - 1·07)
2 doses (days 1, 60)	142	158	109	69	(66	- 73)	1·03	(0·95 - 1·12)
A single dose	130	46	34	74	(68	- 80)	1·10	(1·01 - 1·19)

HPV 18 L1								
3-dose (days 1, 60, 180)	136	114	87	76	(73 - 81)	1·00		
2-dose (days 1, 180)	139	101	76	75	(71 - 80)	0·98	(0·90 - 1·08)	
2 doses (days 1, 60)	142	68	52	76	(73 - 81)	1·00	(0·91 - 1·10)	
A single dose	130	20	17	85	(77 - 93)	1·11	(1·01 - 1·22)	
HPV 6 L1								
3-dose (days 1, 60, 180)	136	294	205	70	(66 - 74)	1·00		
2-dose (days 1, 180)	139	291	199	68	(64 - 73)	0·98	(0·89 - 1·09)	
2 doses (days 1, 60)	142	160	112	70	(66 - 75)	1·01	(0·92 - 1·12)	
A single dose	130	74	48	65	(59 - 72)	0·94	(0·85 - 1·04)	
HPV 11 L1								
3-dose (days 1, 60, 180)	136	391	331	85	(81 - 89)	1·00		
2-dose (days 1, 180)	139	402	339	84	(80 - 89)	0·99	(0·91 - 1·09)	
2 doses (days 1, 60)	142	188	154	82	(78 - 86)	0·97	(0·89 - 1·06)	
A single dose	130	71	63	88	(80 - 97)	1·04	(0·95 - 1·14)	

MFI: median flow intensities; CI: confidence interval; HPV: human papilloma virus; * Proportion obtained from dividing treated MFI (at 5M) by untreated MFI (at 0M) and then multiplied by 100; CI: confidence interval;

[$] Other dose schedules were non-inferior to the 3-dose standard schedule (Days 1, 60 and 180) if the lower bound of the 95% CI for the avidity index ratio was above 0·5 (2 times difference)

Supplementary table 3. Geometric mean neutralization titres of HPV 16, 18 and 6 L1 antibodies at 18 months after first dose among girls who received vaccination per protocol, and those who did not have their complete vaccine schedules

HPV type	Samples tested	Samples with neutralization titres (%)	Geometric mean neutralization titres (95% CI)	Geometric mean ratio (95% CI)* Alternate dose/3-dose
HPV 16 L1				
3-dose (Day 1, 60, 180)	60	60 (100·0)	9906 (7552 - 12995)	1·00
2-dose (Day 1, 180)	59	59 (100·0)	9893 (7754 - 12621)	1·00 (0·69 - 1·45)
2 doses (Day 1, 60)	60	60 (100·0)	2311 (1773 - 3011)	0·23 (0·16 - 0·34)
A single dose	58	56 (96·6)	558 (416 - 750)	0·06 (0·04 - 0·08)
HPV 18 L1				
3-dose (Day 1, 60, 180)	60	59 (98·3)	1951 (1403 - 2713)	1·00
2-dose (Day 1, 180)	59	59 (100·0)	819 (573 - 1170)	0·42 (0·27 - 0·65)
2 doses (Day 1, 60)	60	57 (95·0)	431 (321 - 578)	0·22 (0·14 - 0·34)
A single dose	58	39 (67·2)	156 (113 - 216)	0·08 (0·05 - 0·13)
HPV 6 L1				
3-dose (Day 1, 60, 180)	60	60 (100·0)	13936 (9623 - 20181)	1·00
2-dose (Day 1, 180)	59	59 (100·0)	10867 (7908 - 14934)	0·78 (0·50 - 1·21)
2 doses (Day 1, 60)	60	60 (100·0)	3126 (2365 - 4131)	0·22 (0·15 - 0·35)
A single dose	58	57 (98·3)	787 (592 - 1046)	0·06 (0·04 - 0·09)

CI: confidence interval; HPV: human papilloma virus; * Other dose schedules were non-inferior to the 3-dose schedule if the lower bound of the 95% CI for the MFI ratio was above 0·5 (2 times difference)

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List of PIs and Number Recruited

Site	Name of the PI	Number of enrolled girls	Number of recruited girls
Barshi	Dr. Sylla G. Malvi	8335	7092
Ambilikkai	Dr. Pulikottil O. Esmy	3499	3300
Pune	Dr. Smita Joshi	3720	3018
Ahmedabad	Dr Parimal Jivarajani (until Aug. 8, 2014) Dr Geeta Joshi (from Aug. 9, 2014)	1022	1011
Delhi	Dr Neerja Bhatla	1609	1000
Hyderabad	Dr Usha Rani Reddy Poli	1168	794
Mumbai	Dr Surendra S. Shastri	800	514
Mizoram	Dr Eric Zomawia	545	500
Sikkim	Dr Yogesh Verma	560	500

Detailed procedures

At each study site, dedicated health workers and nurses were recruited and trained to enumerate and interview the eligible girls for socio-demographic and reproductive information, explain the study to the participating girls and their parents, obtain informed consent, administer the vaccines, document adverse events, and carry out follow-up procedures including collection of blood and cervical cell samples. The study was explained to a number of medical practitioners in the study clusters to make them aware of how to manage and/or refer the vaccinated girls if any reported to them with suspected adverse events. A 24-hour committed telephone line was provided to the participants to contact a study clinician in case any need for urgent medical attention arose.

Sample size calculation:

The sample size for the study was calculated to allow observation of 16 cases per group of high-grade cervical intraepithelial neoplasia (CIN 2-3) related to HPV 16/18 infection by 60 months. The required sample size was determined under the following assumptions:

1. The average cluster size of 100 girls

2. Annual drop-out rate of 5%

3. For the intention-to-treat analysis, cumulative incidence rate of 1.3/10,000 in the three-dose group of CIN 2-3 lesions caused by HPV 16 and 18

4. The two doses of vaccine would be considered non-inferior to three doses if the increase in incidence of HPV 16/18-related CIN 2/3 or worse is less than three times the incidence within the three-dose group (i.e.<5.4/10,000). Under assumptions about the incidence in an unvaccinated population[13] with incidence of 40.7/10,000, this corresponds to a drop in absolute vaccine efficacy of less than 10 percentage points

5. A power of 80% and a one-sided 95% confidence (α=0.05)

6. Supposing that the cluster rates are log-normally distributed and that 95% of the clusters will have a true rate within a factor of 2 of the mean rate, using the incidence rate in the

unvaccinated population, the estimated intracluster correlation coefficient would be 0.0008.

7. Clusters of varying sizes with a coefficient of variation of 0.05

Under these assumptions, approximately 120 clusters and 12,000 girls (60 clusters and 6,000 girls per vaccination group) would be required.

However, a subgroup analysis in which the effect of the two- versus the three-dose will be compared in the age at entry categories 10-12, 12-15 and 16-18, is planned. Using Bonferroni adjustment,[20-21] i.e. taking α=1.67% (5%/3) as the level of significance for each of the subgroup analysis in order to be able to remain with a 5% level of significance for the overall effect, approximately 164 clusters and 16,400 girls (82 clusters and 8,200 girls per vaccination group) would be required for the subgroup analysis. This was calculated to allow observation of 22 CIN 2-3 cases per group related to HPV types 16 and 18 by 60 months.

Recruitment of the girls into the 2-dose and 3-dose groups and vaccination of eligible girls was initiated on 9 July 2009 and progressed satisfactorily until 8 April 2010, with more than 95% participation of the invited girls for vaccination, when the Indian authorities suspended all further recruitment and vaccination of subjects in all HPV vaccination trials in India, due to events unrelated to our study.

The suspension occasioned our having four groups of vaccinated girls: on days 1, 60 and 180 or more (three-dose group); on days one and 180 or more (two-dose group); on days one and 60 by default (two-dose/D group); and one dose only by default (one-dose/D group) (Figure 1). We continue to follow-up these cohorts of girls with different doses to evaluate the outcomes in terms of immunogenicity, the frequency of HPV infection and cervical neoplasia.

Each participant is visited annually in her household and her health, well-being and vital status enquired into. Details regarding marriage, medically significant events, pregnancy, ante- and post-natal events, delivery and migration are collected and documented through a network of social workers, medical care providers, hospitals and relatives. Appointments are fixed for blood

and cervical cell collection at designated periods.

To assess the sero-conversion, immunogenicity, antibody levels and durability of the immune response, blood samples were collected at baseline, 7, 12, 18, 24, 36, 48 and 60 months from a sample of the study population (Figure 1).

A sub-set of EDTA plasma at baseline, 7, 12, 18, 24, 36 and 48 months from a cohort of vaccinated girls have been analyzed using luminex-based Multiplex serology[8, 9] to assess HPV-L1 binding antibodies against the major capsid protein L1 of vaccine types HPV 16, 18, 6 and 11 at the Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, India, where a dedicated laboratory was established with technology-transfer and external quality assurance from the German Cancer Research Center (DKFZ), Heidelberg, Germany. Scientists from RGCB were trained at the DKFZ, Heidelberg, and the multiplex serology HPV-L1 antibody assays were carried out at RGCB by trained personnel, blinded to the study groups, under the technical supervision of experts from DKFZ. Briefly, fluorescence-coded bead sets (3000 beads per set per well) carrying different recombinant, affinity-purified HPV-L1 antigens were mixed and incubated with plasma diluted to 1:1000 in 96-well plates. After incubation and washing, biotinylated goat anti-human immunoglobulin antibody and subsequently the fluorescent reporter conjugate streptavidin-R-phycoerythrin were added and the reporter fluorescence of the beads was determined with a Luminex analyzer (Bioplex 200) and expressed as the median fluorescence intensity (MFI) of at least 100 beads per set per well. Final antigen specific net MFI values were generated by subtraction of GST-tag and individual bead background values. MFI as measure of antibody reactivities quantified by HPV multiplex serology are directly comparable to optical densities measured by enzyme-linked immunosorbent assay (ELISA)[8] and MFI values for HPV16 in natural and vaccine-induced HPV16-specific antibody responses are strongly correlated with end-point titration titer in neutralization assay[9] Sero-positivity cut-offs were calculated for each HPV type, based on the MFI values of serum samples obtained from the participants at baseline. The cut-off values were defined after allowing for 5% sero-positivity

among the total base-line samples. The immunogenicity measure was the geometric mean of MFI.[8, 9]

Antibody avidity, which reflects the degree of antibody affinity maturation, was measured in a modification of the HPV-L1 genotype specific binding antibody assay described above. After the first washing step the beads with the antigen-antibody complexes were incubated for 15 minutes with the chaotropic agent urea at 5 M concentration or washing buffer alone and then the standard washing procedure continued. Under this high urea concentration low-avidity antibodies can detach from the antigen and are subsequently washed away which results in lower MFI values as compared to the incubation with buffer alone.

Neutralizing antibodies specific for neutralizing-epitopes in HPV-L1 protein were measured using a highly sensitive, automated, high-throughput pseudovirion-based neutralization assay (PBNA) with excellent repeatability and run-to-run reproducibility.[9] Bovine papillomavirus (BPV) pseudovirion assays were run as control to verify that the test serum is not toxic to the cells, which can mimic neutralization. The Lower Limit of Quantitation (LLOQ) for the HPV-PBNA is a reciprocal dilution of 40. A sample was classified as sero-negative if the PBNA titre was <50; seropositive if the PBNA titre was ≥50 and ≥2 times the BPV titre; or sero-status indeterminate if the PBNA titre was ≥50 and <2 times the BPV titre.

Pelvic examination to collect cervical cell samples is being carried out in married girls 18 months after marriage or 6 months after delivery, whichever is earlier, and annually thereafter for 3 consecutive years.

A dedicated HPV testing laboratory was established at the RGCB, with technology transfer and quality assurance from the Infections and Cancer Biology Group at IARC and the DKFZ. The HPV genotyping method involved HPV type-specific E7 PCR bead-based multiplex genotyping (TS-MPG).[10, 11] The multiplex HPV type-specific E7 PCR utilizes HPV type-specific primers

targeting the E7 region for the detection of 19 high–risk (HR) / probable HR-HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68[a,b], 70, 73, 82), and two low-risk (LR)-HPV types (HPV 6 and 11), with detection limits ranging from 10 to 1000 copies of the viral genome. The method was validated at RGCB under the supervision of scientists from IARC and the testing of cervical cell samples are carried out in RGCB.

The study has so far been monitored and evaluated by outcomes such as sero-conversion, comparison of the immunogenicity over a 48-month period after the first dose, and the frequency of incident and persistent vaccine targeted and non-targeted HPV infections in the different dose groups as the study progresses. In due course, we will document the frequency of CIN 2 and 3 caused by vaccine-targeted and non-targeted HPV types and, in the long-term, invasive cervical cancer incidence in the different dose groups over several years of follow-up by linking with population-based cancer registries.