IMPROVING HEALTH SYSTEMS TOWARDS EQUALITY-BASED CONTROL OF CERVICAL CANCER IN LATIN AMERICA.

Comparing PAP Smear Cytology, Aided Visual Inspection, Cervicography and Human Papillomavirus (HPV) Testing as Optional Screening Tools in Brazil and Argentina.

[1]K. Syrjänen, [2]P. Naud, [3]S.M. Derchain, [4]C. Roteli-Martins, [5]A. Longatto-Filho, [6]S.Tatti, [1]M. Branca, [7]M. Erzen, [2]L. Serpa-Hammes, [2]J. Matos, [3]R. Gontijo, [3]L. Sarian, [4]T.P. Lima, [5]M.Y.S. Maeda, [8]S. Costa, [9]S. Syrjänen.

[1]Unit of Cytopathology, National Centre of Epidemiology, Surveillance and Promotion of Health, National Institute of Health (ISS), Rome, Italy; [2]Hospital de Clinicas de Porto Alegre, Brazil; [3]Universidade Estadual de Campinas, Campinas, Brazil; [4]Hospital Leonor M de Barros, São Paulo, Brazil; [5]Instituto Adolfo Lutz, São Paulo, Brazil; [6]First Chair Gynecology Hospital de Clinicas, Buenos Aires, Argentina; [7]SIZE Diagnostic Center, Ljubljana, Slovenia; [8]Department of Obstetrics and Gynecology, S.Orsola-Malpighi Hospital, Bologna, Italy; [9]Department of Oral Pathology, Institute of Dentistry, University of Turku, Finland.

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ABSTRACT

OBJECTIVES

The main objectives are defined as follows:

1. The traditional tools used in screening of cervical cancer include: a) PAP smear cytology, b) colposcopy, and c) biopsy. Optional diagnostic tools suggested to low-resource-setting DEV countries include: 1) aided visual inspection (AVI), 2) cervicography, and 3) testing for HPV. In this project, the feasibility of the traditional and optional diagnostic tools will be tested in target populations at different risk for cervical cancer in two Latin American countries.
2. To elucidate the epidemiology, biology and pathogenesis of genital HPV infections, as explanatory factors of the different risk of cervical cancer in different populations (research component).
3. Compare the traditional diagnostic techniques 1) with two optional screening tools (aided visual inspection and cervicography) and 2) with HPV typing by Hybrid Capture II and PCR, done a) from conventionally collected samples and b) using self-sampling devices, using the conventional performance tests (sensitivity, specificity, positive- and negative predictive value and ROC analysis).
4. The long-term objective is to improve the health systems in these two LAM countries by designing new strategies for a cost-effective, organised programme for cervical cancer control, particularly covering the most vulnerable groups of high-risk women.

ACTIVITIES

The key activities involve:

1. This study is a combination of a classical population-based, cross-sectional (prevalence) study and a prospective follow-up (cohort) study of women with different (high and intermediate) risk of cervical cancer.
2. During the first 12 months, consecutive series of women (expected n=12.000) attending the partner LAM clinics are screened for cervical HPV infections and CIN, using different diagnostic tools (PAP test, cervicography, aided visual inspection, HPV testing, colposcopy).
3. The women with biopsy-confirmed low-grade CIN (HPV-positive or HPV-negative)(=prevalence data) will then comprise the cohorts to be prospectively followed-up until the end of the 38-month project period (=disease prognosis).
4. High-grade lesions are promptly treated and followed-up for 36 months. These clinics also collect the epidemiological data by questionnaires focused on the known and suspected risk factors of HPV, CIN and cervical cancer.
5. All samples primarily examined in the laboratories of the LAM partners, will be subjected to Quality Control (QC) by the EC partners. Cervical swabs and self-sampling devices (tampons) will be subjected to HPV testing by the Hybrid Capture II, controlled by PCR analysis by the EC partner (=molecular epidemiology). Interlaboratory QC of PAP smear diagnosis will be arranged.
6. Cervical biopsies are analysed for a number of factors with potential pathogenetic and prognostic significance (prognostic biomarkers), using immunohistochemistry (IHC) and molecular biological techniques.

EXPECTED OUTCOME

This type of combined cross-sectional and cohort study is focused on the key epidemiological and biological factors of genital HPV infections and cancer precursors in women living in intermediate- and high-risk regions of Brazil and in Argentina. The differences in the incidence of cervical cancer in these LAM regions could be due to two principal causes1) different natural history of the precursor lesions, or 2) due to different level of exposure to the known risk factors, e.g. HPV, HIV, etc. Elucidating the test performance in populations with different prevalence of HPV and CIN is mandatory for planning feasible cervical cancer control strategies for women at different risk of the disease, based on cost-efficacy analysis which will be completed as the final step of the project.

1.TITLE

IMPROVING HEALTH SYSTEMS TOWARDS EQUALITY-BASED CONTROL OF CERVICAL CANCER IN LATIN AMERICA. Comparing PAP Smear Cytology, Aided Visual Inspection, Cervicography and Human Papillomavirus (HPV) Testing as Optional Screening Tools in Brazil and Argentina.

2.OBJECTIVES

General Objectives

The general long-term objective of the proposed joint research project is to provide simple, accurate and cost-effective tools for the DEV partners to enable early detection, reliable diagnosis and adequate treatment of cervical cancer precursors in populations at different risk in two Latin American (LAM) countries. Proper implementation of these diagnostic tools should enable the design of cost-effective strategies to improve their health systems towards equitable access of all women to an organised cervical cancer prevention programme. Once shown their efficacy by these two LAM partners, these strategies could reach more widespread implementation into their neighbouring countries, which would inevitably lead to decreased morbidity and mortality due to cervical cancer in these South American (high-risk) countries. This reduced women morbidity and mortality, in turn, would certainly have an important impact on the sustainable development in these countries.

Specific Scientific and Technological Objectives

While prevention of cervical cancer by eradicating the etiological agent (HPV) by vaccination is not feasible as yet, attempts should be focused on improving the health care systems to become capable of effectively tracing the women at risk for cervical cancer and treating the detected precancer lesions. The traditional tools used to accomplish these two tasks include: a) PAP smear cytology, b) colposcopy, c) biopsy, and d) measures for an effective treatment of all significant lesions. Optional diagnostic tools suggested particularly to low-resource-setting DEV countries include: 1) aided visual inspection (AVI), 2) cervicography, and 3) testing for HPV (conventional and self-sampling). The feasibility of these diagnostic tools will be tested in target populations at different risk for cervical cancer in two Latin American countries.

Objective #1: With the assistance from the EC, to establish the above listed facilities at the DEV contractors', including the training of professionals. This is the developmental component of the proposal.

Objective #2: To elucidate the extent of the health problem due to HPV infections and cervical cancer precursors in these two countries. Particular attention will be paid to assessment of the reasons for the different risk of cervical cancer between the two countries and in their different regions. This objective is the key research component of this proposal, and can be achieved only after supplementing the available inadequate facilities with the expertise in diagnostic cytology, pathology, colposcopy, and auxiliary diagnostic tools from the EC partners (i.e., fulfilment of Objective #1).

Sub-objectives of #2**:** The assessment of the HPV/CIN problem includes specific sub-objects, focused on exploration of the key data on epidemiology, biology and pathogenesis of genital HPV infections and cancer precursors in the female populations with clearly different risk for cervical cancer. These items include: 1) prevalence, and 2) risk factors of the disease, 3) its pathogenetic mechanisms, and 4) disease prognostication.

Objective #3: Comparison of the traditional diagnostic techniques (cytology, colposcopy, histology) **1)** with two optional screening tools suggested for DEV countries (aided visual inspection and cervicography) and **2)** with the new molecular diagnostic tools (HPV typing by Hybrid Capture II and PCR), done a) from conventionally collected samples and b) using self-sampling devices. The practical value of these tests can be objectively measured by the performance tests for sensitivity, specificity, positive- and negative predictive value and ROC analysis. Elaborating

these data is essential for the assessment of the feasibility of these tests as **cost-effective tools** in **improving health systems** towards an equitable programme for cervical cancer control in these two countries. In addition to this second developmental component, this objective also includes the research component of the project, while applying molecular techniques in an epidemiological study (i.e., molecular epidemiology).

Objective #4: The long-term objective is to **improve the health systems** in these two LAM countries by designing new strategies for a cost-effective, organised programme for cervical cancer control, particularly covering the most **vulnerable groups** of high-risk women. This objective can only be achieved by fulfilment of the objectives #1 through #3. This is the final step of this project, which proceeds from the creation of the basic facilities for HPV/CIN diagnosis for the LAM partners (Objective #1), through the utilisation of these facilities to assess the magnitude of this health problem in different target populations (Objective #2), and by evaluating the feasibility (performance characteristics) of the optional diagnostic tools (Objective #3), ending up with the careful cost-benefit analysis and recommendations for the guidelines how to organise the detection and management of cervical cancer precursors in women at different risk. The efficacy of these strategies can only be measured after several years of implementation under field conditions. The **measurable objective criteria of verification** is the eventual **reduction in the incidence** of and **mortality** due to **cervical cancer** in these two countries. These measurable objective criteria are of two categories: a) long-term, and b) short-term criteria. The former (reduced cancer mortality) can only be seen after prolonged implementation of the organised screening programme, but the latter can be observed relatively soon after such an implementation, e.g. as a reduced incidence of CIN lesions in systematically screened target populations.

3.WORK PLAN

a) Introduction

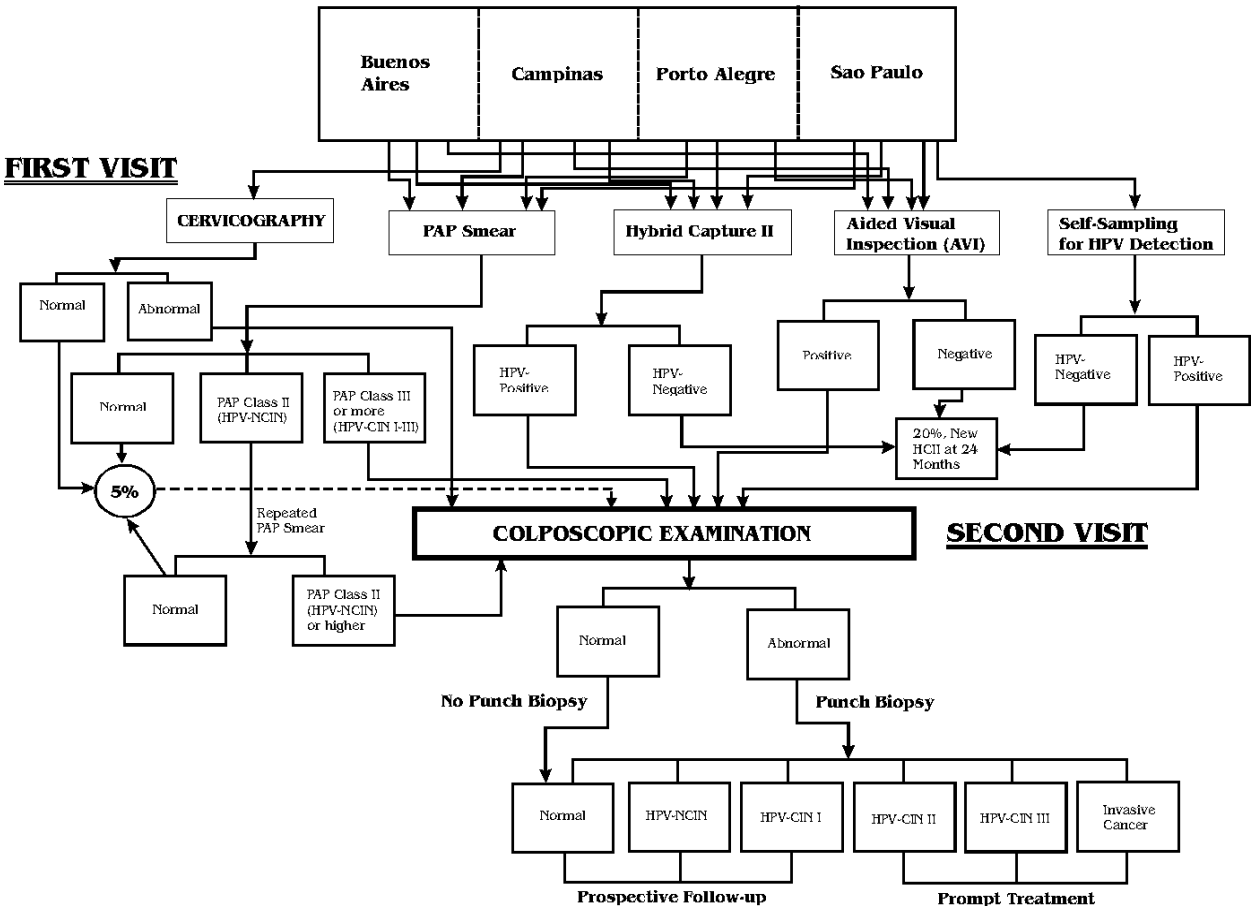
This study is a combination of a classical population-based, **cross-sectional (prevalence) study and a prospective follow-up (cohort) study** of women with different (high and intermediate) risk of cervical cancer. During the first 12 months, consecutive series of women attending the partner LAM clinics are screened for cervical HPV infections and CIN, using different diagnostic tools (PAP test, cervicography, aided visual inspection, HPV testing, colposcopy). The women with biopsy-confirmed low-grade CIN (HPV-positive or HPV-negative)(=prevalence data) will then comprise the cohorts to be prospectively followed-up until the end of the 36-month project period (=disease prognosis). High-grade lesions are promptly treated and followed-up for 36 months. These clinics also collect the epidemiological data by questionnaires focused on the known and suspected risk factors of HPV, CIN and cervical cancer. All samples are primarily examined in the laboratories of the LAM partners, and the **Quality Control (QC) of all the diagnostic tests** will be completed by the partners (#1, 2 and 3) in the EC. Cervical swabs and self-sampling devices (tampons) will be subjected to HPV testing by the Hybrid Capture II (HCII) in LAM countries, controlled by HCII and PCR analysis (of randomly selected samples) by the EC partner (=molecular epidemiology). Cervical biopsies are analysed for a number of factors with potential pathogenetic and prognostic significance (prognostic markers), using immunohistochemistry (IHC) and molecular biological techniques, in part by a LAM (#6) - and an EC partner (#2).

This type of combined cross-sectional and cohort study is focused on the key epidemiological and biological factors of genital HPV infections and cancer precursors in women living in intermediate- and high-risk regions of Brazil and in Argentina. The differences in the incidence of cervical cancer in these LAM regions could be due to two principal causes: 1) major differences in the level of exposure to the known risk factors, or 2) differences in the clinical course of HPV/CIN lesions. Elucidating these data is mandatory for planning feasible cervical cancer control strategies for women at different risk of the disease. The flow-chart of the patient examination, follow-up and treatment is illustrated in **Figure 1**.

Thus, all women attending the clinics of the LAM partners will be subjected to 1) PAP test, 2) AVI

and 3) cervical swabs for HCII (and subsequent HCII & PCR control). Cervicography is done for patients attending the clinic in Campinas only, and self-sampling for HPV (by self-administered tampons) is tested in Sao Paulo only. Women testing positive with any of the techniques will be referred for colposcopic examination (Figure 1; Second Visit), including those with PAP smear abnormality consistent with HPV-CIN. However, women with equivocal smears (ASCUS) will be controlled by a repeated PAP test at 6 months, and in case of persistent abnormality, referral to colposcopy is made. On colposcopy, all abnormal findings are confirmed by directed punch biopsies. The result of the punch biopsy will be used as the gold standard of HPV/CIN diagnosis, to which the other diagnostic tools will be compared in the performance tests. The biopsies will be further examined by other techniques for the prognostic markers, as detailed in Work Packages.

Figure 1. Flow-Chart of the Patient Examination, Treatment and Follow-up



b) Patients and Study Design

To assess the **reasons for the different risk of cervical cancer**, the study is focused on target populations of women **in different regions of LAM countries**: 1) those in South East Brazil (intermediate risk), 2) those in South Brazil (high-risk), and 3) those in Argentina (high-risk). The first 12 months of the project will be used for recruitment of the women with HPV-CIN among the consecutive women attending these clinics in the LAM partners (**first visit**). To elucidate the most **cost-effective tools for cervical cancer control** in these countries, the performance of five potential screening tools will be tested: **1)** PAP smears, **2)** HC II, **3)** aided visual inspection (AVI), **4)** cervicography, and **5)** self-sampling for HPV test. All abnormal test results are controlled by colposcopy and punch biopsy (**second visit**). This enables the calculation of the performance (sensitivity, specificity, positive- and negative predictive value, ROC analysis) of all five diagnostic tools (PAP, HC II, AVI, cervicography, self-sampling), using the histologi

cal diagnosis in colposcopic biopsy as the gold standard.

All patients attending the clinics of all LAM partners will be subjected to **a)** PAP smears, **b)** AVI and **c)** cervical swabs (for HCII & PCR). PAP smear abnormality consistent with HPV-CIN will be referred to colposcopy, whereas women with equivalent PAP smears (ASCUS) will be controlled by a repeated PAP smear at 6 months. In AVI, cervix is covered with 3% acetic acid and, after 1 minute, visualized with a 100W light. At the second step, the cervix is painted with lugol. Positive AVI is recorded in cases with altered acetic acid and/or lugol staining. Cervical swabs will be analysed for HPV DNA by HC II in LAM, and, randomly selected samples will be controlled by repeated HCII test and PCR amplification with consensus primers and type-specific PCR by an EC partner. HPV DNA-negative women will constitute the **Control Group** for all others, to be analysed for the epidemiological risk factors by questionnaires only.

Two other potential screening tools suggested to be used in low-resource settings, **d)** cervicography and **e)** self-sampling for HPV testing, will each be analysed by one partner only, Campinas and Sao Paulo, respectively (Figure 1). Cervicography involves the preparation of the cervix in a similar way as for colposcopy. The cervix is visualized in a self-retaining speculum, and a specifically designed hand-held camera (Cerviscope). An electronic data bank permits accurate identification of the slides (cervigrams) of each patient, classified as normal or abnormal. The latter are referred for colposcopy. Self-sampling for HPV testing is made by special tampons self-administered by the patient. These are subjected to HPV testing by HCII and PCR like the conventional samples (cervical swabs).

To obtain valuable **incidence data**, randomly selected 5% of PAP smear-negative and cervicography-negative women will be invited to colposcopy, to 1) control for false negative PAP- and cervicography tests, and 2) to detect new (incident) positive cases. Similarly, 20% of HCII-, AVI- and self-sampling-negative women will be invited to re-testing for HPV at 12 months (**Figure 1**). This enables the detection of new (incident) cases of both clinical (PAP test-, AVI- and cervicography-positive) and subclinical/latent (HCII-positive) HPV infections among the initially test-negative women.

The Quality Control of colposcopy is arranged through the partner in Buenos Aires, to which the colpophotographs will be submitted from other clinics for evaluation. The result of the punch biopsy will be used as the gold standard of HPV/CIN diagnosis, with all grades from HPV-NCIN to invasive carcinoma being expected. The biopsies will be further examined by other techniques for prognostic markers (see Work Packages for details). Depending on the severity of the lesion on biopsy, the women are allocated to two mutually exclusive categories: **1)** prospective follow-up, and **2)** immediate treatment. Accordingly, women with low-grade lesions (HPV-NCIN & HPV-CIN I) will be followed-up without treatment, whereas those with high-grade lesions are eradicated by any of the conventional treatment methods (laser, conization or LLETZ)(Figure 1).

c) Expected Results

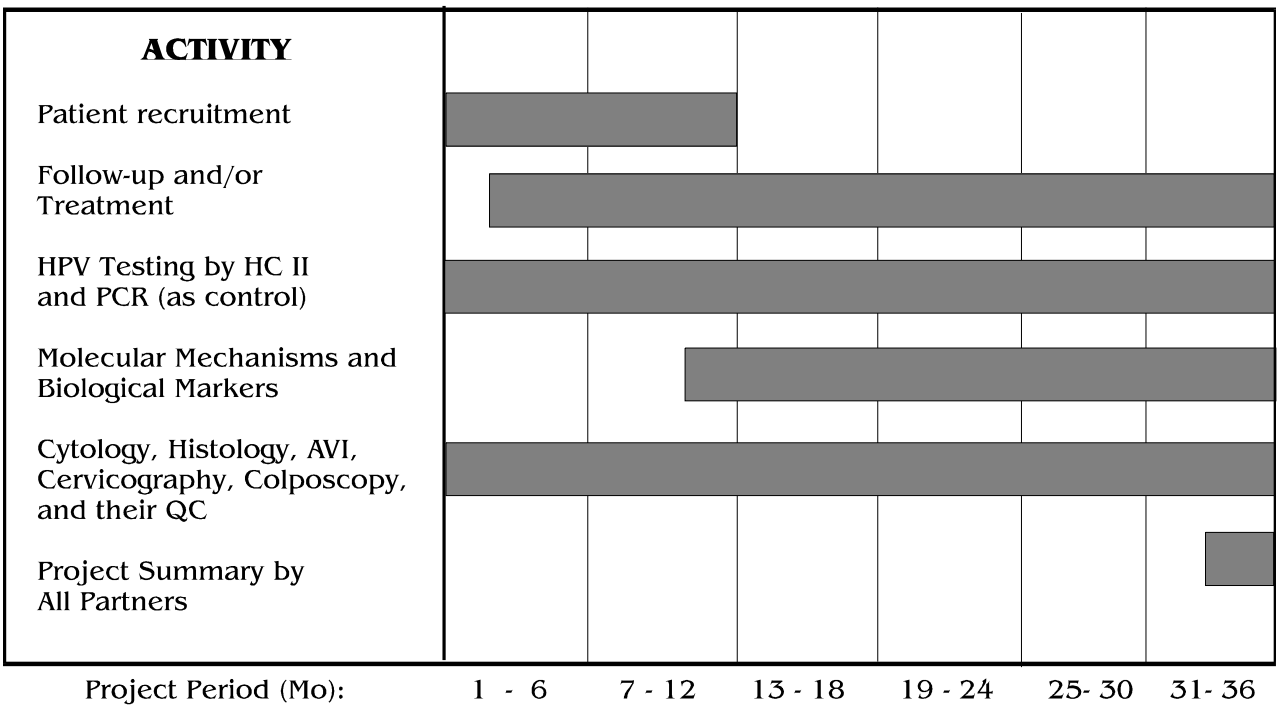
This study design permits an extensive analysis of a number of important issues, necessary for the completion of objective #4 of this project (**designing new strategies for a cost-effective programme to control cervical cancer**). Such key issues include the following: 1) The differences in the prevalence, pathogenesis and clinical course of HPV infections and CIN in different regions of Brazil and in Argentina; 2) How many significant lesions are missed by the five diagnostic arms (=false negative rate)? 3) How many unnecessary colposcopies are caused a) by AMR I (PAP test), b) ARM II (HCII), c) ARM III (AVI), d) ARM IV (cervicography), and e) ARM V (self-sampling)? 4) What is the optimal diagnostic set-up to result in the highest sensitivity and specificity, as well as highest PPV and NPV in diagnosis of CIN lesions in these two LAM countries? These results will be carefully weighted while assessing the feasibility of the different strategies in the control of cervical cancer in Brazil and Argentina. These data are also the mandatory basis for the cost-effective analysis to be completed as the final step of the project.

The prospective follow-up of low-grade lesions (HPV-NCIN & HPV-CIN I) without treatment permits the analysis of a wide variety of prognostic factors with potential impact on disease outcome. An extra dimension is created by the possibility to follow-up cohorts of CIN women in different regions with clearly **different incidence of cervical cancer**. The 24-month period available for this follow-up is justified by the previous cohort studies indicating that practically all lesions predestined to progression do so during the first 24 months since the diagnosis. These follow-up data should also permit identification of the low-risk patients, to be followed-up only, and thus avoiding unnecessary treatment, which should have a major impact on the health economy issues in these countries. Even **more importantly**, this approach enables us to elucidate whether **the different cervical cancer incidence** in these regions is due to 1) **different natural history of the precursor lesions**, or 2) due to **different level of exposure to the known risk factors**, e.g. HPV, HIV, etc.

d) Work Packages

The **Time Table** of the Project activities is depicted in **Figure 2**. These represent at the same time the Work Packages of the project, and their **spatial relationship to each other** is evident from this Figure. Further clarification to their interrelationships is obtained in **Figure 1,** and the explanatory text above. Accordingly, examination of the patients in the LAM clinics will start as soon as the Contract is signed (month 0, starting month). The activities of each partner are explained in more detail in the Work Packages as well as in **Table 1.**

Figure 2. Time Table of the Activities



The Phase 1, patient examination, lasts for a total of 12 months. The Phase 2 (patient enrolment in the follow-up or treatment groups) starts in parallel with Phase 1, as soon as the results of PAP test, HCII, AVI, cervicography and self-testing are available. With the existing capacity of the LAM contractor (#7) responsible for the HCII analyses, the results of HCII will be available within one week from the receipt of the samples in the laboratory. These HPV tests are also an integral part of the follow-up procedures, and thus continue throughout the whole project period. HCII results are controlled by PCR, analysing randomly selected cases in parallel with the HC II tests. This Phase 2 (follow-up) includes a colposcopic examination of the patients and cervical biopsies for histology and other examinations. Quality control (QC) of all the diagnostic techniques is a continuous process throughout the 36-month duration of the study.

Immunohistochemical (IHC) analyses for the biological tumour markers are feasible to be completed when a representative series of biopsies is available. Thus, these analyses will be started during the 2nd year of the project, and will be continued until the end of the project. Cytology and histology are critical diagnostic tools for the successful running of the project also during the follow-up period, and these will be continued throughout the entire study period. The same is true with the QC of these techniques. Towards the end of the 36-month project period, the final activity (Work Package 6) will be pursued jointly by all partners. This is the summary of the obtained results, including the conclusions, cost-effective analysis, and the first draft of the guidelines for an improved cervical cancer control strategy in these LAM countries.

Work Package List:

Work Package No.	Work Package Title	Lead Contract-or No.	Person Months	Start Month	End Month	Deliverable No.
1	RECRUITMENT	4,5,7,8	48	0	12	1
2	FOLLOW-UP	4,5,7,8	96	3	36	2
3	HPV TESTING	7 (2)	72	1	36	3
4	BASIC RESEARCH	2 (6)	48	6	36	4
5	QC AND DIAGNOSIS	1 (3)	252	0	36	5
6	CONCLUSIONS	1	32	32	36	6
TOTAL			458			

List of Deliverables:

Deliverable No.	Deliverable Title	Delivery Date
1	FOLLOW-UP COHORT	12
2	CLINICAL COURSE	36
3	PREVALENCE OF HPV	12
4	MOLECULAR MECHANISMS	36
5	ACCURATE DIAGNOSIS OF HPV-CIN	3
6	COST-EFFECTIVE SCREENING STRATEGY	36

Work Package Description:

WP (1): RECRUITMENT

Objectives

The main objectives of this Work Package is to examine all the consecutive women entering each clinic during the 12-month period of patient recruitment, to get collected and enrolled in the subsequent follow-up a representative cohort of women with diagnosed CIN lesion (with or without HPV).

Description of work

This Work Package is completed exclusively by the partners in Brazil and Argentina. The work includes 1) taking the PAP smear, 2) taking the sample for HCII (and PCR) analysis, 3) completing AVI, 4) making cervicography (one partner), 5) running HPV testing by self-sampling devices (self-administered tampons)(one partner), as well as 6) by collecting epidemiological data using a structured questionnaire (all partners).

PAP smears are routinely taken, processed as usual, and interpreted using the Bethesda System (TBS) terminology. For HCII, see Work Package #3. Epidemiological data will be collected by using a structured questionnaire, previously tested and currently used in the NIS/CCE (INCO-COPERNICUS) Project of the co-ordinator. In completing AVI, the uterine cervix is painted with 3% acetic acid and, after 1 minute, visualized with a 100W light. At the second step, the cervix is painted with lugol. Positive AVI is recorded in cases with altered acetic acid and/or lugol staining. Cervicography involves the preparation of the cervix in a similar way as for colposcopy. The cervix is visualized in a self-retaining speculum, and a specifically designed hand-held camera (Cerviscope). An electronic data bank permits accurate identification of the slides (cervigrams) of each patient, classified as normal or abnormal. The latter are referred for colposcopy. Self-sampling for HPV testing is made by special tampons, self-administered by the patient. These are subjected to HPV testing by HCII and PCR like the conventional samples (cervical swabs).

Deliverables
As a result of this Work Package, a cumulative series of consecutively examined women will be obtained, who comprise the study cohorts to be further examined as detailed in **Figure 1**. At completion of this Work Package, we know the prevalence of HPV infections and cervical pathologies (PAP, AVI, cervicography) in this consecutive series of women examined at each clinic. After this first visit, we do not know yet the severity of this cervical pathology, which is established only by the colposcopic biopsy at the 2nd visit. In practice, however, this will be completed with a delay of days to one-two weeks only.

Milestones and expected results
The milestone after completion of this Work Package is **the cohort of women** (the study subjects). Determined from the inquiry made among the LAM partners, we expect that by completion of this phase, a total number of 26.000 women have been examined, with estimated number of CIN patients in the order of 1.250. According to the best estimates given by the LAM partners, during the 12-month recruitment period, they expect to enrol a cohort of 800-1.000 women with CIN for the subsequent follow-up.

WP (2): FOLLOW-UP

Objectives
To obtain as many as possible of the women who have completed all the examinations of the 1st visit, to become a member of the cohort for subsequent prospective follow-up. Once such a representative series of women with diagnosed CIN (with or without HPV) has been enrolled, the major objective of this Work Package is to keep all these women under close monitoring for a minimum of 24 months.

Description of work
Having completed the examinations and questionnaire of the 1st visit, the enrolment to the 2nd visit will take place as presented in **Figure 1.** This necessitates a fast and accurate feedback to the clinicians from both the cytologists examining the PAP smears and from the partner running the HCII tests for HPV detection. Results of AVI and cervicography are determined by clinicians themselves. As soon as the test results are available, the clinicians invite the women with diagnosed CIN (HPV+ or HPV-) for the 2nd visit. This second visit includes colposcopic examination of the patient. Following the algorithm presented in **Figure 1**, the results of the colposcopic biopsy determine, whether the patient is allocated into the Follow-up group (low-grade lesions) or subjected to treatment and post-treatment follow-up. The proper conducting of the follow-up examinations at 6-month intervals for 24 months is the major part of this Work Package.

Deliverables
By the end of the first 12 months of the project, the Work Package 1 is completed as will be the

first part of Work Package 2, i.e. the enrolment of the women into the follow-up cohort. At this stage, we know the prevalence and distribution of HPV types as well as the severity of the cervical pathology. Similarly, the final size of the cohort as well as the exact numbers of HPV- and HPV+ women will be available.

Milestones and expected results
Work Package 2 continues from this 12-month period of cohort enrolment as a classical prospective cohort study, when all women are controlled at the clinic every 6 months. Thus, every sixth month will be a milestone (=a new control visit) for each individual woman, subjected to repeated examinations (PAP smear, colposcopy, AVI, cervicography, biopsy, HPV testing) until the completion of the minimum of 24-month follow-up. The expected outcome of this type of study design is hard to predict, but it can be anticipated that the follow-up compliance might be less than that obtained in some of the best controlled recent cohort studies in the West. By the completion of the 24-month follow-up, however, we expect to obtain answers to most of the questions listed among the objectives of this project.

WP (3): HPV TESTING

Objectives
To run the HPV diagnostics by Hybrid Capture II and PCR tests during the entire project period. HCII testing is primarily done by the LAM partner (#7), controlled by random sampling by the EC partner (#2). PCR analysis will be done for a representative randomly selected series of samples, to **compare the performance characteristics** of these two HPV detection techniques.

Description of work
The facilities and long-term experience to run these tests is available in one of the LAM partners (#7). The samples will be submitted to the laboratory at two-week intervals by couriers. This system in being tested in a number of ongoing projects and works well, because of the fact that the samples tolerate two weeks storage at room temperature, once in the transport medium. HCII test is a commercial automatic test run by a special machine according to the manufacturer's instructions. The system is also working at the EC partner (#2), where control runs will be done (to test **inter-laboratory reproducibility**). PCR amplification for HPV is done by the EC partner (#2) using consensus primers GP5+/GP6+, and type-specific primers for specific viral typing, confirmed by SB hybridisation. These procedures are everyday routine in the laboratory of this partner.

Deliverables
Deliverables of this Work Package are the results of HPV detection. HCII results include 1) the test result as – or +, 2) the HCII count (varying from 0 to hundreds of thousands), and 3) the HCII-index (RLU/PC), determining as the ratio between the test result and the negative control. In the test, only the oncogenic HPV types will be included. PCR results include the test result as + or -, as well as the specific HPV type confirmed by SB hybridisation.

Milestones and expected results
Role of these two tests is crucial in determining the women as HPV-positive or –negative, which is one of the selection criteria (in addition to CIN) for the prospective cohort. Study design allows the comparison of these molecular HPV tests with the conventional diagnostic tools (PAP test, AVI, cervicography, colposcopy) in terms of specificity, sensitivity, positive (PPV)- and negative predictive value (NPV) as well as ROC (Receiver Operating Characteristics), using histology as the gold standard. The final results of this comparison are impossible to predict, but the results will have a major impact in the final phases of the project, when guidelines for the feasible and cost-effective strategies for cervical cancer control in these two LAM countries are being created. In this setting, the **conventional sampling for HPV** will be compared with the **self-sampling**, done by using self-administered tampons by the women. If shown equivalent in HPV detection,

the latter might result in considerable cost savings as a screening tool.

WP (4): BASIC RESEARCH

Objectives

The key objective of this Work Package is to gain additional information about the **molecular mechanisms** regulating the pathogenesis and natural history of CIN lesions in the presence and absence of HPV. At best, some of the tested biological factors might prove to be valuable **prognostic markers** for the disease outcome(progressive/regressive disease). When analysed in women from distinct regions with different incidence of cervical cancer, another goal is to assess whether this different risk is attributable to different natural history of the precancer lesions. And if so, elucidate the mechanisms underlying this divergent clinical course.

Description of Work

This Work Package is an extensive entity, comprising a wide variety of items to be analysed, using different technical approaches. Both molecular biological and IHC techniques are needed to complete these analyses.

Cell-Cycle Proteins

HPV replication is intimately linked with the normal cell-cycle, with which the virus interferes in a highly complex manner. A large number of these cell-cycle proteins can be analysed and quantified by IHC, including the following: p53, Rb, p21, bcl-2, PCNA, Ki-67, Ki-S2, cyclin-D. When combined with the HPV typing, some of these data might be of prognostic importance in predicting the disease progression. For studies on the expression levels of the transcripts from the cell-cycle regulatory proteins, the new powerful technique of "TaqMan" real-time PCR will be used (see below for viral load).

Biological Prognostic Markers

A wide selection of antibodies are commercially available against different cellular proteins, extensively studied as prognostic markers in human malignancies. Their role in genital HPV infections is inadequately studied as yet. These factors are linked with a wide variety of tumour-host relationships, the main interest being focused on the following three: **1**) the tumour-matrix interactions, **2**) interactions of growth factors and tumour matrix components, and **3**) angiogenesis. Among all these categories, important (and in HPV/CIN lesions largely unexplored) markers exist, which can be analysed by IHC. **The listing of the factors analysed may be subject to changes, according to the emerging results.**

Apoptosis

Apoptosis is a gene-directed programme of cell death implicated in cell turnover in normal adult tissues and it is regulated by a large number of factors, like hormones, growth factors and mitogens. The final common pathway is represented by a peculiar DNA degradation and fragmentation that is morphologically well defined. Apoptosis may be measured by a series of laboratory techniques, including the TUNEL method. So far, few studies have assessed the correlation between apoptosis and type of HPV. This analysis may help clarify some mechanisms by which different HPV types may give rise to lesions with different biological behaviour, i.e. progressive disease or those resolving spontaneously (via apoptotic pathways?) .

HIV-HPV interactions

In case that biopsies from HIV-positive women will be obtained, factors pertinent to interactions of these two viruses (HIV and HPV) will be analysed as well. Accordingly, we will analyse the protein levels (by IHC) of cell proliferation markers (PCNA, polymerase delta), apoptotic markers (TUNEL-technique, bcl-2 and bax), angiogenetic factors, markers signifying local immunodeficiency (Langerhans cells, CD4, CD8, CD3), and inflammatory cytokines (IL-6, TNF, IFN). These are correlated with the expression levels of HPV16 E6, E7, and L1 as well as HIV tat and rev

proteins, with simultaneous detection of mRNA-levels (by RT-PCR).

Viral (HPV) load
Viral load seems to be one of the risk factors for disease progression, and as such a potential marker of prognostic value. For quantitation of the expression levels of the fusion transcripts obtained from integrated HPV as well as transcripts from episomal HPV, the new powerful technique of "TaqMan" real-time PCR will be used. This method is based on the 5' - 3' endonucleolytic activity of Taq polymerase, and allows direct detection of PCR products by release of a fluorescent reporter during the PCR. The nucleolytic activity of Taq polymerase will cleave the hybridised probe (not free probe) during PCR and thereby accomplish the physical separation of the reporter and the quencher dyes. Thus, there will be an increase in fluorescence from the reporter dye, which is proportional to the amount of PCR product accumulated. The method is characterized by a wide linear dynamic range, which is at least five orders of magnitude, contamination-free operation, excellent inter-laboratory agreement and high-throughput capacity without tedious post-PCR processing. The method has been applied successfully to study HPV viral loads as risk factors using clinical samples. **This real-time PCR method (available in partner #2), applied to these clinical samples is a powerful tool in resolving the molecular mechanisms of high-risk HPV types in genital carcinogenesis.**

Deliverables
Deliverables of this Work Package will be the results of the analyses of the colposcopic biopsies (or cone specimens) of the patients. These results become meaningful only when analysed in context with the other parameters recorded from the patients. To be of clinical relevance, any of the observations should have a meaningful correlation with 1) the clinical course of the disease, 2) HPV status, and 3) known risk for cervical cancer. The optimal delivery from this Package would be the discovery of any single marker that could be used as an adjunct in prognostication of the disease, i.e., in decision making whether treatment is needed or not. Another potential deliverable could be a solution of any molecular regulatory mechanism conferring an increased risk for disease progression in those women living in regions with the highest risk for cervical cancer (as compared with those is areas of a lower risk).

Milestones and expected results
It is anticipated that the milestones from this WP can be expected only towards the very end of the 36-month project period. Most importantly, the disease outcome must be available before any statistical analyses can be performed to disclose the potential prognostic markers. It is currently agreed, however, that HPV type alone is not sufficient for malignant transformation, but probably a multitude of other factors are needed. The same is true with the disease progression, although usually linked with the high-risk HPV types. Thus, the results of this Work Package are highly unpredictable, and **the emerging data may necessitate analyses not included in this package description at this stage**.

WP (5): QUALITY CONTROL AND DIAGNOSIS

Objectives:
The primary objective of this WP is to run a quality-controlled (QC) cytological, histological and colposcopy diagnosis as well as validated AVI by all LAM partners, and cervicography by one LAM partner (#7) throughout the project period. In case of any detected deviations from acceptable quality, quality assurance (QA) measures will be promptly taken to restore the diagnostic performance to the acceptable level.

Description of work
Diagnosis of HPV infections and CIN is based on detection of characteristic morphological changes in routine PAP smears and biopsies. In this WP, the primary diagnosis will be made by the

cytopathologists working in each LAM clinic. The exception is Partner #5, to which this service is provided by Partner #6. The diagnostic criteria of CIN and HPV lesions are subjective in nature, and reproducibility of these tests is usually not particularly high. The accuracy of the diagnosis is critically dependent on the training of individual cytotechnologists and cytopathologists. Before the start of the diagnostic work, diagnostic criteria will be agreed in a joint meeting, providing unified quality standards for the LAM partners. Because of the identified deficiencies of both LAM countries in this field, it is mandatory to pursue **continuous QC** of these diagnostic methods, and take appropriate **QA measures,** if necessary. This is accomplished by **re-examining all the slides and all biopsies** by the EC partners #1 and #3. QA measures will be promptly taken to restore the diagnostic performance to acceptable level, whenever necessary. In addition, EC partner 3# will prepare educational material and give appropriate training for the laboratory personnel of the LAM partners. Rigorous measures for both **internal QC** and **inter-laboratory QC** will be taken (by EC partner #3) to ensure the diagnostic accuracy. In accomplishing this, the experience of this partner in another EC Project (Leonardo da Vinci, Cytotrain), will be fully exploited.

Colposcopy is another key diagnostic tool in detection of CIN lesions. Colposcopic diagnosis is based on a subjective interpretation of the abnormal patterns by the colposcopist, and this technique suffers from the same inherent reproducibility problems as the PAP test and biopsy interpretation. QC of colposcopy has received adequate interest only recently. One of the partners (#8) is an established reference centre of excellence in colposcopy, and as such the natural unit responsible for QC of all colposcopic diagnosis of this project. In this work, digitised colposcopic images can be used, submitted for evaluation to the reference centre by the other partners. AVI will be validated by comparing the visual patterns of the patients with colpo-photographs, distributed to the partners by the reference centre. Similarly, the quality of cervicography is controlled by using colposcopy and histology as gold standards, and if necessary, external certified cervicography reviewers (partner #7).

Deliverables
Deliverables of this WP are the correct diagnosis of HPV and CIN lesions in PAP smears and biopsies, as well as adequate performance of colposcopy and accurate interpretation of the colposcopic findings at each examination. The same is true with AVI and cervicography, which are expected to be done according to the highest standards. The production of these deliverables starts immediately when the project is activated, followed with some weeks delay by the first colposcopic examinations. Because these examinations are made also at the follow-up visits, these deliverables will be issued through the 36-month duration of the project.

Milestones and expected results
Role of the three classical diagnostic tests (PAP smear, colposcopy and biopsy), in diagnosis and follow-up of cervical cancer precursors and HPV infections is of crucial importance. In addition to the results of the DNA tests, the PAP smear abnormality is one of the selection criteria to enrol the patient in the follow-up cohort. This study design also allows the comparison of two additional diagnostic tests (AVI and cervicography) with these conventional diagnostic tools and HPV testing, in terms of specificity, sensitivity, PPV, NPV and ROC, using histological biopsy as the gold standard. The final results of this comparison are impossible to predict, but whatever the result might be, it will be of crucial importance in designing the cost-effective strategies for cervical cancer control in these two countries.

WP (6): CONCLUSIONS

Objectives:
The last of the WPs is the process by which all the different data elaborated during this combined cross-sectional and cohort study will be summarised as logical conclusions. The main objective of this activity is to produce sound data for the basis of designing the most feasible and cost-

effective strategy for the control of cervical cancer in these two LAM countries.

Description of work

At this stage, it is somewhat premature to give details on how this activity will be accomplished. However, the project will have a centralised data registry (maintained by the co-ordinator using SPSS 10.0 Programme Package), which is updated on real time. Towards the end of the project, this data file will become increasingly complex, particularly when the results of WP 4 are incorporated. To sort out all the meaningful results with practical importance will be a labour-intense task. That certainly necessitates close consultation with all partners, by taking into account their specific desires and interests. It is desirable to decide the principles of the analyses at earliest convenience, and conclude the technical part of the work as soon as all the data are incorporated in the file.

Deliverables

By combining the results derived from all these analyses, the final conclusions of the project can be reached, which in this case are the deliverables of this last WP. The final deliverable will be the cost-benefit analysis, whereby the produced biological data are transferred into meaningful calculations about the cost-effectiveness of each of the diagnostic approaches tested, when used alone or in different combinations. To accomplish this, the consortium might also consult the local experts available in the participating units.

Milestones and expected results

Because of the decisive nature of this final activity, the milestones and expected results of this Work Package are the same as the main conclusions of the whole project. The ultimate goal is to find out the combination of the diagnostic tools resulting in maximal improvement of the health care system in these two LAM countries, by incorporating a cost-effective, organised programme for controlling cervical cancer precursors, particularly covering the most vulnerable groups of high-risk women. Expectations are high that following the completion of this project, there will be good chances to sort out scientifically valid data to enable the design of effective and sustainable health policy which would make all women in Brazil and Argentina more equal in their access to an organised cervical cancer control.

4.ROLE OF PARTICIPANTS

The composition of the consortium, including the tasks, time table and contributions to be made by individual partners are summarised in **Table 1**. There are 8 contractors; 2 from EC countries (Finland, Italy), 1 representing Associated States (Slovenia), and 5 from two DEV (LAM region) countries (4 from Brazil, 1 from Argentina). **Partner #1** is the Diagnostic Center of Gynecological Cytopathology (SIZE, Ltd)(Ljubljana, Slovenia), where the co-ordinator of this proposal (Prof. Kari Syrjänen) currently works as a part-time consultant. As the leading cytopathology laboratory in its country of domicile, SIZE Ltd. has paid particular emphasis on the uncompromised quality of the PAP test in cervical cancer diagnosis and screening. The second EC **Partner #2**, is MediCity Research laboratory, which under command of Prof. Stina Syrjänen, is responsible for completion of Work Package 3 and 4 (HPV diagnostics; biological markers). **Partner #3** is the Laboratory of Biostatistics, National Institute of Health, Rome, which as the leading QC organisation of cervical cancer screening in Italy, is responsible for the rigorous internal- and inter-laboratory QC measures to be implemented in the LAM partner laboratories.

Partner #4 is Department of Gynecology, Hospital de Clinicas de Porto Alegre (HCPA), the School Hospital of Federal University of Rio Grande do Sul (UFRGS)(Porto Alegre, Brazil), where the local research group (headed by Prof. Paulo Naud) is in key position in this country, as regards to the expertise and experience in cervical cancer screening. According to their given estimates, approximately 750 CIN patients would be potentially eligible for the cohort in one year. **Partner (#5)** is Hospital Leonor Mendes de Barros – Secretaria da Saúde do Estado de São Paulo (São Paulo), primarily serving unprivileged women in downtown Sao Paulo. Under supervision of Dra. C. Roteli-Martins, and Dr. A. L. Filho (Partner #6), this clinic examines some 4.000 eligible women, of which approximately 250 are high-grade CIN patients. **Partner #6** is Instituto Adolfo

Lutz - Secretaria da Saúde do Estado de São Paulo (São Paulo), is a reputable institution located in downtown Sao Paulo, and the regular provider of cytology and pathology services to Partner #5. **Partner #7**, Universidade Estadual de Campinas (Campinas, Brazil), is a major University hospital and well equipped to run adequate HPV/CIN diagnostics. They have informed to contribute around 100 CIN patients into the cohort. **Partner #8**, Primera Catedra de Ginecologia, Hospital de Clinicas Jose de San Martin, Facultad de Medicina, Universidad de Buenos Aires (Argentina)(Prof. Silvio Tatti) is another major University hospital, with excellent facilities for HPV/CIN diagnostics. Being the reference centre for colposcopy in Argentina, this partner is responsible for all QC of colposcopy in this project, and estimates to contribute 200 CIN patients into the follow-up cohort.

5.TRAINING AND EXCHANGES OF SCIENTISTS

As determined from the initial feedback from the LAM partners, the participating clinics and laboratories have variable facilities and readiness to do the work to which they are committed. Some managerial issues as well as training and education is mandatory before the practical work can be effectively started. These issues comprise 1) practical tasks (i.e., co-ordination and management), and 2) work at conceptual level (**i.e., training**)**.** The latter should guarantee all the DEV partners the necessary expertise to fulfil their commitment in the project. Because not at optimal level in all LAM partners at the moment, the basic concepts of diagnosis, treatment and follow-up of women with HPV/CIN must be updated before Phase I of the project. This will be done by organising a **Practical Training Course (PTC)** on HPV and cervical cancer screening before the start of the practical work. The topics to be exhaustively covered include the clinical assessment (colposcopy, cervicography, treatment, follow-up) of the patients as well as the PAP smear diagnosis, histopathology, and molecular detection methods of HPV/CIN. This is most practically done in context with the first co-ordination meeting, and with the representatives of the EC partners as the faculty.

This basic learning is supplemented with the **technology transfer** from the EC- to the DEV partners, aimed to establish HPV testing and other molecular techniques in their laboratories towards the end of the project period. There are two optional ways to accomplish this; 1) scientists from the EC partners make the visit to the units in LAM partners and establish the appropriate technology in situ, or 2) scientists from the LAM institutions make the site visit to EC partners' to study the techniques there. Both options inevitably necessitate **exchange of scientists**, which belongs among the high-priority activities of this project. The latter is usually more cost-effective, and is the preferred mode of scientist exchange in this project.

During the continuation of the project, additional training will be provided by different means, including 1) continuous QC of all diagnostic work in DEV (WP 5), 2) prompt feedback given to the DEV partners, and 3) providing additional practical training (individually or collectively) whenever felt necessary. This is best realised by site visits to the LAM partners, combined with the co-ordination meetings. During **the 1st year**, a **minimum of 3** such **meetings** must be organised, followed by an interval of 6-9 months during the subsequent years.

6.DISSEMINATION ACTIVITIES

Dissemination of the information of this project is of paramount importance in all phases of this project. The purpose of this dissemination is to increase the general awareness of the public as well as health authorities on the importance of cervical cancer as a major health problem, and the great potential of active preventive measures. Fortunately, the LAM partners of this project have substantial previous experience how to find the right channels to do this. During the earlier site visits in these units, co-ordinator has become convinced (by personal experience) about the great potential and major interest of different news media (TV, radio, newspapers) in all the issues related to cervical cancer. In this dissemination strategy, three different levels can be distinguished; a) regional, b) state, and c) federal. The activities proven effective by these partners previously, vary from distributing leaflets to women on the streets of city centres, to interviews of the research leaders and international experts in TV and radio (regional/national channels), and

to articles written in local and national newspapers and women's magazines.

Apart from this type of dissemination activity targeted to the public, distribution of the scientific data is of key importance. In LAM, a lot of opportunities are offered to do this. A huge number of different national and LAM organizations exist in this Continent, covering the disciplines represented in this project. Numerous congresses are organized each year in the fields directly or closely related to the topics of this study. All the LAM partners are key members in a number of these organizations and have substantial previous experience as congress organizers. Invited keynote lectures, special sessions and workshops can be organised on these topics in future national, regional and LAM congresses, offering abundant opportunities for dissemination of the information and results of this project at different stages of its progression. In addition to abstracts, congress proceedings are frequently published from these congresses, opening another channel for dissemination of the data in written form. A plethora of LAM journals exist, which are potential publishing forums for the results. Albeit of key importance in disseminating the results in South-American Continent, their international impact is limited, however. Thus, the major focus of the publishing policy of this project will be on submitting the manuscripts to the high-ranking international journals.

Another important target group of these dissemination activities is the health authorities making decisions on the health policies at the local, regional and federal level in these two LAM countries. Early actions in this field are essential to disseminate among these decision-makers the awareness of the project design and aims, as well as the potential usefulness of its results in the future planning of cost-effective and equality-based strategies for cervical cancer control in these countries.

Finally, as the leading cytopathology laboratory in its home country, SIZE Ltd. (Partner #1) is an SME, and together with its two affiliated companies, ICS, Ltd. (International Cytopathology Services, Ltd. UK) and IMS, Co. (Integral Molecular Screening Services, US) enables prompt worldwide dissemination of the results through its web sites (www.icsglobal.co.uk) as well as commercial exploitation of the results in designing cervical cancer screening strategies in the third countries. Indeed, the design, implementation and monitoring of organised cervical cancer screening programmes is a part of the ICS Concept, and this is another channel for dissemination and exploitation of the research data obtained from this project.

TABLE 1. TIME-SCHEDULE AND WORKING PLAN OF EACH CONTRACTOR DURING THE 36-MONTH PERIOD OF THE PROJECT

Work Carried Out	Year One		Year Two		Year Three	
	1-6	7-12	1-6	7-12	1-6	7-12
1.Diagnostic Center of Gynecological Cytopathology, Ltd. (SIZE, Ltd)(Ljubljana, Slovenia)						
- Project Co-ordination	X	X	X	X	X	X
- Quality control of cytology and histology	X	X	X	X	X	X
- Centralised data registry (SPSS)	X	X	X	X	X	X
2.University of Turku (MediCity & Department of Oral Pathology)(Turku, Finland)						
- Hybrid Capture II (Quality Control)	X	X	X	X	X	X
- HPV PCR typing (as control of HCII)	X	X	X	X	X	X
- Immunohistochemistry (in part)			X	X	X	X
3.Cytohistopathology Unit, Laboratory of Epidemiology, National Institute of Health, Rome (Rome, Italy)						
-Internal Quality Control (QC) of cytology	X	X	X	X	X	X
- Inter-laboratory QC among partners	X	X	X	X	X	X
- Educational material and training	X	X	X	X	X	X
4.Department of Gynecology, Hospital de Clinicas de Porto Alegre (HCPA), the School Hospital of Federal University of Rio Grande do Sul (UFRGS)(Porto Alegre, Brazil)						
- Examination of the patients[1] (n=12.000)	X	X				
- Recruitment of HPV-CIN patients (n=750)	X	X				
- Taking the samples	X	X	X	X	X	X
- Follow-up of the patients	X		X	X	X	X
-Treatment of the patients	X	X	X	X	X	X
5.Hospital Leonor Mendes de Barros – Secretaria da Saúde do Estado de São Paulo (São Paulo, Brazil)						
- Examination of the patients[2] (n=4.000)	X	X				
- Recruitment of HPV-CIN patients (n=150)	X	X				
- Taking the samples	X	X	X	X	X	X
- Follow-up of the patients	X		X	X	X	X
- Treatment of the patients	X	X	X	X	X	X
6.Instituto Adolfo Lutz - Secretaria da Saúde do Estado de São Paulo (São Paulo, Brazil)						
- Cytology and histology of the samples[3]	X	X	X	X	X	X
- Immunohistochemistry (in part)				X	X	X
7.Universidade Estadual de Campinas (Campinas, Brazil)						
- Examination of the patients[4] (n=3.000)	X	X				
- Recruitment of HPV-CIN patients (n=100)	X	X				
- Taking the samples	X	X	X	X	X	X
- Follow-up of the patients		X	X	X	X	X
- Treatment of the patients	X	X	X	X	X	X
8.Primera Catedra de Ginecologia, Hospital de Clinicas Jose de San Martin, Facultad de Medicina, Universidad de Buenos Aires (Buenos Aires, Argentina)						
- Examination of the patients[1] (n=7.000)	X	X				
- Recruitment of HPV-CIN patients (n=200)	X	X				
- Taking the samples	X	X	X	X	X	X
- Follow-up of the patients		X	X	X	X	X
- Treatment of the patients	X	X	X	X	X	X
- Centre for QC of colposcopy	X	X	X	X	X	X
SUMMARY OF THE PROJECT (All Contractors)						
- Prevalence of HPV infections and CIN				X		
- Risk factors for HPV and CIN					X	
- Natural history of the disease						X
- Performance tests of the diagnostic techniques						X
- Guidelines for cervical cancer prevention strategies in these LAM countries						X

[1]PAP test, Aided Visual Inspection (AVI), HCII, histology; [2]PAP test, AVI, self-sampling, HCII, [3]PAP test and histology for Partner 5; [4]PAP test, AVI, cervicography, HCII, histology; ( ) Number of patients expected to be examined and enrolled in the cohort during the first 12 months.