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MERMAID II "Natural history of human papilloma virus infection and cervical neoplasia – Molecular and biomarkers for cervical cancer"

The Final Report

The Independent Audit Committee (IAC)

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has the pleasure to forward the Final Report of the MERMAID II project. This is the fifth and final evaluation from the Independent Audit Committee (IAC) and should be viewed together with our previous four evaluations.

The Final Report has been submitted for evaluation together with 24 papers where 16 are published, 1 accepted, 5 submitted and 2 in manuscripts in the period January 2014 – March 2015.

Cancer of the cervix uteri is the second most common cancer among women worldwide. Apart from Eastern Europe, Denmark has had the highest mortality from cervical cancer and the second highest incidence when age-standardised mortality and incidence rates in Europe are compared. In addition, each year more than 6000 Danish women are diagnosed with severe cervical lesions. And as consequence they have a conisation performed, and require long-term follow-up after the diagnosis. The Mermaid project 2 was launced in 2008 with the goal to study the importance of HPV as a risk factor and a predictive marker for progression of early cervical lesions to CIN2 and cervical cancer.

The project design was very ambitious and has been divided into four main categories studied: Viral factors, Epidemiological / clinical factors, Genetic factors, and Viral / host interactions which have all –with the exception of Genetic factors-led to important new knowledge which will be instrumental for future design of screening projects relevant for prevention of cervical cancer. The overall project has also led to results of great importance for future monitoring of the Danish women vaccinated since the vaccine was offered for free in 2006. The four major topics of the project were studied in the ten subprojects I-X listed below.

I. To assess the overall as well as type-specific prevalence, incidence, and persistence of HPV infection in large cohorts of Danish women from the general female population, and to assess the prevalence of HPV among Danish men from the general population.

II. To identify lifestyle risk factors correlated to the risk of acquisition of HPV and persistence of HPV.

III. To examine the risk of cancer following an HPV infection measured as occurrence of genital warts (associated predominantly with HPV6 and 11) by comparing the incidence of cancer among men and women from Denmark diagnosed with GW with that of the general population.

IV. To a examine the relationship between HPV type, variants, viral methylation patterns, the prevailing HPV type in multiple infections, viral load, physical state and viral transcripts and the risk of persistent HPV infection or progression to high-grade CIN or cancer.

V. To identify germline genetic markers and variants that are associated with persistence of HPV infection or predict the initiation and progression of high-grade cervical neoplasia in women infected with HPV.

VI. To examine the effect of potential cofactors (such as smoking and parity) on the risk of high-grade cervical neoplasia in women infected with HPV.

VII. Identify gene expression 'signatures' that are associated with persistence of HPV infection or predict the initiation and progression of high-grade cervical neoplasia in women infected with HPV.

VIII. To examine the value of HPV DNA testing in the identification women at risk of relapse or residual disease (≥CIN2) after loop conisation.

IX. To identify determinants for non-screening and non-subscription to the cervical cancer screening program.

X. To examine the effect of the HPV vaccination on the burden/incidence of genital warts and high-grade cervical intraepithelial lesions at a general population level.

The overall study was based on specimens collected from different cohorts of women beginning with the collection in 1991 and has later led to establishment of eight different cohort of women as well as two cohorts of Danish men which were not sponsored by the Mermaid 2 project but which confer important information about risk of infection by sexual transmission of HPV. (The report gives the detailed information of the different cohorts in the Figure 2.).

Part 1. Viral factors.

HPV DNA and prevalence

The analysis of the prevalence of HPV and of the individual HPV types in the specimens from Young Danish cohort, the LBC cohort, the conization cohort and the male cohort has led to 17 publications in very prestigious international journals which show the importance of the findings. The data are summarized in an extensive final report, which is well written and clearly present all findings.

Only some of the major contributions will be highlighted here in the evaluation report. The overall results clearly show that persistent HPV16 infection is the highest risk factor for development and progression of cervical cancer. Data show that HPV persistence is the best predictive factor and that a negative HPV test is a strong assurance against later development of CIN3 or worse. Screening in the future should therefor include HPV analysis. Most importantly, it is also noticed

that the data support the conclusion that the vaccines against cervical cancer should only contain the high-risk HPV types.

HPV is also a risk factor for development of male cancer and for transmission of HPV among sexual partners. The prevalence of HPV in penile swabs was found to be as high as 42% when the most sensitive DNA test is used and 22% with the often used HC2 test. HPV16 and 51 were the most prevalent types found. These data are not really part of the Mermaid 2 study but contribute with important information to the natural history of the virus infection.

Genital warts

Based on the registries available in the Nordic countries an analysis of a large population of both men and women with genital warts were followed from the initial diagnosis until a possible cancer was reported in the cancer registries. The patients with a previous genital wart diagnoses had an increased risk of both genital and head and neck cancer development (the last category mostly related to HPV16 infections. Reviewers note). The Danish male study also checked for HPV in the genital specimens and showed that HPV6 and 11 were the most prevalent types in the genital warts, which add important knowledge to the possible future strategy for vaccination of males. (The studies are presented by three publications.)

Viral variants

The importance of different variants of HPV16 has been discussed over many years why it is nice to see the data from the Mermaid 2 study. Two European virus strains are the most prevalent and both leads to a high percentage of persistent HPV infections (in total 24% over two years) with an absolute risk of progression to CIN3 >50%. The data are important for the screening and treatment strategy and one publication is highlighted for this study.

HPV viral load and HPV physical state

To obtain further information on the risk of progression of HPV infections from normal to CIN3 based on cytology the investigators compared data for viral load and integration from two cohorts: the young (20-29y) and the LBC cohort (14-90y).

The data for the correlation between cytology and copy number of viral genomes in persistently infected women showed that the HSIL specimens had a higher copy number of the viral genome than specimens with normal cytology. The HSIL specimens also had a higher amount of integrated genomes.

The data for disease progression are still preliminary but differences are seen between data from the two cohorts when data from HPV persistently infected women with normal cytology at enrolment were analysed after later knowledge about disease progression or maintenance of normal cytology. The follow-up time was up to 13 years. For the young cohort, the viral load was not significantly higher in specimens from women with later progressed disease whereas in the LBC cohort, a significantly higher number of HPV copies were measured in specimens from women with disease progression. The integration of the viral genome in the specimens from the two cohorts also differed. It thus remains an open question if the copy number of HPV genomes and the integration are solid parameters for prediction of disease progression. The Mermaid 2 specimens will according to the investigators be analysed further to clarify the significance.

However, the investigators do conclude that it is unlikely that the copy number will be a useful clinical marker for disease progression risk. (Five publications are presented to document the important findings).

Expression of viral transcripts as markers for progression

It is well known that transcription of the viral oncogenes is instrumental for viral transformation. It is therefore interesting to see the comparison between transcriptional activity in specimens collected from women with persistent HPV16 infection and normal cytology with or without later progression. The report summarizes the results obtained from a very limited number of samples from the young cohort. The findings are interesting as there is no difference between the levels of E7 transcripts from the two series of samples. Only the transcripts from the non-oncogene E4 are increased in the group of "progressors", which is surprising and it will be interesting to see the results when published.

Methylation analysis of the viral genome

Modifications of the viral genome are studied extensively with respect to differences related to integration in cervical cancer specimens. The Mermaid 2 studied the methylation modification as a possible marker for viral persistence, viral load and viral integration. The data are obtained from 99 HPV16 positive specimens belonging to the young cohort. Interestingly, the correlation of the position/s of the genome methylated is different for the three parameters of interest. Only one methylated position in the L1 correlated to HPV persistence whereas methylation of several positions in the open reading frames of the E6, E7 and L1 correlated to the viral load and integration. The investigators do not try to interpret the data in the final report as they will need further investigations of a larger number of specimens. A manuscript is in preparation with the current data.

Stability of cervical samples in specimen transport medium for high-risk HPV detection

The information presented by the study group in the final report is useful. DNA stored under alkaline conditions is not stable and not valid for analysis in the HC2 test after three months of storage. The more frequently used PCR tests are not influenced after storage even for years, which is in agreement with data from other laboratories. The transport medium used for the Danish specimens collected for the Mermaid study is hopefully changed for later cohorts. A manuscript is in preparation.

Investigation of transcriptionally differences in different HPV-types

It is not quite clear if this study is part of the Mermaid 2 study as it would only be relevant to compare the transcriptional activity of the different upstream regulatory sequences (URR) in one cell line as authentic as possible which would be a human cervical epithelial cell line. The analysis of the URR from the oncogenic types HPV16, 18 and 31 as well as the weakly or non-oncogenic types HPV53, 66, 70, and 82 are not all types commonly found in cervical specimens. Professor Iftner initiated many years ago analysis of transcription from the URR regions in the presence of the E2 protein and it is per se a very interesting project. The conclusion that the transcriptional activity from the URR region does not correlate to the carcinogenecity of the different virus types is surprising but interesting. One publication is highlighted.

Part 2: Epidemiology

Sexual habits/factors

The careful analyses of sexual behaviour using several cohorts of women from the Nordic countries are valuable for interpretation of possible influence of the HPV vaccine on sexual behaviour. The findings were that the young women did not change sexual habits after vaccination,

which is a very positive result. Sexual habits of Danish men are also important for understanding of transmission of infection and risk factors for later disease development and this was studied in a randomly selected cohort of 22,979 men. Ten % of the men had sexual debut at the age of 14 or before, which increases the risk of later risky sexual behaviour. (Six selected publications are listed).

Cofactors for HPV persistence and progression

The Mermaid 2 study focussed at the risk of persistent HPV infection and progression of infection to higher grade lesions. For this the young cohort was very valuable and the women with *persistent HPV infection* were used for analysis of other risk factors. The studies confirmed that tobacco and child birth were risk factors but not use of OC or IUD contraceptives. It has been discussed if Clamydia Trachomatis infection was also a risk factor in HPV positive women and the study showed that repeated CT infections led to increased risk of CIN3 development. Analyses of risk factors in the HPV HR-positive women from the LBC cohort added information on the importance of the immune response in preventing persistent infection. (Four selected publications are listed).

Co-morbidity, socio-economic factors and cervical cancer

Very interesting and important findings show that the risk of developing cervical cancer is very much related to socio-economic factors such as education, single life and age. The diagnosis of cancer showed a higher grade of lesions in women with less education, living alone and >60 years old. The same influence was also seen on survival from the disease - most likely because of the late diagnosis. (Three selected publications are listed).

Treatment of high-grade CIN (conization)

Mermaid 2 has included a cohort of women who are treated for high-grade CIN with conization. The data are new and very interesting. Conization leads to a risk of preterm delivery. The risk depends on the depth of the cone and on the number of cone surgeries performed. The other important result is that after conization there is a risk of residual or recurrent disease (5%-35%) as well as of HPV persistence.

The HPV persistence after conization was 9.4% and for the women who cleared the infection 2.2% showed HPV re-appearance.

With a follow-up time of 11 years the risk of acquiring CIN2+ in HPV negative women increased with time to 5-6% - a study where cytology vs. HPV testing is also evaluated.

These studies are very valuable for clinicians where the follow-up can be optimized based on such findings although the investigators suggest a larger study to be conducted before clinical implications can be drawn. (Five publications are selected presenting these studies).

Incidence of cervical cancer and precancerous lesions

The cervical cancer incidence is fortunately decreasing as measured from 1997-2011 in women >45 years old. However the adenocarcinoma increased in women < 44 years. CIN3 and adenocarcinoma in situ (AIS) also increased.

Based on these finding it is very important to high-light the effect of the quadrivalent vaccine on CIN3 and AIS in the vaccinated women where there is a significant reduction. In the young women 18-20 years old the effect of the vaccination on the incidence of both atypia and CIN2+ was a significant reduction. These data are very important and it will be very interesting to follow the effect of the vaccine in the future. (3 publications are selected).

Population effect of HPV vaccine

The effect of the vaccine measured by the effect on genital warts is very profound particularly in the younger study groups where the coverage of the vaccination is high. Interestingly there is a significant reduction also in genital warts in men as an effect of herd immunity. The study has also analysed the effect of vaccination with two vs. three dosages and shown that for protection against genital warts two dosages might be as effective as three dosages if they are given 6 months apart. This is very important new information.

The effect of vaccination on atypia, atypia+, CIN2 and CIN2+ in the birth cohorts 1991-1994 also showed that the vaccination reduced the risk of acquiring these premalignant lesions. Although the vaccine is offered for free there are still 10-13% of the girls who have not wanted the vaccination.

These studies are instrumental in the understanding of the success for the prevention of hopefully also cancer. (Seven publications are listed).

Cervical cancer screening

The Mermaid 2 program also focus at the preventive screening offered to Danish women and the projects designed -and in part finished – will illustrate the acceptance as well as the non-acceptance of this offer among women. The study will also present data on the acceptance of a self-sampling method, which might reach out to a larger group of women. The data processing is in progress.

Genetic factors

Genome sequencing was planned as part of the Mermaid 2 project but the technical problems has not been solved why the project is postponed.

Virus-Host interactions

Biomarkers for progression to CIN3+ in women with persistent HPV16 infection

Mermaid 2 has led to identification of three molecules, which might be important for risk management in the future. The genes were TMEM45A, SERPIN5, and p16INK4A. The genes were transcribed to a higher level at baseline in women from the young cohort with HPV16 persistent infection and normal cytology who later showed disease progression.

The molecular studies of TMEM45A are summarised in the report but there is also submitted a manuscript with the data. Although the molecular data shows that the mRNA level of this gene is high in normal keratinocytes and decreased in HPV positive cell-lines it is still puzzling that the mRNA level is high in HPV positive specimens collected as early as 19 years before CIN3+ lesion occur. There is still information to be gathered before the gene can be evaluated as a molecular marker for clinical risk and progression management.

The other molecular marker is SERPINB5 –where the mRNA was found increased in HPV positive specimens with normal cytology at baseline, which later showed disease progression. SERPINB5 is a protease inhibitor and considered to be a tumour suppressor. In the current study the investigators do not find any correlation to HPV positivity but this does not exclude that SERPINB5 could be related to cell cycle progression and tumour development. (Other members of the SERPIN family (B3 and B4) are considered as *cervical squamous cell antigens* and lost in HPV16 cell array studies. Reviewers note). Publication is in progress.

The analysis of these molecular markers is a very interesting part of the possible identification of markers for future use in risk management or as targets for therapy but there is still a way to go. The investigators also consider the quality of the RNA analysed from the young cohort as questionable.

Biomarkers for persistence of an HPV16 infection

To optimize the quality of samples for mRNA and array analysis the investigators have used specimens also from a German cohort and compared data to results obtained using the young cohort from Denmark. Focus was to identify genes of importance for HPV persistency. The preliminary results clearly show that there are technical problems as the two datasets gives contradictory results although the specimens are of similar origin. It will be interesting to see if future analysis will identify new molecular markers of interest for the understanding of both HPV persistency but also for the risk of disease progression.

miRNA expression as marker for progression to CIN3+ in persistently HPV16 infected women

The investigations were done with specimens from both the German cohort and the Danish young cohort. The study was designed to look for differential expression of selected miRNAs (miR-9, 16, 96a, and 25) in specimens from HPV persistently infected women who had normal cytology vs. specimens from women who had progression to CIN3+. The selected miRNAs were found differentially expressed in the literature from abroad but in the current study no differences were observed. There are few data available also from Danish cervical cancer specimens and may be it could be worth to look for a possible differential expression of some of these miRNAs in future studies.

Filagrin

A study was performed in order to investigate a possible correlation between mutations in the differentiation marker filagrin and the risk of cervical cancer development. Blood specimens from the young Danish cohort were used for typing filagrin and control data were available from 8050 controls. The investigators did not find any correlation between the presence of selected mutations and the risk of cervical cancer.

In summary, the analysis of molecular markers has not been solved during the time the Mermaid 2 has been conducted. The search for and the analysis of such markers are an on-going process which will most likely lead to new, important markers to be identified in future studies using the very valuable specimens collected in the large Danish cohorts.

In summary:

The Final Report of MERMAID II is well written and provides a comprehensive review of the research.

The high-lights of the Mermaid research program can be summarized as follows:

- The program has allowed the study of series of parameters necessary for the understanding of the natural history of HPV infection and progression to cancer.
- The program has analysed social parameters and sexual behaviour (risk behaviour) among men and women related to risk of cancer development.

- The program has allowed a study and a follow-up of the effect of the vaccine introduced in Denmark as a free offer to girls and young women since 2008 with very valuable and solid data on the prevention of GW and so far on the reduction of premalignant lesions.
- The program has initiated the ambitious search for molecular mechanisms and possible molecular markers, which can be of use for future identification of HPV infected women and men at risk for disease progression.

The IAC can conclude that the Mermaid II program has provided important new knowledge on the natural history of HPV infection and cervical neoplasia. The investigations have resulted in an impressive number of publications, which all are published in high-ranking international journals. The results are of clinical importance and are useful for both patient treatment strategy and as a base in the ongoing development of preventive strategies. Together the cohorts and the collection of specimens make up a unique platform for future studies.

Mermaid II has contributed significantly to the international knowledge on the natural history of human papillomavirus infection and cervical neoplasia.

On behalf of The Independent Audit Committee (IAC)

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