60 years of the Department of Cytopathology at the Institute of Oncology Ljubljana

60 let Oddelka za citopatologijo Onkološkega inštituta Ljubljana

Živa Pohar Marinšek

Department of Cytopathology, Institute of Oncology, Zaloška 2, SI-1000 Ljubljana, Slovenia

ABSTRACT ► Cytopathology was introduced at the Institute of Oncology, Ljubljana (IO) in 1951 by prof. Božena Ravnihar and was applied to routine practice a year later. In 1959 prof. Marija Us-Krašovec became the first head of the cytology laboratory. She introduced fine needle aspiration biopsy (FNAB) as well as many ancillary techniques. Cytopathology became an important diagnostic tool and the cytology laboratory grew into the Department of cytopathology which was the only centre for FNAB in Slovenia for many years. According to the number of FNABs the cytopathology at the IO is still one of the largest cytology laboratories in Europe. Two modern, indispensable ancillary techniques which have been developed through the years are immunocytochemistry and flow cytometry. We have achieved a good quality of immunocytochemical staining as it is evidenced by high marks received in external quality control. Immunophenotypisation by flow cytometry for diagnosing lymphomas in cytology took many years to develop. We had to adapt protocols for sample preparation and the interpretation of results to suit the needs of cytopathology. Today, the multi-parameter measurements make it possible to use this ancillary technique in various samples including those with poor cellularity. In addition to routine diagnostic work the Department of Cytopathology was involved also in research and education on the undergraduate and post graduate level. All the cytopathologists working today in Slovenia, as well as many pathologists from abroad, received their training in cytopathology at our department. During the last few years we also train cytotechnologists in cervical screening for the need of the whole country.

Key words ► cytopathology, history, Institute of Oncology

IZVLEČEK ► Na Onkološkem institutu v Ljubljani je citološko diagnostiko uvedla prof. Božena Ravnihar leta 1951. Metoda je v polni meri zaživela leto kasneje. Leta 1959 je postala vodja citološkega laboratorija prof. Marija Us-Krašovec. Uvedla je aspiracijsko biopsijo s tanko iglo (ABTI) in mnoge druge dopolnilne tehnike. Cytopatologija je postala pomembna diagnostična metoda in citološki laboratorij se je razvil v Oddelek za citopatologijo, ki je bil dolga leta edini center za ABTI v Sloveniji. Glede na število vzorcev ABTI smo še vedno eden največjih citoloških laboratorijev v Evropi. Dve moderni in danes nepogrešljivi dopolnilni metodi, ki smo jih razvili z leti sta imunocitokemija in pretočna citometrija. V imunocitokemiji smo dosegli visoko kvaliteto kar se odraža na dobrih ocenah, ki jih prejemamo ob zunanji kontroli kvalitete. Imunofenotipizacijo s pretočnim citometrom za diagnostiko limfomov smo razvijali dolga leta. Potrebno je bilo namreč prilagoditi protokole za pripravo vzorcev in za analizo rezultatov meritev potrebam citologije. Danes lahko izvajamo multiparametrne meritve na različnih vzorcih vključno s takimi, ki vsebujejo majhno število celic. Poleg rutinskega dela smo bili delavci odelka vselej udeleženi tudi v raziskovalnem delu in v izobraževanju dodiplomskega in podiplomskega študija. Vsi citopatologi, ki danes delamo v Sloveniji ter veliko patologov iz tujine smo se izobraževali na Oddelku citopatologije OIL. V zadnjih letih smo prevzeli tudi izobraževanje presejalcev brisov vratu maternice za potrebe cele države.

Ključne besede ► citopatologija, zgodovina, onkološki inštitut

The early period of cytology at the Institute of Oncology

Young people are seldom interested in the history of the organization for which they work. Such interest is usually not aroused until one has to prepare a speech or an essay in honour of some anniversary. It is then that we realize how little we know about our past and that we missed the time when we could have asked our elders. This statement is certainly true for me and for my younger colleagues, cytopathologists, since we know very little about the first eight years of cytopathology at the Institute of Oncology in Ljubljana (IO).

Fortunately, some old records have been spared from the occasional floods of the institute's archives in the basement. Among them was a draft for a publication entitled History and Development of The Institute of Oncology written in August 1963 by dr. Leo Šavnik and dr. Božena Ravnihar (1,2). On page 29 there is a short paragraph in which it is stated that cytological diagnostics was introduced at the Institute of Oncology in 1951 by dr. Božena Ravnihar. The same paragraph also contains the information that dr. Leo Šavnik brought the dyes for Papanicolaou staining from the United States as a gift to the IO from the United Yugoslav Relief Fund in New York. At that time dr. Šavnik was the director of the IO while dr. Ravnihar was acting as the scientific director and working also as a clinician, manager of the Cancer registry, and as the only doctor in the histology laboratory. Among the few written documents from the early period of cytology at the IO is also the index book containing patients' names and numbers of specimens for the period of years between 1952 and 1959. Since we were not able to find any cytological reports from 1951 we assumed that during the first year the method was still being tested. Therefore, we named the year 1952 as the official birth date of cytology when 224 cytological samples have been recorded.

During the following years the number of cytological specimens ranged from 200 to 400 until 1959 when the number rose to 707. All cytological samples belonged to exfoliative cytology, including cervical and vaginal smears, sputum and effusions. During these first years cytological samples were processed and stained in the histology laboratory by Svetozar Nečak and Jožica Kovačič, laboratory technicians whom prof. Ravnihar trained also as the first screeners. Prof. Ravnihar was examining the smears herself. She was aware that pathology and cytology would not flourish unless new people could be hired who would devote all their time to one speciality only (1). In 1953 Dr. Pavla Mavec was hired for the histology laboratory but she also examined cytological samples. Due to sever financial restrictions and the lack of available space in the old military facility of Šempeter, further expansion was not possible until six years later when dr. Marija Us (later prof. Us-Krašovec) was hired in the second part of 1959 and made the head of the new cytology laboratory. Janez Škrk, a biologist joined the cytology laboratory in the same year. However, he worked in cytology only five years and moved to the radiobiology unit in 1964.

The arrival of prof. Us-Krašovec marked a new era for cytology at the IO. She expanded the exfoliative cytology by including urinary samples, cerebrospinal fluids, breast discharges, scrapings of skin lesions and bronchial lavage samples into cytological examination. Most importantly, prof. Us Krašovec introduced fine needle aspiration biopsy (FNAB) in 1959. She learned about FNAB from prof. Berta Jereb who has just returned from Karolinska hospital in Stockholm, Sweden where FNAB was already in use by dr. Zajiček and dr. Franzén. All the pioneers of cytology in Slovenia had to learn the method more or less on their own with the aid of few available books like the Diagnosis of uterine cancer by the vaginal smear written by Papanicolaou and Trout. In exfoliative cytology, prof. Us-Krašovec received some help also from prof. Rravnihar and from dr. Jule Kovačič from the Clinic of Gynecology. Three years after her arrival at the IO prof. Us-Krašovec was able to go to the Sabbatsberg hospital in Stockholm where she learned more about exfoliative cytology but had to earn her keep by working as a laboratory technician and teacher for screeners. However, the application of FNAB she had to learn all by herself, using systematic comparison of FNAB samples and classical histology slides from the same tumours.

Exfoliative cytology and fine needle aspiration from the sixties onward

Fig. 1 shows the dynamics in the number of cytological specimens through the years while Fig. 2 demonstrates also the relationship between fine needle aspirates (FNAs) and the exfoliative cytology samples from 1965 onwards. The number of cervical smears experienced much more fluctuations compared to the other cytological samples. There was a steady increase in the number of gynaecological samples until 1975. In 1976 we noted an increase in the number of samples by 150% since annual gynaecological check-ups were introduced in Slovenia. For the next 13 years there were only minor fluctuations in the order of 200 to 400 samples. A major decline in the number of cervical smears started in 1989 and during the next three years the number of smears dropped by 56% due to opening of new cytological laboratories in other Slovenian cities. The second extreme increase in the number of cervical smears happened in 2005. This year marked the beginning of the organized screening programme for cervical cancer in Slovenia (ZORA) and new regulations were imposed on the cytological laboratories for cervical cytology. Stricter regulations resulted in closing of nine cytology laboratories for cervical screening and the remaining ten laboratories had to take on a bigger workload.

The number of non-gynaecological cytology sample continued to climb from 1960 until the mid -nineties when they reached a plateau at about 3500 samples per year. The only exception was the sputum cytology which represented the main number of cytological samples until 1967. Sputum cytology started to lose its importance during the seventies and by the end of the nineties the number of sputum specimens dropped for 300% due to new techniques for diagnosing pulmonary carcinoma.

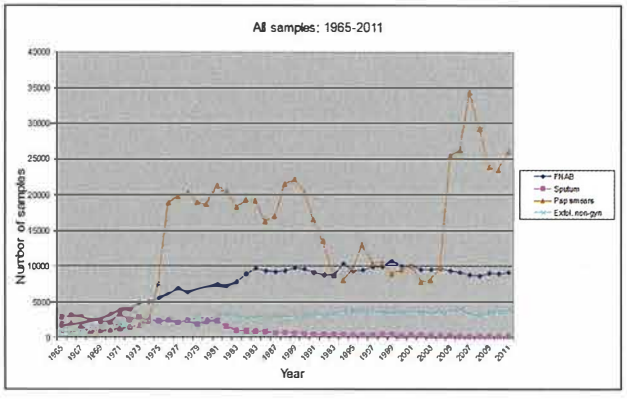

Figure 1. *The number of FNAs and exfoliative non-gynecological samples has been increasing for 20 years before reaching a plateau in the might eighties; The number of sputum samples was high until the eighties and dropped sharply afterwards; The number of Pap smears experienced several sharp increases and falls between 1965 and 2011.*

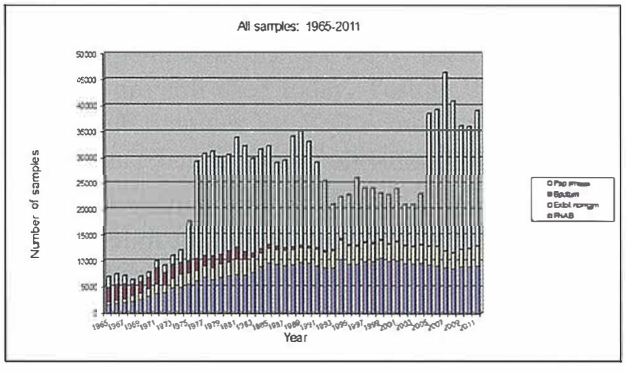

Figure 2. *Similar representation of the number of cytological samples as in Fig. 1. The columns demonstrate the relationship between various types of cytological specimens.*

In 1960, a year after FNAB was introduced at the IO, there were already 236 cases. There was a continuous rise in the number of FNAs until 1989 when we noted 9700 samples. Later on the number of FNAs fluctuated between 8541 and 10561.

First FNAs at the IO were performed by needles mounted on glass syringes which were awkward to handle and did not produce enough vacuum. Plastic syringes were introduced much latter, after an accident when the syringe exploded in the hands of prof. Us-Krašovec. The syringe holder was introduced in 1969, after prof. Us Krašovec returned from her second visit to Sweden. Further advancement in the technique of FNAB came in the second half of the eighties with the introduction of rapid on-site evaluation of specimens obtained by the cytopathologists. The practice of quick staining by toluidine and checking the cellularity of samples under the microscope at the patients' site decreased the number of unsatisfactory samples. Later on we substituted toluidine with hemicolor.

At the end of the sixties the cytology laboratory obtained an automatic stainer, manufactured by Shandon. It was used until the mid-eighties when it had to be substituted by a new one. However, the new machine used a different technology which was not compatible with the glass slides used at that time. The size and thickness of slides varied which caused many of them to break. The cytotechnologists had to return to manual staining, a practice which lasted almost 20 years.

Until 1987 the cytopathologists performed the majority of FNAs. For a while a few sample were collected also by the clinicians at the Breast cancer clinic at the IO. Such practice was later discontinued due to the significant difference in the percentage of unsatisfactory samples obtained by the clinicians and the cytopatologists. In 1987 the radiologists started to perform ultrasound- guided FNAB and therefore "blind" aspirations of palpable deep seated lesions in the abdomen were no longer performed by the cytopathologists. We have been able to monitor the quantity and the quality of FNAB performance on a regular basis since 2006. The cytopathologists of our department performed between 5000 to 5700 FNAs per year which was 60% of all FNAs processed. Approximately 20% of FNAs were performed by radiologists under ultrasonographic control, 70% of them were performed at the IO. The rest of the FNAs came from breast disease clinics outside Ljubljana. Comparison of the quality of aspirates obtained by cytopathologists and specialists of other professions showed that the number of unsatisfactory samples is lower when FNAB is performed by cytopathologists.

The use of new technologies and ancillary techniques

Many new ancillary techniques were introduced at the Department of cytopathology during its sixty years of operation. Some of them are no longer in use because they became outdated with new technological advances. The two ancillary techniques which re-

present milestones in the further development of our department are immunocytochemistry (ICC) and flow cytometry.

Ancillary techniques in the early days

In 1960 the cytology laboratory obtained the first binocular microscope, the Anoptral contrast while in 1961 the IO was able to buy a microscope named Zetopan (3) which had multiple functions: light, phase contrast and fluorescent microscopy as well as photomicroscopy. Phase contrast microscopy was introduced already in 1961. It was used mainly for intraoperative assessment of effusions for the presence of malignant cells. The technique enabled immediate viewing of smears without staining since quick stains were not available at the time. The fluorescent microscopy came into use in 1963 and was applied only in research with the aim of eventual application to routine use. The photomicroscopy function was in use the longest, until the mid-eighties and a large body of microphotographs has been accumulated at the department for educational purposes. Zetopan was located in the cytology laboratory but was used also by people from other laboratories. Some pathologists even came from other Slovenian cities in order to use the photomicroscope.

Cytochemistry was introduced in the cytology laboratory in 1962, an ancillary technique which is seldom used nowadays since it has been mostly replaced by ICC.

The electron microscopy was introduced in 1976 but the IO did not have its own microscope at the time. Prof. Us-Krašovec used the microscope at the Institute of biology, Faculty of Medicine University of Ljubljana. The IO bought an electron microscope in 1986. However, it was not a modern machine and working with it was time consuming. Preparation of specimens was laborious and their examination in total darkness was stressful for the eyes. Specimen preparation for microscopy was performed by laboratory technician Vesna Gril (later Žitnik). Electron microscopy was useful for approximately15 years, mainly in classification of sarcomas. The method was judged important enough at the time for dr. Pohar Marinšek to spend one month learning electron microscopy in the Norwegian Radium Hospital in Oslo in 1989. However, electron microscopy was eventually replaced by ICC and the molecular techniques.

Modern technical advances and ancillary techniques

Among the new technologies and ancillary techniques which were introduced in the eighties and nineties were ICC, flow cytometry, image cytometry, filtration of urinary samples and the introduction of Delaunay fixative. Image cytometry, introduced in 1993, was used only in research projects.

Delaunay fixative

A novelty introduced in the eighties was the substitution of ether-alcohol with the Delaunay fixative. Prof. Us-Krašovec learned about this fixative from dr. Dalquen at the European congress of pathology in Athens in 1985. Dr. Dalquen from Basel, Switzerland sent the recipe and the new fixative was introduced into routine practice at our department in 1987 after it was tested by comparing the quality of Papanicolaou staining between specimens fixed in ether-alcohol and in Delaunay. Delaunay proved to be an excellent fixative also for ICC staining ant it is still used today.

Filter imprint technique

In 1994 the Department of cytopathology acquired the apparatus for filtering urine specimens. The new filter imprint technique greatly increased the cellularity of preparations compared to classical smearing of urinary sediment. The original method was changed by using immediate spray fixation of imprints thus eliminating the need for cold slides (4). The method is still used today.

Immunocytochemistry

The introduction of ICC as an ancillary technique represents one of the most important milestones in cytopathology. The Department of Cytopathology at the IO introduced this method in 1986. The method was primarily developed for the use on histological sections and there were no protocols for the use in cytopathology. Therefore, it was necessary to adapt the method for the use on cytological material. Many parameters of the staining procedure had to be tested including fixation protocols, antibody dilutions, transport media for cell preservation and the appropriate type of cytological preparation. The implementation of a high standard ICC with good diagnostic accuracy took many years. In order to start with the new technique prof. Us-Krašovec asked for help outside our

department. Between 1986 and 1988 the ICC was performed by prof. Ida Eržen at the Institute of Anatomy, Faculty of Medicine University of Ljubljana since our department did not have anybody with the necessary technical knowledge for the application of the procedure. With the arrival of Srebotnik Kirbiš, an engineer of biotechnology, and after some initial instructions from prof. Eržen we started to perform the ICC at our department. At the beginning we performed ICC on smears and were not satisfied with the results. Drawbacks of such practice were heavy background staining with some antibodies and a limited number of suitable samples for ICC as well as uneven cell distribution on slides. These difficulties were progressively remedied by meticulous testing, the use of positive and negative controls and through the collaboration between the cytopathologists and Srebotnik Kirbiš who received some additional training in ICC at the Division of Cytopathology, University of Basel, Switzerland in 1990. A great advantage for ICC was the introduction of cytospin preparations and the use of the transport medium, the washing solution. Dr. Bizjak Schwarzbartl brought the recipe for the transport medium and the information on how to prepare cytospins from her visit to the immunology laboratory at the Slotervaart Hospital in Amsterdam in 1988 (5). The original recipe and the procedure for preparing cytospins from specimens suspended in the washing solution have been modified later in order to suit better the cytological samples with small number of cells. The new recipe and the method of cytospin preparation for ICC are still used today. Testing the suitability of various fixatives for ICC demonstrated that Delaunay, an ethanol based fixative was suitable for the demonstration of many antigens. However, methanol proved to be a universal fixative especially suited for nuclear and cell surface antigens (6).

In 1986 only 27 ICC reactions were performed and only eight different antibodies were in use (6). In 1999 we performed 1752 ICC reactions for diagnostic purposes and used 40 antibodies (6). During the last ten years we have been performing between 2500 and 3500 ICC reaction per year for diagnostic purposes. The number of different antibodies used is increasing because new, tumour specific antibodies are constantly emerging. Currently, we are using 61 various antibodies mostly for diagnostic purpose. The determination of predictive markers, the hormonal receptors on primary and metastatic breast cancer samples, has been in use for ten years.

During the first 16 years the ICC was performed manually and the procedure took 24 hours. Since the method was complicated only two people performed the ICC staining. The year 2002 marked a new period in ICC of our department because we switched from manual to automatic ICC staining on the Ventana immunostainer. Since 2005 we have been progressively educating more cytotechnologists in ICC staining and today there are four people who master the procedure: Nataša Nolde, Milena Petelin, Brigita Šturbej and Marijana Matić.

In addition to routine ICC staining we have to perform a large number of ICC reactions for the purpose of quality control. During the last five years the number of reactions performed on control samples has increased greatly. In 2011 the number of control slides has been three times higher compared to the figure in 2002. Simultaneously with the introduction of automated ICC staining we also took a step forward in the quality control by joining the external quality control scheme organized by UK NEQUAS. Fig. 3 shows the scores received for ICC staining during the last ten years. High scores which we have received during the last few years are proof of good performance in ICC.

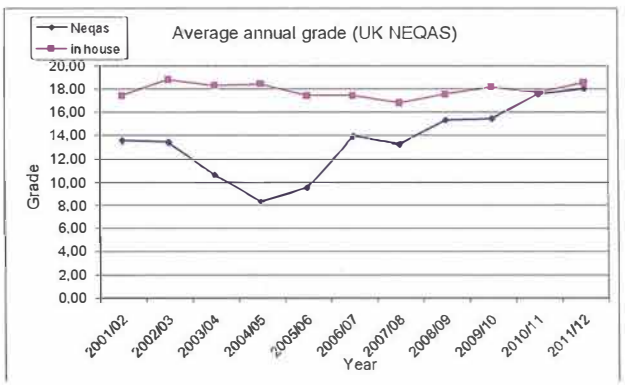

Figure 3. *The Department of cytopathology has been receiving high grades for the ICC staining of the in-house control slides. The grades for the ICC staining of slides provided by UK NEQUAS have been somewhat lower initially and have improved during the last few years.*

Flow cytometry

DNA measurement

The measurement of cellular DNA quantity by flow cytometry was introduced in 1988 on a flow cytometer manufactured by Partec in Munster, Germany. During the first few years a couple of instruments have been used in succession due to quick technical improvements. Since such flow cytometer was the first of its kind in Slovenia it was necessary to learn the whole technical procedure from sample preparation, measurements and interpretation of results. For this reason, prof. Us- Krašovec and laboratory technician Brigita Šturbej visited Partec headquarters a few times in order to familiarize themselves with the

new method. Four people are currently involved in DNA measurements: cytotechnologists Brigita Šturbej and Marijana Matić and biologists Jaka Lavrnčak and Andreja Brožič. The prognostic parameters extracted from these measurements are ploidy and proliferative activity. During the nineties the cytometer was used mainly in research projects. Determining DNA ploidy in bladder washings of patients after treatment for urothelial cancer in order to detect early recurrence used to be a routine practice for a while but has been discontinued. The method is currently used for the determination of proliferative activity in breast cancer FNA samples and for the determination of ploidy in blood and bone marrow samples of children with leukaemia and in rare childhood tumours.

Immunophenotypisation

Immunophenotypisation (IFT) by flow cytometry was introduced in 1998 and it represents today one of the most important activities of our department. At the IO the method is used for differentiating between lymphadenitis and lymphoma and for the subclassification of lymphomas. An additional use of flow cytometry is quantitative determination of certain antigens like CD20 and CD 52 which have predictive value for planning specific therapy. At the end of the nineties IFT by flow cytometry was not an established ancillary method for diagnosing lymphomas in cytology and few centres in the world had any experience with it. Furthermore, the protocols for sample preparation and analysis of the results of measurements were originally designed for peripheral blood and bone marrow and were not best suited for FNAs of lymph nodes or body fluids. Therefore, a lot of time and effort was put into adapting the protocols to suit our needs. The adapted techniques were the result of our own expertise mainly introduced by dr.Vera Kloboves Prevodnik. In order to familiarize herself with the necessary technical aspects of the flow cytometer, dr. Kloboves Prevodnik visited the flow cytometric laboratories at the University of Newcastle upon Tyne, United Kingdom (1999) and at the BC Cancer Agency in Vancouver, Canada (2001). In addition, prof. Us-Krašovec invited a few experts in flow cytometry to visit our department. (dr. B. Shenton, Dr. I. Broderick and dr. M. Ormerod from England.)

The sensitivity of flow cytometer depends on the number of lasers used. At the beginning we performed only two-colour flow cytometric measurements. In order to increase sensitivity and specificity of results and to make IFT possible on samples with very few tumour cells we progressively introduced multi-colour IFT and modified the protocol for sample preparation. Three-colour IFT was introduced in 2001and four-colour IFT in 2005. Due to such modifications we could perform IFT not only on lymph node samples but also on body cavity fluids, cerebrospinal fluids and vitreum. The new method of sample preparation raised the number of suitable specimens for IFT from 36% to 76% (7). In 2007 it was necessary to replace the old flow cytometer and the IO bought the FACCanto which enabled six-colour IFT. This new development was necessary in order to detect small populations of lymphoma cells in samples of peripheral blood and bone marrow from lymphoma patients. The introduction of this new technique took approximately two years to develop in a stepwise manner from five-colour to six-colour measurements. The six-colour method was put into use in 2009 for IFT of T-cell lymphomas, plasmocytomas, peripheral blood and bone marrow samples. The four-colour IFT is still used when B-cell lymphomas are suspected.

In 1998 when IFT was introduced, only 70 samples were analysed. Four years later, the number of samples increased to 470. In 2011, thirteen years after introducing flow cytometry, IFT was performed in 1098 samples. The sensitivity and specificity of diagnosing lymphoma in our department using the combination of morphology and flow cytometry is 0.99 and 0.92 respectively (8).

The progress in IFT by flow cytometry was made possible mainly through the work of dr. Kloboves Prevodnik. The sample preparation and measurements were performed by laboratory technicians Brigita Šturbej and Marijana Matić until 2005 when biologist Jaka Lavrenčak joined the team. Flow cytometer is a complicated apparatus which needs the attention of highly skilled personnel with a feeling for computer technology. The progressive improvement in IFT went hand in hand with more complicated computer software. The ability of Lavrenčak to handle the ever more demanding software was invaluable for the successful development of the method. The introduction of IFT of peripheral blood and bone marrow increased the workload due to more demanding measurements and result interpretation. Therefore, dr. Ulrika Klopčič joined the flow cytometry team as well as a new biologist, Andreja Brožič. Dr Klopčič is currently involved also in developing a protocol for subclassication of low grade lymphomas by flow cytometry.

Sentinel lymph nodes

The intraoperative examination of imprints from sentinel lymph nodes in patients operated for breast can-

cer was introduced in 2001 when dr. Pogačnik was head of the Department of Cytopathology. The number of sentinel lymph node imprints was growing rapidly during the first two years and in 2003 we examined imprints from 674 patients. Due to regular US examination of axillary regions in women with breast cancer many metastatic lymph nodes are aspirated under US-control thus eliminating the need for sentinel node biopsy. Therefore, the number of examined sentinel lymph node imprints decreased. During the period between 2007 and 2011 we examined from 307 to 355 sentinel lymph node imprints per year.

HPV testing

Determination of high-risk HPV viruses in cervical brushings using the Hybrid capture 2 method was introduced in our department in 2010 when dr. Pohar Marinšek was head of the Department of Cytopathology. This molecular technique was introduced in Slovenia as an additional method in cervical screening for the identification of women with abnormal Pap smears who have a higher risk for developing cervical cancer and have to be followed more regularly. Four people are currently performing the HPV testing: biologists Nataša Nolde and Mojca Lešnjak, as well as laboratory technicians Simona Uhan Kastelic and Vesna Žitnik.

Improved method of cell acquisition from liquid samples

In 2011we improved the technique for the preparation of liquid samples. In addition to classical centrifugation we routinely make samples also by direct smearing of fluids, by cytocentrifugation and by filtration of haemorrhagic fluids. Using several techniques simultaneously raised the percentage of detecting malignant cells in fluid samples by 10%.

Licence for operation

In 2009 the Department of Cytopatholoy received an operational licence issued by the Ministry of health after we successfully passed the external quality control audit. This achievement may be regarded as a technical advancement in quality control. The preparations for the audit lasted several years and included courses in quality control and the preparation of a large volume of documentation. The personnel of the whole department was involved in the preparations, however, most of the work was performed by biologist Nataša Nolde, the head of the laboratory in the Department of cytopathology.

Research activity at the Department of Cytopathology

The people working at the Department of Cytopathology have constantly been involved in research in addition to the heavy routine workload. Prof. Us-Krašovec remembered that prof. Ravnihar has insisted from the start of the cytology laboratory that the IO has to be involved in research in all its fields in spite of the overall poor conditions under which the IO was operating during those early times. Through the years research in cytopathology was part of various projects financially supported by the Foundation of Boris Kidrič, Research Community of Slovenia, IO, Ministry for education, science and sports and the State programme for early detection of cervical cancer (ZORA). All cytopathologists and several medical associates were involved in research projects. Twelve of them were headed by the cytopathologists of our department. The knowledge accumulated during these sixty years has been published in many doctors' and masters' of science dissertations, in numerous articles published in medical journals in Slovenia and abroad as well as presented at various scientific meetings. The fields of research activity can be divided into a few categories. One of them is the study of morphological characteristics of various neoplasms in order to gain knowledge of the variability of cytological appearance of neoplasms and to improve the diagnostic accuracy. Studies which also included accuracy in diagnosing a specific entity served also as quality control. The second field of research involved testing new techniques for its practical applicability in cytology. The third field involved the use of cytology for monitoring response to treatment.

It is not possible to mention all the works. I selected only a few representatives of each field of research.

One of the first research projects performed in the cytology laboratory involved testing the applicability of new technique, namely fluorescent microscopy. Prof. Us-Krašovec tested the potential applicability of various fluorochromes for the purpose of diagnostics in oncology (9). Another area of her early research involved monitoring response to therapy. Her first such research was performed on cells in tissue cultures obtained from body fluids before and after chemotherapy and radiotherapy (10). The changes were monitored using light, phase contrast and fluorescent microscopy. Growing tissue cultures was also one of the tasks of the cytology laboratory at that time.

Another important research in the same field coordinated by prof. Us-Krašovec in cooperation with prof. Marija Auersperg was performed during the nineties. The effects of chemotherapy were monitored on samples from thyroid tumours, squamous cell carcinomas and on some sarcomas. Dr. Pogačnik, dr. Ruparčič Oblak and dr. Pohar Marinšek were also participating in this research with sample collection and morphological evaluations. The timing of chemotherapy applications were adapted to each patient according to the changes observed on morphology and on DNA measurements in cytological tumour samples. Such individualized approach to therapy produced good results in patients with large and mostly inoperable tumours that normally did not respond well to chemotherapy. The results of various aspects of this research have been shown in many scientific meetings (11-13). In spite of the encouraging results the practice was discontinued for several objective reasons: inability to demonstrate the advantage of such treatment in comparison with a control group, impracticality of frequent FNAs which were a burden to the patient and the cytopathologists, insufficient evidence for the cost benefit of such treatment (14).

The research in the field of cytomorphology of neoplasms produced some dissertations and many articles. In the era when ICC was already established as the valuable ancillary technique the morphology and ICC were evaluated together. One of the first such works was the evaluation of cytomorphology of samples from fibroadenoma (15). Two early works were performed in the field of lymphomas (5,16). Many similar morphological evaluations were performed on series of breast neoplasms (17), thyroid tumours (18), sarcomas (19,20), childhood tumours, bone tumours, lymphomas, salivary gland tumours. In addition to the morphological and ICC evaluations some works contained also an evaluation of diagnostic accuracy obtained by correlation between cytopathology and histology. (21).

Testing of new ancillary techniques applicable to cytological samples included ICC, flow cytometric DNA measurements, IFT with flow cytometry and image cytometry. Early research with fluorescent microscopy has already been mentioned above.

Most of the research in ICC was not part of some broad research project. It was the necessity in order to apply this ancillary method in the way that it would give reliable and reproducible results. An example of such research is ICC demonstration of hormonal receptors in FNAs of breast cancer. Before the method was introduced into routine practice we tested it for two years on breast cancer cells in tissue culture and subsequently on tumour cells obtained by FNAB of breast carcinoma in resected specimens (22). Similar testing was performed for ICC determination of Her2 (23).

Flow cytometric DNA measurements for monitoring therapy have already been mentioned above. A few other important works in this field were concerned with the standardization of DNA measurements on the basis of which other studies were possible (24). The results of the study investigating the usefulness of ploidy and proliferative activity as prognostic markers in breast carcinoma are nowadays used in routine practice (25). Research in IFT with flow cytometry was very practically oriented and constituted part of the development of this new method for diagnosing and subclassification of lymphoma (8).

Research in image cytometry can be designated as basic research. The predecessor of this method was image analysis of cellular morphology which was attempted already in 1986 (26). The purpose of image cytometry, which is a quantitative method, was the search for new diagnostic and prognostic markers in oncology. The principle is based on computer analysis of nuclear images photographed with a high resolution camera in order to determine DNA quantity and variations in chromatin structure and organization. In 1993 dr. Margareta Strojan Fležar and dr. Breda Škrbinc, two young researchers were engaged to work in this new field under the mentorship of prof. Us-Krašovec and in collaboration with Branko Palčič and his team of co-workers from the British Columbia Cancer Agency in Vancouver, Canada. The first works with the image cytometer CytoSavantTM at the IO were concerned with technical aspects of specimen preparation (27) and staining as well as with development of software for the analysis and presentation of nuclear features (28). Later works included investigation of nuclear features in some normal tissues and in tumours of breast, thyroid, soft tissues, lung, head and neck and cervix. Research in the field of cancer detection was concerned with determining nuclear features in normal cells of cancer patients, the so called malignancy associated changes (MAC). The search for these nuclear features was investigated in buccal mucosa cells of patients with lung and breast cancer (29).

Other professional activities of the Department of Cytopathology

The Society for Cytopathology was founded in 1964. For 43 out of the 48 years the Society was led by prof. Us-Krašovec, dr. Pogačnik, dr. Bizjak Schwarzbartl, dr. Pohar Marinšek and dr. Kloboves Prevodnik. The aim of the Society is to contribute to the education of its members, to the organization and development of medical service in Slovenia and to contribute to research. The Society organized three to seven meetings annually, seminars in electron and flow cytometry as well as tutorials with microscopy sessions covering cervical smears, breast, thyroid, respiratory pathology, cytology of body fluids and lymphoma. Among the most important events organized by the Society of Cytopathology were the organization of the 4th and the 24th European Congress of Cytology held in Ljubljana.

The education of cytotechnologists and screeners was also the responsibility of the cytopathologists. Four doctors and one engineer of biotechnology from the Department of Cytopathology at the IO were involved in teaching cytology at the Secondary School for Pharmacy and Health Care for 40 years (1962– 2002.)

In 2005 the Ministry of Health of Slovenia authorized the Department of Cytopathology at the IO to organize on a regular basis six-months courses in cervical screening for the needs of the whole country. In preparation for these courses we asked for help dr. Jasenka Matisic who was the head of the Cytopathology department at the BC Cancer Agency in Vancouver, Canada where the school for cervical screening has been in practice for many years. The Canadian colleagues sent us their curriculum as well as two head screeners, Brenda Smith and Gwen Ross for a period of four months to help in the teaching process. The Canadian curriculum was revised to suit our use by dr. Strojan Fležar who was the head of the school programme for screeners in Slovenia and was also overseeing all the preparations.

The preparations for the organized screening programme for cervical cancer lasted eight years and all cytopathologists of our department colaborated. Among the cytopathologists from the Department of Cytopathology who were most intensely involved in the preparation and execution of the screening programme was dr. Pogačnik.

An important contribution to an organized education of cytopathologists was the effort of prof. Us-Krašovec towards the acceptance of the specialization in cytopathology into the curriculum of the specialization in pathology. Her first proposal in 1965 was refused and then accepted in 1975. The curriculum for cytopathologists which is valid today dates from 1990. All the cytopathologists working in Slovenia today received their education in cytopathology at the Department of Cytopathology at the IO. All cytopathologists and some cytotechnologists of our department had the privilege of continual education through the attendance of pathology and cytopathology courses and congresses as well as occasional visits to the cytology laboratories abroad. Some of the visits to foreign institutions have already been mentioned in the text above. In addition, dr. Pogačnik visited the Institute Curie in Paris in 1978 and in 1986. The second visit was intended for learning the ICC determination of hormonal receptors in cytology. Dr. Pohar Marinšek was the guest of the cytology laboratory at the Radiumhemmet Institute of Karolinska hospital in Stockholm, Sweden in 1986. The main advantage of the visit to Karolinska was learning cytomorphology from the extensive set of collected cytology samples from various organ systems. The visit was also an initiative for the preparation of our own collection of cytology samples which we use nowadays as a reference set in rare cases and for teaching purposes.

The administration and the laboratory information system

The cytology laboratory did not have any administrative help until 1976. The doctors had to type the cytological reports by themselves while the cytotechnicians and the screeners helped in all other administrative tasks. At first, the diagnoses were recorded in the form of report sheets. Later on, all reports had to be typed directly into patients' case history folders. For a short time cytological reports were typed on special green cardboards. All cytological reports for the same patient appeared together on one cardboard. From the mid-seventies on the cy-tological report sheet was introduced again and stayed until the present. The style of the report changed a few times.

In 1965 the cytology laboratory already possessed the first databank for the cytological material they have examined. Using the Cordonnier's method of filing, data were recorded on the filing cards by perforating them in specific places on the top margin. The method enabled a quick sorting of data but the perforating took some time since it was done manually. In 1977 the cytology laboratory was able to

use the only computer at the IO which was in the radioisotope laboratory. From 1984 we used the computer located at Intertrade and in 1986 we received our own computer. In the same year Gorazd Noč, a computer engineer, wrote the first programme for registering patient's personal data, type of cytological material, tumour location, diagnosis. From 1994 we use a computer programme which enables the whole cytological report to be saved in the electronic form. The programme, which has been updated a few times, enables many data to be retrieved. However, it is not practical enough for today's needs and we are eagerly awaiting a modern laboratory information system whose application is long overdue already.

Human resources at the Department of Cytopathology

As already mentioned, the first people working with cytology samples were prof. Ravnihar, dr. Mavec and laboratory technicians Nečak and Kovačič. In 1959 when prof. Us-Krašovec was made the head of the cytology laboratory there were four people working in the laboratory: prof. Us- Krašovec, Kovačič, Janez Škrk and the orderly Marica Podržaj.

During the first half of the sixties the workload was increasing fast and four new people joined the laboratory. Majda Škrk and Mimica Herakovič who joined the laboratory in 1962 and 1964 respectively were working as laboratory technicians and screeners. Dr. Milica Bolka was working in the cytology laboratory for a short time during the sixties. She encouraged dr. Marija Bizjak (later Bizjak Schwartzbartl) to join the cytology laboratory in 1966. Dr. Bizjak helped in the laboratory by performing FNAs even before she finished all her undergraduate courses.

During the next five years the total number of cytological samples increased by almost 50% and new help was needed. Jožica Bedenk, a laboratory technician was hired as screener in 1971, dr.Ana Pogačnik and a laboratory technician Marta Trojner joined in 1972. In the next three years the number of exfoliative cytology specimens was so high that the screeners no longer had time for helping with specimen processing, staining and administrative work. Therefore, two laboratory technicians were hired, Milena Petelin as the substitute for Trojner in 1977 and Polona Butara in 1978. Mojca Černe joined in 1976 as the first administrator. She was substituted by a much more competent Fanika Cesar in 1978. Džula Begulić, an orderly came to our department in 1974.

The eighties and the nineties witnessed the arrival of new technologies and an increasing number of cytological samples which necessitated hiring additional personnel. Dr. Ljudmila Ruparčič Oblak, dr. Živa Pohar Marinšek and a laboratory technician Vesna Žitnik (later Gril) came in 1980. Sanita Halilović substituted Begulić in 1983. Four additional laboratory technicians were hired in the second part of the eighties: Brigita Šturbej in 1987, Irena Srebotnik Kirbiš in 1988 and Marijana Matić in 1989. Irena Srebotnik Kirbiš, an engineer of biotechnology, who was hired as a laboratory technician later advanced to the position of the head of the technical sector in the Department of cytopathology. This was the first time that our department had a university educated person with good knowledge in laboratory technology who could devote most of her time to the development of laboratory techniques. Such a position became a necessity in the new era of ICC which was rapidly gaining acceptance as the new ancillary technique in cytopathology.

The nineties witnessed a considerable change of generations. Jožica Kovačič, the first screener in cervical cytology retired in 1988. In 1992 prof. Us-Krašovec gave up the leadership of the department after holding the position for 33 years. She was succeeded by dr. Ana Pogačnik who was the head of the department until 2004, close to 13 years. Prof. Us-Krašovec, dr. Bizjak Schwarzbartl, dr. Ruparčič Oblak and biologist Majda Škrk retired in 1997. The administrator Fanika Cesar retired in 1999.

The new generation of people who joined the department in the nineties were: dr. Vera Kloboves Prevodnik (1990), dr. Margareta Fležar (later Strojan Fležar) and administrator Erika Pavlinič (1993), biologist Jaka Lavrenčak (1995), biologist Brigita Medle (1997), cytotechnologist Simona Laknar, later Sem-primožnik (1998), dr. Ulrika Klopčič and administrator Nadežda Bajec (1999).

The last change in the leadership of our department came about during the twenties when dr. Pohar Marinšek became head of the department in 2004. The additional workers whom our department gained during the twenties were mainly screeners of cervical cytology due to the retirement of Heraković and Bedenk in 2005 and due to an increase in the number of Pap smears by 160% in the same year. The new screeners were biologists Tanja Planinšek (2004), Janja Zalar (2005) and Mojca Lešnjak (2008). Simona Uhan Kastelic, a cytotechnologist replaced the scree-

ner Medle in 2006. Dr. Strojan Fležar and Srebotnik Kirbiš left the department in 2007. Srebotnik Kirbiš was replaced by Nataša Nolde as the head of the cytotechnologists. Two new administrators were also hired during the twenties. Milanka Janež replaced Pavlinič in 2005. Barbara Horvat was hired in 2006 since the cervical screening programme necessitated sending the results of Pap tests to ZORA headquarters which increased the workload in the administration beyond the capacities for two persons. The last addition to our department in 2010 was biologist Andreja Brožič, a young researcher in the flow cytometry unit.

During the course of the sixty years there were many people who worked in our department for a short time of one to three years. They were doctors Peter Fras, Janez Lamovec, Dušanka Marn, Andrej Vrabec, Matjaž Šebenik, Karmen Stanič, Breda Škrbinc, and Biljana Grčar Kuzmanov; cytotechnologists and biologists Ljubica Šmid Reja, Majda Mihelak, Natalija Horvat, Zorica Miličević, Helena Malnar, Stanka Savenc; Vlasta Medvešček, Jana Žnidaršič, Lidija Salobir and Irena Kranjec.

Working facilities at the Department of Cytopathology

During the first few years the cytological and histological laboratory shared premises in the attic of the old military facility of Šempeter, later called tract A of the IO. Specimens were prepared for microscopic examination in one room; the other room was shared by doctors, biologists and screeners. Both laboratories moved to the ground floor of the same building in the mid-sixties where they stayed for twenty years. The premises were adequate for a few years. In the beginning of the eighties they became too small for all the activities and the growing number of personnel. Therefore, temporary new rooms were acquired for the screeners and for two doctors in other parts of the IO. Finally, the whole laboratory moved in 1986 to the first floor of newly built tract D. For the first time in 25 years the cytology laboratory resided in a new, modern and spacious location.

The years between 2003 and 2005 will be remembered for the renovation of the tract D at the IO. The management of the IO decided that the Departments of Cytopathology and Histopathology will not be moved for the time of renovation. Nobody complained because we did not realize what we were getting into. For almost two years both departments were operating in the middle of the construction site enduring dust, noise and cold during winter since central heating was disconnected. Finally, in 2005 we moved a floor up in the tract D to newly constructed and newly furnished laboratories and offices. In addition, the department received also some new equipment including a ten-head discussion microscope, some new microscopes for the cytopathologists, an automated stainer for Giemsa and Papanicolaou staining and a digital photomicroscope. Dr. Pogačnik was the head of the department during the renovation and therefore involved in planning the layout of the laboratory and in the selection of the new equipment.

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Literature

1. Šavnik L, Ravnihar B .History and development of the Institute of Oncology. Aug. 1st 1963. Archives of Slovenia, Box 2525. (in Slovene)
2. Institute of Oncology in Ljubljana, 1978, Published by the Institute of Oncology for internal use only. Library of the Institute of Oncology. (in Slovene)
3. Ravnihar B. A report to the Scientific committee of Slovenia, 1961. Archives of Slovenia, box 2535. (in Slovene)
4. Srebotnik Kirbiš I, Petelin M, Žitnik V, Butara P, Us Krašovec M. Filter imprint for urinary samples. 9th international meeting of the Adriatic Society . 1994, Book of abstracts.
5. Bizjak Schwarzbrtl M. Cytomorphological, citochemical, and immunological characteristics of non-Hodgkin malignant lymphoma in aspiration biopsy of lymph nodes. Disertation, 1992, Ljubljana. Library of the Institute of Oncology. (in Slovene)
6. Srebotnik Kirbiš I. Immunocytochemistry, 1985-2002. Power point presentation at the 50th aniversary of the Department of cytopathology, 2002. Archives of the Department of cytopathology, Institute of Oncology.
7. Kloboves Prevodnik V, Strojan Fležar M, Pohar Marinšek Ž. Improved method for flow cytometric immunophenotyping of FNA samples. ISAC. 22nd International Congress, Montpellier, 2004.

8. Kloboves prevodnik V. Cytopathologic diagnostics of lymphomas in lymph node samples. Oncology 2010; 14: 139–43. (in Slovene)
9. Us Krašovec M, Mermolja M, Kovači J. Cytodiagnostics of pulmonary carcinoma using fluorochrom Acridin-orange. Tuberkuloza 1968; 20: 354–60. (in Slovene)
10. Us Krašovec M. Changes on malignant cells caused by cytostatic therapy. Docotor's dissertation. 1972, Library of the Institute of Oncology. (in Slovene)
11. Auersperg M, Us Krašovec M, Pogačnik A, Pohar Marinšek Ž, Goehde W, Jaffe N. DNA flow cytometry and cytomorphology for evaluation of chemotherapy. 15th international cancer congress, Hamburg 1990. Part I: Poster abstracts. J Cancer Res Clin Oncol 1990; 116 (Suppl): 478.
12. Auersperg M, Us Krašovec M ,Goehde W, Pogačnik A, Pohar Marinšek Ž, Oblak Ruparčič L, Bešić N. DNA and protein flow cytometry in human solid tumors used for planning chemotherapy.13th European congress of pathology, Ljubljana 1991. Pathol Res Pract 1991; 187: 651.
13. Auersperg M, Us Krašovec M , Pohar Marinšek Ž , Oblak Ruparčič L, Zorc R. Role of DNA measurements and cytomorphology in the development of chemotherapy for rare tumors in the head and neck. 5th international conference on advances in regional cancer treatment, Rosenheim 1991. Reg Cancer Treat 1991; 4: 5.
14. Personal communication with prof. Marko Hočevar and prof. Nikola Bešić, Institute of Oncology, 2012.
15. Pogačnik A. Cytomorphology of fibroadenoma of breast. Master's of science dissertation 1978. Library of the Institute of Oncology. (in Slovene)
16. Bizjak Schwarzbrtl M. Cytomorphological characteristics of non-Hodgkins lymphoma in lymph node FNA samples. Dissertation for academic specialization, 1984. Library of the Institute of Oncology. (in Slovene)
17. Pogačnik A, Us Krašovec M. Analysis of routine cytopathologic reports in 1,598 histologically verified benign breast lesions. Diagn Cytopathol 2004; 30: 125–9.
18. Us Krašovec M, Auersperg M, Bergant D, Golouh R, Kloboves Prevodnik V. Medullary carcinoma of the thyroid gland: diagnostic cytopathologic characteristics. Pathologica 1998; 90: 5–13.
19. Pohar Marinšek Ž, Zidar A. Epithelioid sarcoma in FNAB smears. Diagn Cytopathol 1999; 11: 367–72.
20. Pohar Marinšek Ž, Lamovec J. Angiosarcoma in FNA smears: diagnostic accuracy, morphology, immunocytochemistry and differential diagnoses. Cytopathol 2010; 21: 311–9.
21. Pohar Marinšek Ž, Bračko M: Rhabdomyosarcoma: Cytomorfology, subtyping and defferential diagnostic dilemmas. Acta Cytol 2000; 44: 524–32.
22. Srebotnik Kirbiš I, Pogačnik A. Reliability of estrogen receptors assessment on routinely prepared Papanicolaou stained samples of breast carcinoma. 2nd Conress of the world society of breast health, Butapest, Hungary 2003. Book of abstracts, 50.
23. Nolde N, Drev P, Kloboves Prevodnik V, Pohar Marinšek Ž, Frković Grazio S, Gazić B. Immunocytochemical assessment of HER2 protein on cytological specimens of breast cancer. Cytopathol 2009; 20 (Suppl 1): 128.
24. Stanič K. Standards of flow cytometric DNA in the determination of tumour ploidy and monitoring the effects of chemotherapy. Master's of science dissertation, 1994. Library of the Institute of Oncology. (in Slovene)
25. Gazić B. Prognostic significance of cytometric DNA measurements in FNAB of breast cancer. Doctor's dissertation, 2006. Library of the Institute of Oncology. (in Slovene)
26. Pogačnik A. Morphological characteristics of breast carcinoma in aspiration biopsy samples. Doctor's dissertation 1987. Library of the Institute of oncology. (in slovene)
27. Strojan Fležar M. The effect of fixation and duration of hydrolysis on nuclear features measured by image cytometry. Master's of science dissertation, 1996, Library of the Institute of Oncology. (in Slovene)
28. Strojan Fležar M.Ten years of the laboratory for image cytometry. Onkologija 2005; 1: 15–6. (in Slovene)
29. Us Krašovec M, Eržen J, Žganec M, Strojan Fležar M, Lavrenčak J, Garner D, Doudkine A, Palčič B. Malignancy associated changes in epithelial cells of buccal mucosa: a potential cancer detection test. Anal Quant Cytol Histol 2005; 27: 254–62.