These questions were posed by webinar attendees and subsequently responded to by the speakers Renske Steenbergen and Megan Clarke.

1. Recent data from PLATO trial, where all participants were tested for HIV, shown only 3% of participants with invasive anal SCC were HIV positive. How do we reconcile anal cancer screening these populations and other high-risk groups (which probably also account for less than 10% of invasive SCC) ignoring all the rest.

   - Renske Steenbergen - This is a great point, getting at risk of anal cancer vs. the fraction of anal cancer cases attributable to strong risk factors like HIV (see Deshmukh et al. IJC 2023). Most of the cases occur among women without HIV. The challenge is how to identify those at high risk who we could target for screening. Anal cancer is rare in the population, there is limited expertise/availability of anal cancer screening/HRA, and the harms would not outweigh the benefits for population-based screening.

2. In your risk analysis, did you consider instead of using the positive or negative outcome with one threshold, to subdivide the assay result into “no risk”, low, medium, “high risk”, cancer?

   - Renske Steenbergen - Thanks for your nice suggestion. For HGAIN treatment guidance methylation results will most likely be considered in the context of other clinical parameters. While a positive / negative outcome may be easier, a scale may indeed also be considered valuable. We are currently planning to define our threshold settings with several experts taking different clinical settings into account.

3. Thanks so much for this session. What’s the advantage of methylation testing in comparison to the routine HPV assays currently available?

   - Renske Steenbergen - HPV testing detects all HPV-induced lesions, both LSIL and HSIL. Methylation is associated with cancer development, which is a later step in the carcinogenic process (i.e. Development of advanced HGAIN with a higher cancer risk in need of treatment). In other words, methylation reflects the risk of cancer development. In the MSMLWHIV population most are HPV positive, so HPV testing has a rather low specificity. Methylation can be used to define which HGAIN need treatment.
4. Dr Steenbergen, did you consider including HPV DNA methylation patterns in MARINE study or any other? does HPV methylation correlates to lesion progression?

- Renske Steenbergen - Our MARINE study is aimed at clinical validation of the ASLC1/ZNF582 methylation test, but in follow up samples can also be tested for HPV methylation. 2nd Q: To the best of my knowledge there is no data yet on viral methylation in anal lesions being related to progression, but I expect such data to come from the studies of Dr. Clarke.

5. Gary Clifford. Epidemiologist, IARC, Lyon. Thank you. Question to Renske please. Do you think the best set of methylation markers in anal swabs will be similar to those that best discriminate disease risk from biopsies.

- Renske Steenbergen - We are currently performing a marker discovery study on anal swabs. Additional promising markers have been found but are still awaiting further testing on anal swabs. I hope to show some data next year.

6. I have HPV and was told I would have to see an anal surgeon to get an anal swap for HPV/cancer. This was after discussing it with my gynecologist and gastroenterologist. Could the home fecal testing be expanded to include this?

- Renske Steenbergen - This is an interesting suggestion. We have no data on this, but methylation markers do well for colorectal cancer detection in stool.

7. Second question to Megan. If I understood correctly, one slide suggests that a HSIL lesion in different populations (e.g HIV MSM versus HIV women) might have different risk. Is there evidence for this?

- Megan Clarke- The figure in the slide was showing that disease (HSIL) prevalence (baseline risk) varies across different groups, being highest in MSM with HIV. There are limited data on risk of progression from HSIL to cancer by different populations. The Machalek et al., 2012 study that Dr. Barroso mentioned modeled a higher progression rate from HSIL to anal cancer among men with HIV compared to those without. In general, this is very difficult to study. Data on anal HSIL recurrence suggest similar rates between MSM with HIV and women living with HIV (Stier et al., 2020)

8. I agree that methylation can be a marker for progression or regression, are you also planning to test it on swabs? Because biopsy is already treatment in a way.

- Renske Steenbergen - This is currently ongoing, and I presented some of our initial data showing the feasibility.

9. Is HPV DNA methylation being studied in HPV-related head and neck cancers?

- Renske Steenbergen - HPV 16 methylation has been tested for the detection of oropharyngeal cancers in gargles (doi: 10.1002/cam4.4757)
10. Would the risk of HGAIN or anal cancer be similar in case of positive methylation and HPV-16 or non 16 HPV-HR? In other worlds is the risk of cancer due to the presence of methylated genes, or is there a residual effect of the 16 genotype? For a screening strategy, that would justify taking into account all HR, rather than 16 alone.

- Renske Steenbergen - Present data show that methylation can be increased in HPV 16 and nonHPV16-positive HGAIN. Moreover, we have quite some cancers that are positive for HPV types other than HPV 16, all are also methylation positive. While HPV16 does indeed impose a higher cancer risk, particularly in immunocompetent patients, methylation is a biological event that adds to the cancer risk. Our study on risk groups other than MSMLWHIV showed no difference between HPV status and methylation (Mol Oncol. 2021 Nov;15(11):3024-3036. doi: 10.1002/1878-0261.12926). In our ongoing study on anal swabs we find that testing for hrHPV and methylation is better that testing for HPV16 and methylation in terms of advanced HGAIN detection.

11. Are there any immunology-based markers on the horizon that might be of use to assess cancer progression?

- Renske Steenbergen - This is an interesting suggestion and we are in the process of analysis the immunological profiles in HGAIN. Hopefully we can present some results next year.

12. What do you think about to combine methylation test with E6/E7 mRNA, taking into consideration the function of the protein related?

- Renske Steenbergen - Indeed as also pointed out by Dr. Clarke we should also test marker combinations for identification of (advanced) HGAIN. One reassuring issue is that data so far demonstrate that all HGAIN progressing to cancer and all anal cancers are methylation positive. Yet further longitudinal studies are needed for confirmation.