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Hospital Practice Report

Hospital Pharmacy

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Hradec Králové, January – March 2015

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1. Introduction

This report describes the work that I have done during my internship at University Hospital in Hradec Králové in collaboration with Faculty of Pharmacy of Charles University in Hradec Králové. It was developed since January to March of 2015 and it includes some theoretical and practical concepts that I have learned in the different departments where I have been. The report is divided in seven parts, each corresponding to a different department.

The first part is related to the Faculty of Pharmacy, the Charles University and the University Hospital in Hradec Králové. Here I will do a brief introduction.

The second part of this report is about my stay in the Hospital Pharmacy. In this chapter, I will explain the different tasks that a pharmacist can have in the Hospital Pharmacy and how important is their work for the well-being of the patients. I will also explain the work that is done, specially, in Geriatric, Pediatric and Oncology Department and also some explanations about the preparation of Cystostatics.

The third part explains the work developed by the Department of Clinical Immunology and Allergology as well as some techniques that are made and that I have learned about it. It is also supported by some theoretical concepts.

The fourth part includes some explanations and concepts that I had the opportunity to learn about the Tissue Bank and about their main goal that it is cryopreservation based on cryotechnology.

The fifth part is about III. Internal Gerontometabolic Clinic – Laboratory, where I introduce this department and explained the High Performance Liquid Chromatography (HPLC).

The sixth part concerns to Department of Hematology, where I could see the phases of hematological analyses and where I could learn how it is done.

The seventh part includes my visit to the Biomedicine Center. I will explain their organization and their main work performed in the present.

Finally, the eighth part of this report is about my passage to the Faculty of Pharmacy, specifically in the Department of Analytical Chemistry. I will explain two different techniques: Capillary Electrophoresis and Flow Injection.

2. University Hospital / Charles University / Faculty of Pharmacy

Fig. 1 - University Hospital of Hradec Králové

University Hospital Hradec Králové belongs to the largest medical facilities not only in eastern Bohemia, but also in the Czech Republic. University Hospital is also a major research and teaching facility closely associated with the Faculty of Medicine and Pharmacy in Hradec Králové. Modern hospital medical care has been developing successfully in Hradec Králové since the end of the 19th century, but the academic roots of today's University Hospital Hradec Králové date back to the 1930s. There are the most complicated surgical procedures and technologies used in the diagnosis and treatment. [1, 2]

Charles University is the oldest university in central Europe and it was founded by King Charles in 1348. At present, it counts with 17 faculties in Prague, Hradec Králové and in Plzeň. It is also renowned as a modern, dynamic, cosmopolitan and prestigious institution of higher education. [3]

Fig. 2 – Charles University in Prague

Fig 3 – Faculty of Pharmacy of Charles University

The Faculty of Pharmacy of Charles University is located in Hradec Králové and it was established in 1969. Pharmacy is a medical branch, which is focused on the research, production, control, supply, and dispensing of medicines and drugs. It also deals with information about drugs and pharmaceutical functions, organizations, with the management of pharmaceutical functions within the health service system (social pharmacy), and with the historical development of the branch. [4]

The University Hospital works in close cooperation with the Faculty of Pharmacy and Medicine in Hradec Králové, Charles University in Prague and Military Medical Academy Association.

3. Hospital Pharmacy

Hospital Pharmacy provides basic and specialized pharmaceutical care for the hospitalized and ambulatory patients. This department counts with 70 employees, being 20 pharmacists, 20 technicians and the rest assistants. It is open 24 hours a day but only from 7h30 to 16h00 the patients can be served by a pharmacist, after this the drugs are transferred by wicket. During my stay here, PharmDr. Martina Maříková taught me everything that I need to know about the Hospital Pharmacy. [5]

The Hospital Pharmacy can be divided in different areas:

1- **Department of Dispensing** that includes inpatients (the pharmacy has to ensure that the medical products are shipped for the all hospital in the right quantities) and outpatients (it is provided a whole range of drugs, including preparation of medicines, that requires sterile dosage forms, and they also provide medical devices);

Fig 4 – Storage area for medicines that are dispensing to the patients

2- **Department of Preparation of Drugs** that includes preparation of normal medicines and sterile products, such as parenteral nutrition (20/30 bags per day that are specific for each patient – critical patients can change it everyday) and cytostatics. In this department is also prepared medicines that are involved in clinical trials, namely for multiple sclerosis;
3- **Department of Quality Control** where organoleptic properties are determined and analytical tests are done;
4- **Clinical Pharmacy** that works as a Drug Information Center. It cooperates with the Faculty of Pharmacy and started 2 years ago. There are two types of information that can be given: one is about how much the patient is going to pay for the medicines and the other is about pharmacological information such as doses for specific patients. This way the pharmacotherapy is optimized. This area of Hospital Pharmacy has a big importance since it can help to prevent medical errors and drug interactions. [5]

The critical care units for the Hospital Pharmacy are metabolic diseases, where it is included different areas such as geriatric, kidney transplant and diabetes disease, also with cooperation with gastroenterology and hematology; pediatrics where it is important to be aware of the doses and pharmacokinetics properties; and oncology.

During my stay in Hospital Pharmacy, I could see the storage of all medicines, either for inpatient or for outpatient. The other departments in the hospital order the medicines once or twice a week, depending on the need. However, it is possible to come every day to the Hospital Pharmacy to pick up some special and urgent medicines that the department did not ordered. Before sending the medicines for each department, the pharmacist has to check each package to see if everything is according to the order.

In relation to outpatients, they can come to the Pharmacy and order any medicine or medical device according to their prescription. The medicines that the patients order more are the medicines for cardiovascular diseases. Every day, the prescriptions are reviewed to check if everything is right with the medicines dispensed. The same person that dispenses the medicines cannot do this reviewing. This is done to minimize the incidence of errors.

I have also been in two types of laboratories inside the Pharmacy. One is for drug control, where is tested the solutions that are made in big quantities and are used to divide and store. The others were to prepare drugs. One laboratory to prepare pills, another to do solutions (eye drops and disinfectants), another for ointments and finally laboratories for sterile products. These last laboratories can be for parenteral nutrition (the volume is according to the patient weight), where I could see the preparations for adults and for neonatal babies and it can also be for sterile solutions to use in the hospital (ethanol 70 or 96%, color solution (panteblau)) for gastroenterology in endoscopy and for radiology. These solutions are sending to a company that tests them in relation to sterility and just then, the hospital can use them, if they were approved. The laboratories for sterile products are a high clean space with grade B.

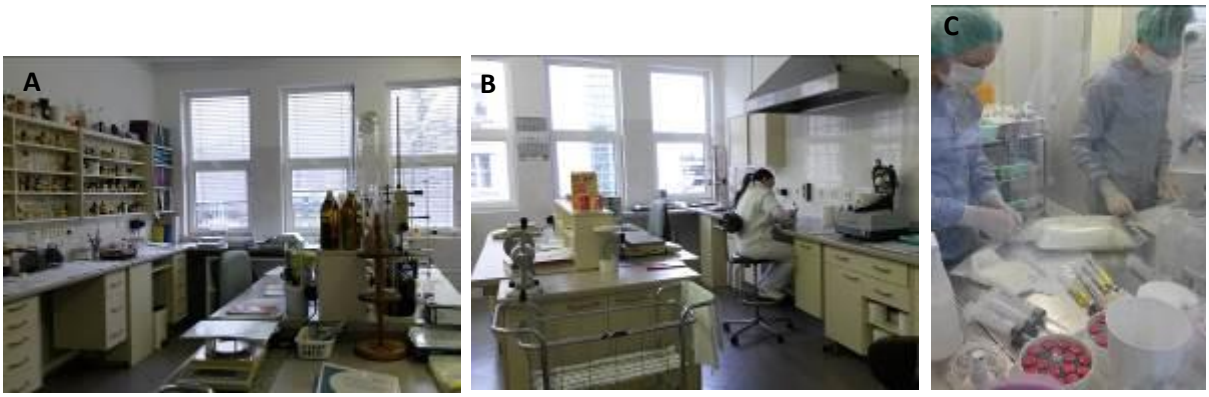

Fig 5 – Different Laboratories that belong to the Pharmacy Hospital. A: Drug Control Laboratory; B: Laboratory to prepare pills; C: Sterile Laboratory to prepare parenteral nutritional

Clinical Pharmacy

✓ Geriatric Department

The Geriatric Department has only 22 beds and, because of this, only patient with more than 78 years old are admitted there. The patients need a special care in relation to liver and kidney function, to check if they are working properly. If not, the dose must be adjusted to the patient. Besides this, and not forgetting the innumerous co-morbidities that these patients have, the main goal is to stabilize the patient to the same status that he had before hospitalization. There is no time limit to remain here in this department, the patients just go home when they are improved. Nevertheless, most of the cases are acute situations.

Since the Geriatric Department does not have enough beds for all cases that appear, there are other places where the patients can be in recovering, like nurse's home and institutions for long-term care. The pharmacist has an important role in the cooperation with this department, institutions and with the family, explaining to the responsible person for the patient every care that he needs to have with the medication and what non-pharmacological measures can be made to keep the well-being of the patient.

The main work of the pharmacist in Geriatric Department is reviewing the medication. This means that he needs to make sure that every medicine is correct for each patient. Before the visit to patients, the pharmacist has to check all the parameters, and he does this everyday. It is important to see if the medicines are according to the disease, if the doses, the time of taking them and the way to take them are correct and also the risks, problems and interactions that can occur with the medication that the patient is taking. In the case that there is an interaction, it has to be seen if there are any manifestations and according to this, change or not the medication. Besides all this, it is important to check the compatibility between chronic and new medication. In addition, it is needed to check the biochemistry and hematology analyses. A marker that has a great importance in this area is C-reactive protein, which is an indicator of infection. In cases of patients with infections it is also checked the microbiological analysis.

During my stay in this department, I visit, with the pharmacist and the medical doctors, every patient. It is needed to see his status, how he feels, review the medication, check if he is responding well to the treatment and hear his complains. In addition, when it is necessary, the medical doctor have to ask some basic questions like the name, age, where he lives etc. to see the mental state of the patient. For this to be possible, it is very important the communication between the medical doctor and the pharmacist. There is a dossier for each patient with all his information.

In this department I could see patients with a lot of different diseases, however, the ones that I contact more were: broken arm, bronchitis, pulmonary embolism, hypotension, atrial fibrillation (the patient has also edema and fever), urinary infection (with high temperature and confusion; the results show that the patient has a high C-reactive protein), ischemia heart disease, thrombosis, pneumonia, pulmonary infection, gastrointestinal bleeding, Helicobacter pylori infection, chronic obstructive pulmonary disease and upper gastrointestinal candida. According to these diseases, the medicines that were used more were corticoids, anticoagulants (warfarin), antibiotics, antifungals, painkillers and triple therapy with proton pump inhibitors (pantoprazole) + amoxicillin + clarithromycin.

After the visits I could realized that there are several different cases, from older patient in good conditions, to younger patient in worst conditions.

✓ Pediatric Department

Like at Geriatric Department, my stay here was to review the medication. However, it is important to be much more carefully with the determination of the doses because the patients are babies and children, so every single dose of medicines has to be calculated for each patient based on the weight and sometimes based on the body surface area. The principal sources that are used are "Lexicomp" online and the book "BNF for Children" for the doses of medicines and "UpToDate" to consult the pathology of the diseases. During my stay here, Dr. Petra Thomson explained me everything.

This department accepts people since they are born until 19 years old and it is divided in two parts: one of intensive care with high level of monitoring and other called standard wards where the cases are not so serious than the first one, but with equal level of care. [6]

Before every visit, a multidisciplinary team with medical doctors, pharmacists and nurses get together to discuss each case, reviewing what happened, what made the patient come to the hospital, the patient medical history and, finally, it is important to go through the prescription, not forgetting to check the correct doses, schedule and the correct way to administrate it. This way, the cooperation between the pharmacist and medical doctors have been having a lot of success and continues in development to become better, since the main goal of this cooperation is the well-being of the patient. As these patients are babies and children, they need a redouble care.

The cases that I had the opportunity to see and to review the medication, calculating the doses were fever and seizures, thoracic insufficiency syndrome, multiresistant Pseudomonas aeruginosa

infection in a child with Pierre-Robin syndrome, pneumonia by Streptococcus pneumoniae, obstructive acute pancreatitis, hypoglycemia crisis in a patient with Diabetes I, cystic fibrosis and hematuria.

According to these diseases, I contact more with the reviewing of the follow medicines: antibiotics (cephalosporin and aminoglycosides), benzodiazepines, proton pump inhibitors, β2 adrenergic agonist, antihistamine, corticoids, painkillers (metamizole), antipsychotics and insulin.

✓ Oncology Department

The Department of Oncology and Radiotherapy in University Hospital in Hradec Králové has been leading the implementation of new methods of cancer treatment in Czech Republic. For inpatients, it has capacity to 64 beds and for outpatients it includes patients from six clinics and hospices for chemotherapy and biological therapy. The treatment of cancer is based on a system of treatment standards and interdisciplinary collaboration. [7]

The difference between inpatients and outpatients is that the first ones have bad status with renal and hematological problems or they are having radiotherapy for 30 days, and in this case, they are admitted. Also, the patients that do chemotherapy (stay for six or seven days) are admitted, and then they go home. The outpatients spend the all day in the department when they receive chemotherapy because first they need to do blood analysis to see the blood count and the kidney function. Then they have to wait until the results and interpretation of them are done. After this, they need to wait for the preparation of cytostatics and just then they receive the medicines through IV infusion that also takes a while. If the cytostatics that they take are tablets, they can go home and take them there.

The role of the pharmacist is very important in this department, since they prepare the cytostatics, they review the medication of each patient and they report the adverse effects of chemotherapy to the National Institute in Prague. Besides the preparations of cytostatics, many medicines are prepared by pharmacists to help preventing the complications associated to chemotherapy and radiotherapy. Some examples of these medicines are vitamin K for skin problems, morphine gel as a painkiller, suspension of omeprazole that is used in patients with mucositis (administrated through a tube into the stomach because it dissolves there), benzocaine emulsion as a painkiller before meals, lidocaine gel as an anesthetic to relieve the pain, capsaicin crème for the skin, caphosol with phosphorus and calcium to prevent mucositis, fentanyl and buprenorphine as painkiller and artificial saline to help patients that have xerostomia.

To discuss every case of patients a multidisciplinary team is formed by the chief of chemotherapy, the chief of radiotherapy, the head of the clinic, a senior nurse, medical doctors and a pharmacist. They review the medication and analyze the progress of the patient, by seeing the medical history of today to prevent what is going to happen in the future, what are the effects that he is going to suffer and when is the best time to stop with chemotherapy (when the renal function is too low). The nurse tells how the patient is doing and how he feels because she has more contact with them. The pharmacist gives to medical doctors information about the news from the medicines and recommendations about changes that have to be made (higher the dose when it is not making effect or lower the dose because it is too high; change the schedule of taking the medicines; change the formulation). To do all this they have a dossier for each patient that contains the level of pain that he is feeling, the temperature that he has, the levels of blood glucose, pressure and pulse. They also have the control of nutrition, the results of hematology, biochemistry (level of potassium) and when there are infections the results from the microbiology (they do an antibiogram to see the resistances of the bacteria to the antibiotics). They also check the levels of hemoglobin that has to be high (the patient has to have a good transport of oxygen) to the radiotherapy works. When the patients do not have this value of hemoglobin, they need to do first a blood transfusion.

The use of chemotherapy or radiotherapy it depends on the recommendations that exists on guidelines that are updated every six months and are based on clinical trials. There are different types of chemotherapy that depends on the purpose for which is used. Chemotherapy it can be used to reduce the tumor size so then it can be operated to remove it; it can also be used as an adjuvant after the surgeries and as a palliative chemotherapy.

In relation to chemotherapy, usually it is used a combination of cytostatics to eliminate the tumor and not just one. However, in hematology it is usually just one. The medical doctors are the ones that decide this combination of cytostatics. As some drugs can destroy the small vessels, the chemotherapy can be administrated using a central line to inject the medicine in vena jugulars because if it was in the small vessels the treatment will destroy them.

The side effects of the radiation depends on the area that is affected. If the radiation goes to the neck and the face, the normal problem is mucositis. If it goes to the chest, appear skin problems. If it goes to the stomach the most frequently side effects are nauseas and vomiting and if it goes to the anus the problem is diarrhea. On the other hand, the side effects of chemotherapy are essentially vomiting and diarrhea.

In this department, I had the opportunity to review the medication and visit the patients. I saw different types of cancer, such as esophagus, kidney, gastric, brain, neck, uterus, vulva, rectal colon,

testicles, pancreas, anus, lung and triple negative breast cancer (which is the worst type of breast cancer). The last two types of cancer can only be treated with radiotherapy. Due to this, I saw mucositis and skin problems as a side effect of radiotherapy.

As a result of my visit, I could realize that these patients use almost as much support medicines as they use cytostatics. Neoplugen is an example and it is used when the patients have lower number of leukocytes due to the chemotherapy, because this medicine increases the production of leukocytes. Another example is the use of physiological solution (with KCl and $MgSO_4$) before and after the administration of Cistoplatin (cDDD) because this cytostatic is dangerous for the kidney so it is necessary to control the renal function by hydration the patient and it also leads to the loss of magnesium. It is also very common the use of anti-emetic drugs to prevent the vomiting, like grarisetron and dexamed, cetrone, corticoids in small doses, aprepitant (NK agonist) and in some special cases benzodiazepines when the vomiting is due to psychological effects (when the patients see red solution they associate with doxorubicin and they start vomiting and also when they see people with the white coat). It is also used corticoids in high concentrations as an anti-edemic for brain metastases and in this case, it is needed to use proton pump inhibitors such as omeprazole to protect the stomach. Antidepressives are also used when the patient as psychological problems due to his situation.

Cytostatics

The usual treatment for patients with cancer includes cytostatics. Cystostatics are anti cancer drugs that have the ability to prevent growth and proliferation of cells and, because of that, they do not only destroy cancer cells, they also destroy normal cells, being dangerous and having many unwanted effects. For this reason, the preparation of them has to be very carefully done, so only pharmacists or pharmacists' assistants can prepared them. These medicines can also be used in neurological, rheumatologic and eye diseases. I had the opportunity to visit the part of the Oncology Department where these medicines are prepared thanks to PharmDr. Zuzana Ducháčová Woidigová.

In this department, it is prepared cytostatics mainly for outpatients, being less the ones that are inpatients. The preparation has to be done in aseptic conditions and with safety techniques because of the negative effects. To ensure the aseptic preparation it is needed to have all materials sterile and disinfected. It is also indispensable to do this procedure in clean rooms with air filtration and just by qualified people. These rooms have a periodic monitoring and a cleaning plan and also a periodic

rotation of cleaning agents because of the innumerous resistances. To enter here, it is needed to wear special outfit made from polystyrene that avoids emission of particles.

The preparation has to be done in rooms with grade A (highest grade of cleaning) with background of grade B or C. It has to be made a validation by an outside company once a year. In relation to microbiological self monitoring and sterility test, it has to be made once a month. It is very important the safety of preparation since these medicines can cause acute toxicity and delayed toxicity. It can also cause carcinogenicity, embriogenicity and teratogenicity. To avoid this it is necessary to wear protective equipment such as respirator and protective overall; the waste disposal can only be by incineration. The preparation of cytostatics has to be done in an isolator, to have grade A that is a close system with negative pressure to protect the staff.

Fig 6 – Preparation of cytostatics

For reviewing the prescription and checking the preparation, it exists a program called Cato. There it exists the diagnosis, data of patient and parameters that are important to adjust the dose to each patient. The doses depend on the weight, body surface area and renal clearance. The chemotherapy cycles depend on the diagnosis, age and status of the patient. Usually, more than one cytostatic is administrated and it could be by intravenous infusion or intravenous, intramuscular or sub-cutaneous bolus. The preparation could be a solution (diluted with physiological solution or glucoses) or a powder (it has to be reconstituted before administration). After the preparation, the cytostatic has to be packed into a plastic bag (black bag if the product is photosensitive) and put it into a box identified. Every preparation has a unique number to prevent the errors.

Fig 7 – Packing the cytostatic in a plastic bag.

In the department, usually, are produced 120 cytostatics per day. The most common medicines are Fluorouracil, Doxorubicin, Rituximab, Trastuzumab and Cyclophosphamide and the most common cancers are colorectal and breast cancer. After a day of work, the room has to be cleaned.

Besides preparing the medication for patients, this department also has the responsibility to do cytostatics that are included in clinical trials. This clinical trials are namely for breast cancer, multiple myeloma, acute leukemia and lymphoblastic leukemia.

4. Department of Immunology and Allergology

The department of Immunology and Allergology in University Hospital in Hradec Králové was founded in 1921. This department provides complex diagnostic and therapeutic care for patients suffering from diseases in which abnormal functions of the immune system are involved (immunodeficiency, infections (HIV), autoimmune diseases, allergies). It also provides diagnostic and consultative support for clinics, departments from Hospital and other medical facilities in the region. [8]

During my practice, PharmDr. Doris Vokurková, Ph.D. explained me theoretical concepts and showed me the most widely used technique in this department – Flow Cytometry. In addition, she taught me its applications in immunology and some functional tests realized here. I really need to thank her a lot for all the things that I have learned.

Despite the large number of analysis performed in this department, all are based in the same principle – binding of a monoclonal antibody associated with a fluorochrome to the surface receptors (CD) of the blood cells.

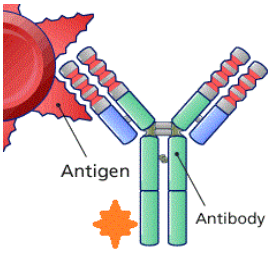

Fig. 8 – Interaction between an antigen and a conjugated antibody [9]

The measurement is done in the Flow Cytometry and has to be as soon as possible, since the cells have to be viable. It is important that the blood used is uncoagulated, for example with heparin or EDTA. In addition, the temperature, centrifugation and pH of the blood sample has to be controlled. The main task of this department is immunophenotyping of blood to distinguish B, T and NK cells and functional tests of granulocytes (phagocytic activity).

Cluster of Differentiation (CD)

CD means cluster of differentiation, which indicates a defined subpopulation of cellular surface receptors (epitopes). They are used to identify cell type and stage of differentiation since antibodies recognize them. Because of this, CD system is used as cell markers. This allows cells to be defined based on what molecules are present on their surface. These markers are often used to associate cells with certain immune functions or properties. While using one CD molecule to define populations is uncommon (though a few examples exist), combining markers has allowed identification for cell types with very specific definitions within the immune system. [10, 11]

Cell populations are usually defined using a + or a – to indicate whether a cell expresses or lacks a CD molecule. It exists more than 300 CD markers. [10, 11]

Type of Cell	CD Markers
Stem Cell	CD34+
Leukocytes	CD45+
Granulocytes	CD45+, CD15+
Monocytes	CD45+, CD14+
T lymphocytes	CD45+, CD3+
T helper lymphocytes	CD45+, CD3+, CD4+
T cytotoxic lymphocytes	CD45+, CD3+, CD8+
B lymphocytes	CD45+, CD19+, CD20+
Natural Killer Cells	CD16+, CD56+, CD57+, etc.

Table 1 - Clusters of Differentiation markers according to the type of cell

Fluorochromes

The fluorochrome is a functional group in a molecule, which absorbs energy of a specific wavelength and re-emits energy at a different, but equally specific, wavelength. When a fluorescent dye is conjugated to a monoclonal antibody, it can be used to identify a particular cell type based on the individual antigenic surface markers of the cell. [12]

To analyze the phenotype of cells is important to use monoclonal antibodies stained with different fluorochromes (thus different emission) to recognize them. That way every antibody against each type of CD marker is labeled with a different fluorochrome, so thus it is possible, in one single sample, to see all the different types of cells that exist in the sample.

It exists different fluorochromes; however, fluorescein isothiocyanate (FITC) and phycoerythrin (PE) are the most widely used.

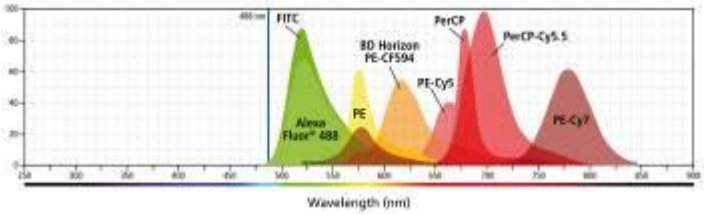

Fig 9 - Emission spectra for some dyes used to label antibodies [13]

Flow cytometry

Flow Cytometry is a powerful technique of studying single particles, usually cells, to determine their many physical or biological characteristics, while they flow in a fluid stream through a beam of light. In Flow Cytometry cell suspension are loaded into a vibrating flow chamber with a nozzle that expels them in droplets, each containing a single cell. The droplets pass a laser beam and scatter light as the beam strikes them. A photomultiplier tube (PMT) detector measures this scattering. The properties measured includes size, relative granularity or internal complexity and relative fluorescence intensity due to the dispersion of the laser light. It is a reliable and quick method that can analyze thousands of cells in just one small sample and has a wide range of clinical applications. The material that it is used in flow cytometry is blood, suspension of cells from biopsy, bone marrow and cerebrospinal fluid. [14, 15]

A flow cytometer is composed by three main systems: fluidics (which transports particles from one flux to the laser beam for the point in the sample stream at which the laser light is focused, the cells are measured on this point), optics (that has lasers which illuminate the particles in the sample stream and optical filters to direct resulting light signals to the suitable detectors) and electronics (able to convert the detected light signals in the electronic signals to be processed by the computer). [14, 15]

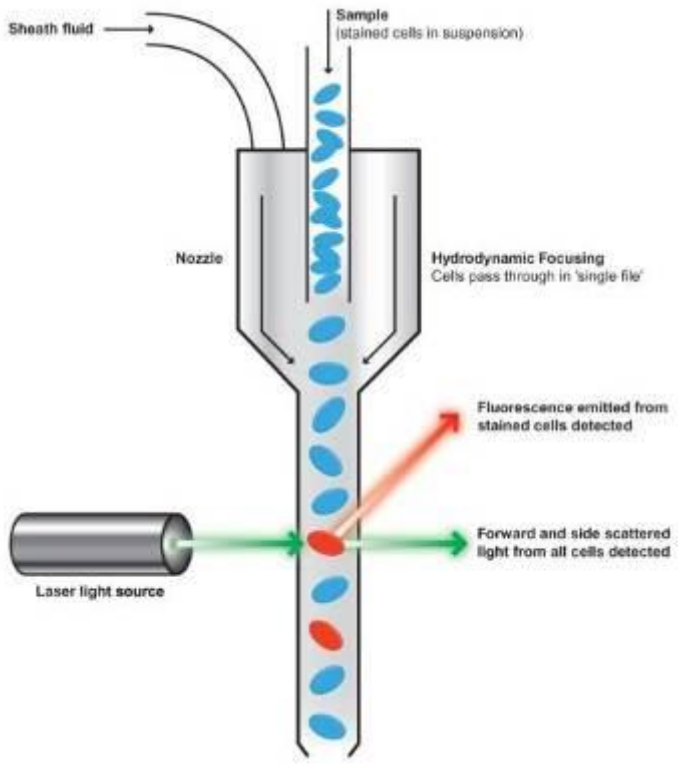

Fig 10 – Basic features of a flow chamber [16]

Usually, the light scatter is measured at two different angles – side and forward scatter. Forward-angle light scatter (FSC) correlates with cell size. Orthogonal light scatter or side scatter, defined as 90° light scatter (90°LS) with respect to the beam axis, correlates with cellular granularity and with the plasma/nucleus ratio of the cells. Data are usually shown as two parameter correlated plots, often called cytograms. In a dot plot cytogram, each cell recorded is shown as a single dot. [14, 15]

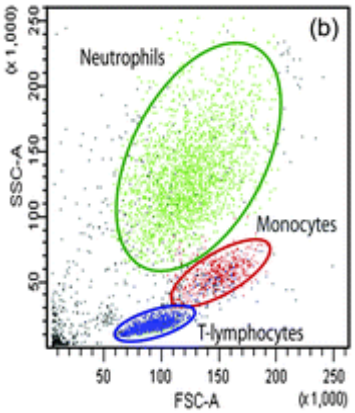

Fig 11 – Relation between side scatter and forward scatter in an analyze of leukocytes [17]

This method permits differentiation between large cells with a high plasma/nucleus ratio and a granular cytoplasm (granulocytes) and small cells with a large nucleus (lymphocytes). Monocytes have intermediate properties.

The sorted cell population can also be analyzed for other parameters, such as fluorescence. The immunofluorescence of lymphocytes and monocytes in the sample can be separately analyzed. The intensity of cell fluorescence correlates with the antigen density on the cell surface and can be quantified using a PMT detector. A number of antibodies directed against different antigens and conjugated with different fluorescent dyes can be analyzed simultaneous because after his contact with blue laser beam they emit different peaks of fluorescence. [14, 15]

Assuming that there are two different CD markers of interest, it is incubated the sample with two different monoclonal antibodies against this CD markers (for 50 minutes) and each with a different fluorochrome. This way it is possible to see if the cell express both of the target antigens (emit both fluorescence at different wavelength) or only one of the target antigens (emit just one fluorescence) or even antigen-negative cell population that do no express either of the CD markers (no fluorescence). After the incubation is needed to lyse the erythrocytes with ammonium chloride just for 10 minutes and then stop the reaction with physiological saline, to not destroy the leukocytes with the osmotic pressure.

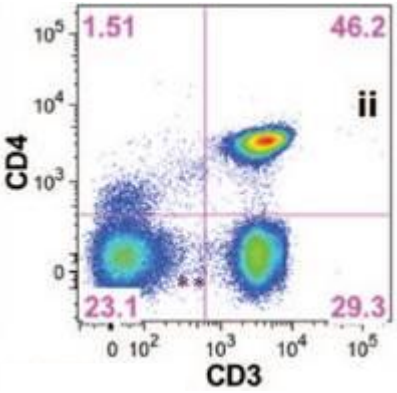

Fig 12 - Cytogram of a flow cytometry with two color analyzes: four populations can be detected: CD4+ (helper T lymphocytes), CD4- (most will be CD8+), CD4+ CD3- cells (most will be monocytes) and double negative cells (including B lymphocytes, NK cells and some monocytes

Flow Cytometry in Clinical Applications

✓ Cytocount

It is possible to determine the absolute number of leukocytes population of interest in the sample (for example CD3+ and CD4+) using the flow cytometry. For that is used a suspension of small polystyrene fluorochromes beads with an exactly known number (usually 982 beads/µL).

The follow expression is used to know the exactly number of lymphocytes:

$$\frac{\text{Number of lymphocytes} \times 982}{\text{Number of beads}}$$

✓ Immunophenotyping

Immunophenotyping is the analysis of heterogeneous populations of cells, which has the purpose of identifying the presence and proportions of the various populations of interest. Antibodies are used to identify cells by detecting specific antigens expressed by these cells (CD markers). These markers are usually functional membrane proteins involved in cell communication, adhesion or metabolism. Therefore, this technique is an indispensable tool since it is used to distinguish healthy from abnormal cells (they could have over expression of some markers and under expression of others), such as myelomas, lymphomas and leukemias. It can also be used to monitor the effectiveness of clinical treatment.

I had the opportunity to do this procedure and analyze the results of patients by measuring the percentage of CD4+ cells (T helper lymphocytes) and CD8+ cells (T cytotoxic lymphocytes).

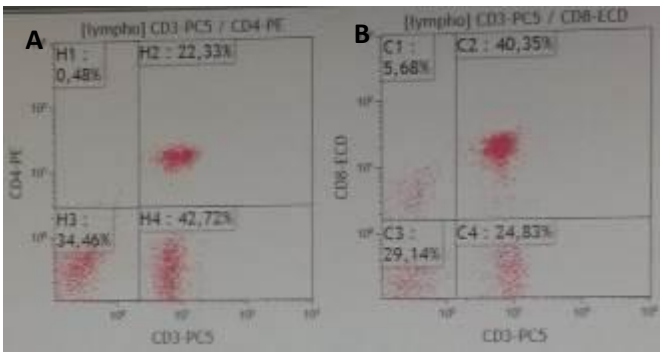

Fig 13 – Cytogram A: Population of T helper lymphocytes of 22.33% (CD3+ and CD4+); Cytogram B: Population of T cytotoxic lymphocytes of 40.35% (CD3+ and CD8+)

There are many applications of immunophenotyping. One of them can be the count of CD4+ plus CD8+. Usually this number has to be the same as CD3+. When this do not happen, it is because there is a subpopulation of receptors in T cells that can be αβ or γδ. If this last subpopulation is higher, that can mean that the patient has tuberculosis.

Another very important application of Immunophenotyping is the **diagnostic and monitoring of cancers**, like leukemia and myeloma. I had the opportunity to learn about this with Mgr. Ondřej Souček. For this type of diagnostic, the samples can be from bone marrow, peripheral blood, liquid from the lungs, brain and stomach tissue. To be used in Flow Cytometry they have to be in an emulsion of cells.

Immunophenotyping is needed to diagnostic this type of cancers, so it is indispensable to know the markers of Plasmatic cells (CD138+ and CD38+), Stem cells (CD34+), Myeloid progenitors (CD117+) and also T lymphocytes (CD3+) and B lymphocytes (CD19+). This department needs to cooperate with Pathology and Hematology department to diagnostic the exactly type of cancer.

Leukemia is a neoplastic disease in which precursor cells (in every stage of development cells can start the procedure of cancer) of the bone marrow become unable to differentiate and have high proliferative activity. The uncontrolled growth of neoplastic cells suppresses the normal hematopoiesis. After the clinical suspicion, the biological sample that can be peripheral blood, bone marrow or cerebrospinal fluid come to this department and several analyses are done to confirm the pathology and to discover what type of leukemia is. The sample is prepared (dilute in case of bone marrow and concentrate in case of cerebrospinal fluid) and it is incubated for 15 minutes with antibodies. Then the sample is analyzed by flow cytometry. It is possible to distinguish acute myeloid leukemia by the higher number of progenitors B cells and chronic myeloid leukemia by the higher number of grown B cells.

B lymphocytes produce antibodies that in their light chain can be Kappa or Lambda. In normal patients the proportion is 3:1 but in patients with leukemia the proportion is very different. Higher kappa (correlated with B proliferation) or kappa negative are indicators of leukemia. Since this happens, it is used monoclonal antibodies with fluorochromes against kappa and lambda to see the proportion and diagnose leukemia.

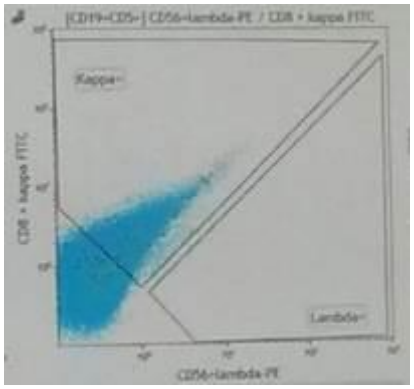

Fig 14 – Cytogram of Kappa and Lambda antibodies. We can see that Kappa is much higher, comparing to Lambda, which is indicative of Leukemia.

✓ Viability of Stem cells

Stem cells are undifferentiated cells that can differentiate into specialized cells. They act as a repair system for the body, replenishing adult tissue, maintaining the normal turnover of regenerative organs, such as blood. These cells have an important role in transplantation. To keep the patient safe the percentage of viable stem cells has to be at least 80%. To know the percentage of viable cells (not in apoptotic process) propidium iodide is used as an intercalating agent and a fluorescent molecule. If the cell is not viable and thus the membrane is permeabilized the propidium iodide permeates the cell and binds to DNA, emitting fluorescence. However if the cell is viable there is no fluorescence because the propidium iodide cannot permeates the intact membrane so it cannot binds to DNA. Therefore, if there is an increase in apoptotic activity, the fluorescence is higher than in the normal cells.

It is used CD34+ to identify stem cells (not CD45+ because they are immature cells so they have this marker in less concentration). If the cell is dead the antibody do not bind to CD34.

✓ Functional Tests for Lymphocytes

Blastogenic Transformation Lymphocyte Test (BTT)

To execute the functional test of lymphocytes the DNA must be analyzed and measured using propidium iodide (dye that has red fluorescence and can be excited at 488 nm) in a flow cytometry. The fluorescent intensity of dye in each cell is a direct measure of the amount of nuclear DNA, since the propidium iodide intercalates with the DNA. With this dye it is possible to determinate the precise number of cells in each phase of the cell cycle (G0/G1, S and G2/M) and calculate the proliferative activity.

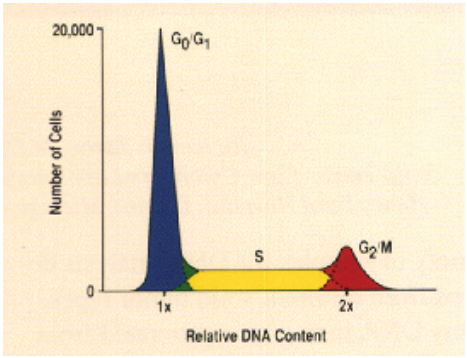

Fig 15 – DNA histogram in a normal patient [18]

This test is based on the fact that lymphocytes, which have been sensitized by a certain mitogen or antigen, transform into blasts and proliferate. This proliferation is determined by measuring the incorporation of propidium iodide, which bounds to double-stranded DNA (direct proportion of the content of DNA in cells). This way it is possible to determinate the various stages of cell cycle (G1, S, and G2).

After 3 days of stimulation with antigen (72 hours to form new DNA), the intensity of fluorescence must be increased (particularly in phase G2), because a large amount of newly DNA

have been formed, so more propidium iodide binds to them. This means that the proliferative activity of blast cells are right, so they are functional and viable. To do this procedure, it is used Phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) to stimulate the T and B cells, respectively.

This test is commonly used in cases of immunodeficiency and after chemotherapy because there are low proliferation of lymphocytes and, also, in cases of organ transplantation when the higher number of proliferation could mean that the organism is rejecting the organ.

Fast and Later Activation Test

This test has the same role as the last one; however, it takes less time to see the activation of lymphocytes. It can be divided in two main parts: in the first one it is searched the marker CD69 in cells (marker of fast activation of lymphocytes). This marker appears 4 hours after stimulation and decrease after 24 hours. To identify T lymphocytes it is necessary to look for CD3+ and CD69+; to identify B lymphocytes it is needed to look for CD19+ and CD69+.

The second part of this test takes place after 48 hours and it is searched different markers for different subpopulation of cells, through antibodies marked with fluorochromes. For T lymphocytes, it is used antibody against CD25 that is marked with fluorophore PE and antibody against CD3 marked with fluorophore PC7. For B lymphocytes, it is used antibody against CD23 marked with fluorophore F and antibody against CD19 that is marked with fluorophore PC5.

To do this test is necessary to have three tubes for each population of cells. One of them works as a negative control and it just contains x-vivo (medium to dilute the blood). The other contains PHA to stimulate T lymphocytes. The final one contains PWM to stimulate B lymphocytes. Every tube has the blood sample to be analyzed.

After incubation for 24 hours (fast activation) or 48 hours (later activation), it is added the antibodies against the markers that are wanted and wait for 15 minutes. After this is done the lyse of erythrocytes with formic acid just for 10 seconds and then it is added physiological saline to stop the reaction. After this, it is possible to measure the fluorescence in Flow Cytometry. To be able to analyze this sample for 1 week it is added formaldehyde that is going to fix the sample.

✓ Functional Tests for Granulocytes

Granulocytes are an important category of white blood cells that have an essential role in the host defense against bacterial or fungal infections. The phagocytic process can be separated into several major steps: 1 – Chemotaxis; 2 – Intake/Invagination; 3 – Oxidative Burst; 4 – Bacteria digestions (oxygen-independent mechanisms).

Fig 16 – Scheme of phagocytic process [19]

It is possible to analyze the phagocytic activity in three from the four steps. First of all, the intake step can be observed in an optical microscope. For that, it is needed to incubate a suspension of *Candida albicans* with blood from the patient during 1 hour at 37°C and under agitation. After this, is counted, in the microscope, the total number of granulocytes (100%) and the number of granulocytes that are in step two (at least they need to have three microorganisms inside the cell). Finally, it is calculated the percentage of phagocytosis.

In relation to Oxidative Burst step, to analyze this it is necessary to measure the respiratory burst of granulocyte after the stimulation with E.coli bacteria. During the process of bacteria ingestion, phagocytes activate the NADPH oxidase producing reactive oxidative intermediates (respiratory burst) resulting hypochlorite ions inside phagocytes strongly oxidize dihydrorhodamine 123 (DHR 123) into fluorescent rhodamine 123, which is detected by a flow cytometer. These reactive oxidative intermediates are the responsible to kill the pathogens. A positive control sample is stimulated using PMA (Phorbol 12-myristate 13-acetate) which activates respiratory burst of granulocytes without adhesion and ingestion of the pathogen. This test allows the quantitative determination of leukocyte phagocytosis (ingestion of bacteria). It measures the percentage of granulocytes that have ingested bacteria and their activity.

In the Burst test, is needed three tubes. All have blood sample and DHR123 and they are incubated for 45 minutes at 37°C with 5% of CO2. One of them works as a negative control and it just contains physiological saline, so the intensity of fluorescence is going to be very low. Other tube works as a positive control containing PMA, so is going the have the higher intensity of fluorescence. The final tube contains E.coli and it should have intermediate intensity of fluorescence because it is not so strong stimulator as PMA, being the normal values between 75-100%. After incubation, it is necessary to lyse the erythrocytes and just then analyze in flow cytometry.

In the tube that contains E.coli, if there is not any fluorescence it could means that the patient has Chronic Granulomatous Disease (CGD) or Myeloperoxidase (MPO) deficiency. CGD is clinically

and genetically a diverse group of hereditary diseases with deficiency in multiple enzyme of the NADPH oxidase cascade generating reactive oxygen radicals used for the pathogen killing. MPO deficiency is a genetic disorder of a downstream myeloperoxidase enzyme generating hydroxyl radical and hypochlorite, which is the most effective killing agent produced during a respiratory burst.

Finally, in relation to digestion step, the final one, it is possible to analyze it by doing a cultivation of the pathogens with the blood during 24 hours. If colonies growth, it means that the phagocytic activity is not working properly.

Separation of Lymphocytes

Lymphocytes are the main cells involved in pathological situation. Increased lymphocytes suggest viral infection, chronic infection, drug and allergic reactions. They are also increased in autoimmune diseases or after some transplantations. However, decreased lymphocytes suggest immune deficiency syndrome, after some oncologic treatments and during the treatment with immunosupressors. In this department, separations of lymphocytes are very common techniques for identifying some pathologies and, also, research for new signals pathways involved in specific pathologies.

For lymphocytes separation it is performed the gradient separation technique using Histopaque 1077, which is a high density solution. Anticoagulated peripheral blood diluted with medium is layered into Histopaque 1077. During centrifugation, erythrocytes and granulocytes are aggregated by Histopaque compound and rapidly sediment. The lymphocytes and other mononuclear cells remain at the plasma-Histopaque 1077 interface. Erythrocytes contamination is negligible. Most platelets are removed by slow speed centrifugation (20 minutes) during the washing steps.

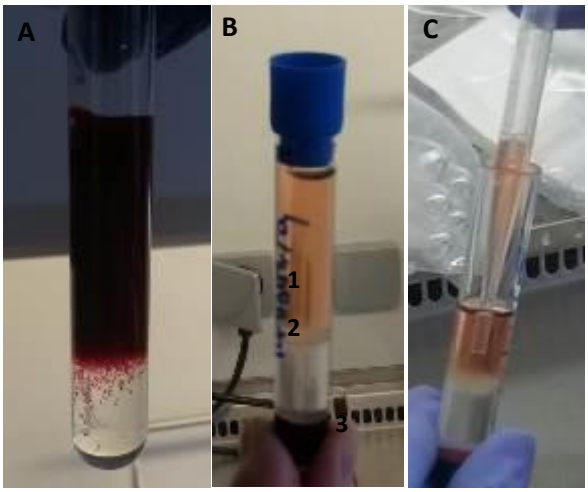

Fig 17 – Process of Separation of Lymphocytes. A: sedimentation of red blood cells when Hitopaque is added to a blood sample; B: distribution of the cells after centrifugation (1 – Plasma layer; 2 – Lymphocytes and Monocytes Layer; 3 – Erythrocytes Layer); C: removal of plasma layer to be easier to separate the lymphocytes and monocytes layer.

In just one step is not possible to separate the lymphocytes because Histopaque 1077 solution just makes the separation between granulocytes and mononuclear cells possible, the lymphocytes and monocytes stay in the same layer. So it is needed a second step of the isolation process and it consists in separate these two kinds of cells by the "adherence method" which consists in putting the mononuclear cells layer in a plastic wells plate and wait for the monocytes adhere to the plate. In cells suspension it will only be lymphocytes.

In this department, the isolation of these cells is important because they are participating in a study developed in Belgium to evaluate the importance of the vaccine against *Herpes zooster* after transplantation.

Reproductive immunology

Nowadays, infertility is a problem that occurs to more and more couples and because of that, more tests have been made to discover the reason why this happens. In this department, it is done three different tests, either to diagnose the problem or to monitoring the treatment. The samples for these tests are woman's blood, man's blood and sperm. After the collection of samples, the tests must be done as soon as possible, keeping the temperature at 34°C.

✓ Sperm Flow

Flow Cytometry has become an important technique in sperm evaluation and is increasingly used both for routine assessment and for infertility's research. A number of characteristics of semen can be assessed, like sperm count, leukocyte count, sperm viability, sperm acrosome integrity and presence of intra-acrosomal protein in sperm.

These parameters are important since the acrosome contains digestive enzymes that breakdown the outer membrane of the ovum called zona pellucida, allowing haploid nucleus of the sperm penetrates into the ovum. Besides, the presence of leukocytes in semen is a mark of an actual inflammation or a venereal disease.

To evaluate these five parameters it is used four tubes with semen diluted 10 times and physiological saline.

Tube A – sperm count and leukocyte count: addition of an internal standard (fluorescent beads with known concentration) after incubation to the semen sample. Detection of leukocytes is performed by staining with labeled antibody against human CD45 antigen. To know the exactly number of sperm cells it is possible to use the follow expression:

$$\frac{Number\ of\ sperm\ cells \times 1000 \times 10\ (dilution\ factor)}{Number\ of\ beads}$$

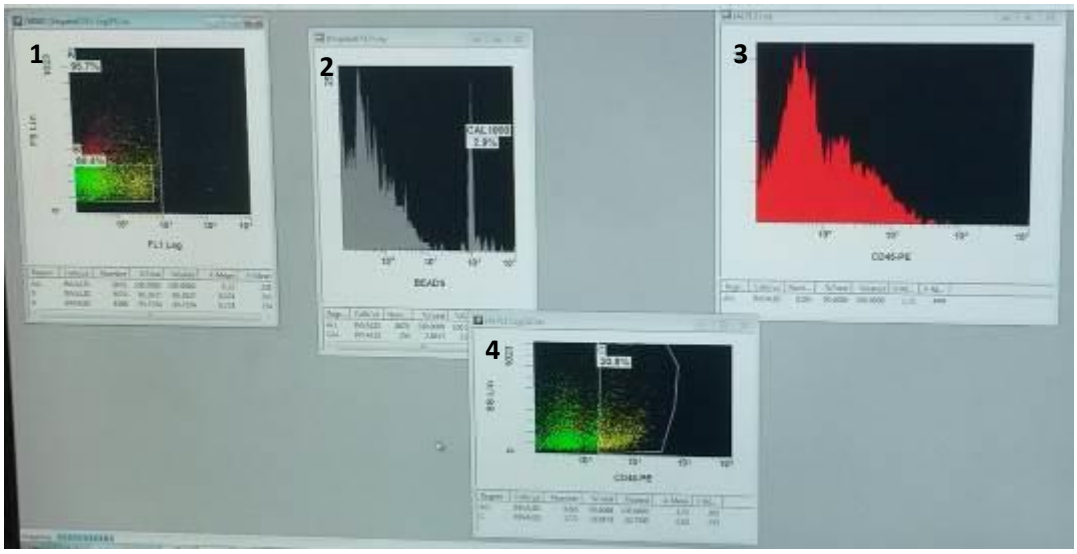

Fig 18 – Graphic 1: Count of sperm, being the green dots the mature sperm cells and the red dots the immature sperm cells; Graphic 2: Calibration; Graphic 3 and 4: Count of leukocytes

Tube B – sperm viability: is examined using propidium iodide, which permeates through damaged membranes of dead cells and binds to their DNA.

Tube C – acrosome integrity: is based on the detection of intra-acrosomal protein (IAP) which can be found inside the acrosome. If the sperm acrosome is intact, it is unable to detect IAP. Sperm with damaged membrane has IAP exposed and therefore, accessible to the antibody against IAP, so the protein is detected. In the normal cases, the protein is inside the cell because the membrane is intact, so the expression of the protein is very low.

Tube D – presence of intra-acrosomal protein: after permeabilization of sperm membrane, IAP is exposed to the antibody IAP and thus is detected. In case that sperm does not contain IAP, the protein is not detected after permeabilization. It is used ethanol as permeabilization reagent and the expression of the protein should be more than 75%.

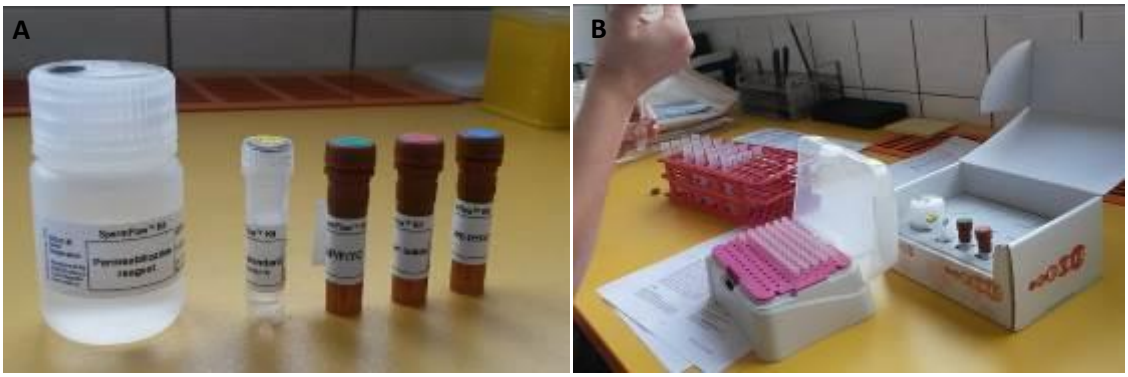

Fig 19 – A: Kit used to do the Sperm Flow Method; B: Preparation of the four tubes for each patient to then run the samples in the Flow Cytometry

✓ Inhibition of migration

Inhibition of migration is another test that can be made to see if there is any infertility problem. For this test is needed two plates with agarose gel with small holes, one for the man and the other for the woman. The both plates have to be incubated for 16 hours.

For man the plate is divided in two: one part for putting a suspension of leucocytes and medium (negative control) the other part for putting a suspension of man leukocytes plus sperm. If the migration is inhibit is because the lymphocytes produced cytokines. This happens when they are more sensitive to the sperm antigen.

For woman the plate is divided in three parts and all have a suspension of woman's leukocytes. In one part is to put the sperm of the partner, in another trophoblastic cells (obtain by cells banks that have them frozen) and in the last part is to put medium that works as a negative control. If the cells do not migrate, the surface of the hole is smaller and that means that the woman is sensitive to the sperm antigen of her

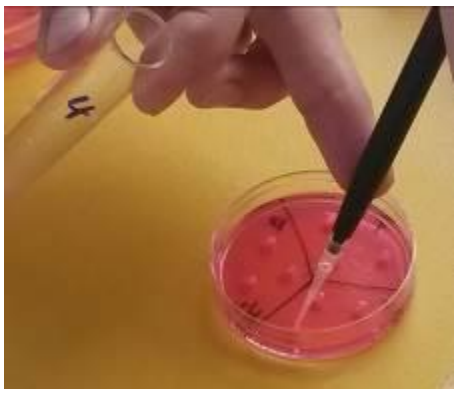

Fig 20 – Preparation of the plate for woman samples: adding leukocytes to every part of the plate

partner. This could be a reason of infertility because the woman cells produce antibodies against the sperm antigen of the partner.

The longer the woman is with her partner more probability exists for the development of these antibodies against the sperm. In addition, the long term of taking contraceptives can also lead to this. To solve this problem of inhibition of migration, it is used corticoids as a treatment, to decrease the activity of immune system, and, also, condoms for three months. If this does not work, the only solution is in vitro fertilization. If the problem is with the sperm, the solution is more complicated because there is no treatment. They only have some preventions measures like not smoke, not use computer or mobile phone in the legs and have a careful alimentation.

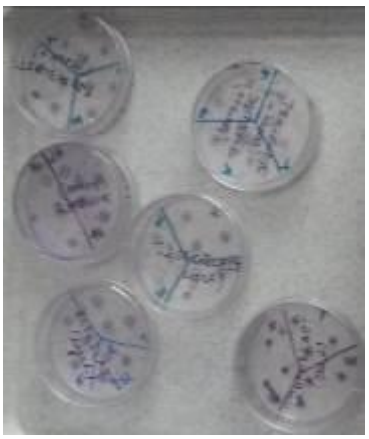

Fig 21 – Some results of Inhibition Test in five women and in one man

✓ Activation of NK cells

Activation of NK cells is another interesting test that is being carried out in unsuccessful pregnancy because a higher number of NK cells could be a reason for not getting pregnant since they act against sperm and trophoblastic cells. The aim of this test is to see the activation of peripheral blood natural killer cells in infertile woman.

To analyze the activity of NK cells it is incubated in a 96-well plate the woman's blood with medium (negative control), sperm and trophoblastic cells, during the night. After the incubation it is added the antibodies against CD3, CD56 and CD69 markers since the NK cells are identified by CD3-, CD56+ and CD69+ (early activation of leukocytes maker) in the flow cytometry.

If the result is more than 30%, it means that there is activation of NK cells and these cells are going to attack sperm and trophoblastic cells. The treatment with corticoids used to decrease the woman's immune system can also lead to the increase of NK cells.

ELISA

In this department, I also had the opportunity to do an ELISA with Mgr. Martina Koláčková, Ph.D. who I want to thank for all the explanations. The aim of ELISA that I have done was to found antibodies, present in the blood samples, against peroxidase located in thyroid to identify Hashimoto's disease.

Enzyme-linked immunosorbent Assay (ELISA) is a quantitative method for substances like peptides, proteins, antibodies and hormones, in which one of the reagents is labeled with an enzyme. This may be either the antigen or the antibody. In a well plate is coated the antigen corresponding to the target antibody. If this antibody is present in the blood sample, it will bind to the antigen. An enzyme-conjugated secondary antibody binds to the target antibody in the subsequent reaction step. Between every addiction is necessary to wash it three times to make sure that there are no free antibodies (will give us a false positive). In the presence of the enzyme, the substrate is transformed in a color staining reaction. The antibody concentration in the test can be determined by comparing the color reaction product with that of standards of known concentration, in a spectrophotometer, since the intensity of color is directly proportional to the concentration of antibodies present in the blood sample. A positive and a negative control is always needed when this test is done and, also, four standard. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction. [20]

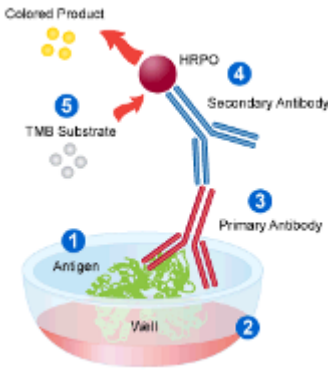

Fig 22 – Scheme of what happens in an ELISA [21]

Fig 23 – Procedure of an ELISA. A: Adding the conjugated antibody; B: After adding the substrate; C: Results after time of incubation

5. Tissue Bank

The Tissue Bank is a very important department in the University Hospital and it is specialized in collecting, preserving, testing, storing and distributing cells and tissues of human origin used for clinical transplantation. It is a member of the European Association of Tissues Banks and one of the oldest Tissue Banks (it was established in 1952). It becomes part of the European network of accredited cell and tissue establishments as expected by the European Union legislation (Directives 2004/23/EC, 2006/17/EC and 2006/86/EC) and it is able to produce advanced medicinal products, cultured autologous chondrocytes for reconstructing articular surfaces. However, for each type of tissue, the department has to have a license according to the legislation. It includes, in addition to tissue banks, also laboratory for cell therapy, organ bank and human milk bank. The responsible person of the tissue bank establishment is MD. Pavel Měřička, Ph.D. who showed me the organization of this department and explained me some theoretical concepts. [22]

The Tissue Bank activity includes preparing solid tissue grafts for reconstruction in neurosurgery, traumatology and orthopedic surgery, burn medicine and plastic surgery. Tissue culture laboratory work includes preparing cultured tissue replacement for articular cartilage reconstruction. The bone marrow bank works on cryopreservation of hematopoietic progenitors cells for clinical transplantation and cord blood banking as part of the Cord Blood Bank of the Czech Republic. [22]

The concept of the tissue bank is based on a combination of cryotechnology and clean room technology. Because of this, there exists a big container with 10 tons of liquid nitrogen, enough to supply the department for one month. The use of clean rooms is an important tool to prevent secondary contamination of samples. In addition, to prevent cross contamination during storage, double bagging is used, and the tissues from donors with markers of active severe infection (hepatitis, toxoplasmosis, etc.) are stored separately at a different place, usually a freezer or liquid nitrogen container. [22]

Cryopreservation

Cryopreservation is based on the use of very low sub-zero temperatures to preserve structurally intact living cells and tissues. After controlled-rate freezing the samples are stored in liquid nitrogen, at the temperature of -196°C (boiling point of liquid nitrogen) or preferably in the vapor phase of liquid nitrogen. Under these conditions, the cells and tissues remain viable during several years. At these low temperatures, any biological activity, including the biochemical reactions that would lead to cell death, is effectively stopped. However, it is very important to use cryoprotectant solutions,

because when they are not administrated, the cells are often damaged due to freezing during the cooling to low temperatures or having to room temperature. These cryoprotectants inhibit the formation of intra and extracellular crystals and hence prevent the cell death. [23, 24, 25]

The first cryoprotectant discovered was glycerol and it was used in sperm, bone marrow, tissues and skin because of its protective action against crystal formation during the freezing process. However, glycerol has some disadvantages. The bone marrow cells cannot be used immediately in transplantation because of the osmotic effect of glycerol that could lead to the influx of water into the cells resulting in cell rupture. To avoid this it is necessary to remove all glycerol step by step (deglycerolization) that takes a long time (more than 1 hour). For this reason, nowadays the Tissue Bank uses glycerol only in cryopreservation of sperm and skin. [23, 24, 25]

Another cryoprotectant that is nowadays most frequently used is dimethylsulphoxide (DMSO). It permeates more quickly into the cells and it is removed more quickly than glycerol. The cells cryopreserved by DMSO can be infused into the blood stream without removal. However, DMSO is a toxic substance that accumulates in the organism, so the limit dosage per day is 1 gram per 1 kg of patient weight. It is necessary to be more careful with patient that have kidney and cardiac diseases. If the patient undergoes the chronic dialysis program, it is necessary to remove DMSO by dialysis after each infusion of cryopreserved cells. In case of cardiac amyloidosis, removal of DMSO from thawed cell concentrates is performed in any case. [23, 24, 25]

Before adding to the cell suspension, the cryoprotectant should be diluted in sodium chloride or other solution. Through this is possible to minimize the potentially deleterious effects of chemical reactions, and it is also possible to ensure more uniform exposure to the cryoprotectant when it is added to the cell suspension. The potential toxic effects of concentrated DMSO are also reduced.

In the Tissue Bank, there are two types of freezers: liquid nitrogen freezers, at -196°C, which are used for storage of blood progenitors cells and sperm, but in different containers; and conventional mechanical freezers, at -80°C, that are used for storage of ligaments, bones, tendons, skin, grafts, etc. For each freezer it is necessary to control continuously the temperature. For the temperature control, the freezers have an alarm system, that if the temperature achieve the warn set point (in liquid nitrogen freezers is at -160°C) the liquid nitrogen enter in the freezer and restore the temperature.

Fig 24 – Room with the freezers

Clean Rooms

The preparation of tissues, grafts and cells for transplantation should be carefully done in the qualified areas: clean rooms that assure aseptic conditions. These rooms have to be of grade A (work under a homogenous laminar flow) with background of grade B. To enter these rooms it is necessary to pass through an entrance with controlled areas impervious (air lock). Also, it is important to have restricted standards of cleanliness and appropriate system of filtered air (removal of 99% of the particles). In these rooms, the difference of pressure is maintained at least 30Pa to prevent the passage of particles to the critical processing area. The clean room operators working in the room of the grade B need to wear sterile antiemission clothes.

The pharmacist in this room has an important role since it is he who controls the quality of the procedures. The air systems and filters, maximum number of particles and microbiology contamination are parameters evaluated for this department. In addition, the function of devices and facilities is regularly verified by validation performed by an independent company with EU accreditation.

I had the opportunity to see a room with grade B that is used for chondrocytes culture (it is needed to wear blue clothes) and to be in a room with grade C (use for less critical steps) that is used for culture of stem cells for diagnostics purposes (we had to use yellow clothes that avoid the emission of particles). In this last room, I had the opportunity to see the stem cells that were growing in the lab for a few days.

Clinical Applications

In this Tissue Bank there are many different types of tissue, that go from peripheral hematopoietic cells, bone marrow, umbilical cord blood, tissue grafts like bone, ligament, cartilage to vascular tissue. These cell grafts are used primarily in hematology and oncology. However, tissue grafts are used during reconstruction surgery in neurosurgery, traumatology, vascular surgery, orthopedics, burn medicine and ophthalmology.

There are two types of transplantation of hematopoietic progenitor cells for malignant: Allogeneic and Autologous. In the University Hospital Hradec Králové autologous transplantations are done about 30 times per year, mostly in cases of multiple myeloma and malignant lymphoma. First of all, the patients need to take out their own cells, after that, they undergo high-dose chemotherapy or radiotherapy that destroys the bone marrow and finally the patient is transplanted with the

previously cryopreserved cells. The advantage of this type of transplantation is that the patients do not receive immunosuppressors since the graft is from them. Nevertheless, it is not possible to use this procedure for everything. To do this procedure first it is needed to do separation and concentration of cells, and finally cryoprotection and freezing.

The allogeneic transplantation is when the patient receives grafts from another person. Normally the cryopreservation of these grafts is not performed as it is immediately used. For doing this kind of transplantation the HLA from the donor has to match with the patient, if not, the host destroys and rejects the tissue. For preventing this, the patients need to take immunosupressors.

Solid tissues are obtained from deceased and living donors. In the last case, they can be used for autologous or allogeneic transplantation. In case of the living donors the tissue has to be preserved until all laboratory tests are done (first after the collection and the second one after 6 months). In addition, it is necessary to do a bacteriological test to prove that all results are in compliance with the standard operating procedure of the method of processing and preservation. For decontamination, betadine is used, with except of cardiovascular tissue where a mixture of antibiotics is used.

In this department, it is also possible to have some cases of autologous **transplantation of bones**, for example, when people suffer from a contusion in the brain and it is needed to remove a part of the skull bone to decrease the intracranial pressure. This bone is frozen until the patient is in condition to be able to undergo transplantation.

During my stay in the Tissue Bank, I could observe the processing of a tibia bone before deep freezing.

Hematopoietic progenitor cells collected from peripheral blood are being increasingly used in treatment of hematological malignances such as leukemia, lymphoma or myeloma. These cells can be also used in supportive care after intensive chemotherapy. If autologous transplantation is done, the hematopoietic stem cells collected from the peripheral blood are cryopreserved. Autologous transplantation can be repeated. The amount of progenitor cells transplanted must be sufficient for a complete restoration of hemopoiesis after myeloablative chemotherapy.

Fig 25 – Cryopreservation of Hematopoietic progenitors cells

Another type of tissue that this bank cryopreserve are **cells from breast and ovary cancer**. They collect the cells at the beginning of the cancer and store them to research purposes (comparison of

the properties of cancer cells collected in the same patient in the future). This has the purpose to see if the treatment is working. In addition, these cells can be used in studies with new medicines to see if they act against the cancer cells.

Veins and arteries can be also cryopreserved. They have to be used clinically according to the type of blood group (A, B, AB or 0). With this tissue is very important to freeze and thaw slowly to prevent rupture of the vessels. These tissues are widely used in cases of ischemia of lower limbs or in case of infection of artificial vascular prosthesis.

It is also possible to cryopreserve **umbilical cord blood** to future use, if it is necessary. However, it is just chosen if there is not any compatible donor and because of this the probability of using the umbilical cord blood from the bank is just 1%. Some time ago it was claimed that umbilical cord blood, theoretically, have cells that can transform in any kind of cells. Nevertheless, no evidence of such possibility exist now. For this reason the interests of married couples to undergo this procedure is low. There are only 10 cases per year, and the insurance company only pays this if the first child had any disease that is potentially curable by cord blood transplantation.

Sperm can be also cryoconserve and normally it is done in patients that have cancer and are going to receive chemotherapy or radiation therapy because they can become temporarily or permanently infertile, so they store the sperm for a long term. After, if it is needed it can be done fertilization in vitro with the cryopreserved sperm.

In relation to the woman, the freezing of oocytes is not done because after thawing them they do not be as viable as the original ones, so there may be problems with in vitro fertilization and embryo culture. The alternative is to freeze the **embryo.** After implantation of embryo, the woman has higher probability of becoming pregnant.

Organ Bank

The Organ Bank makes part of the Tissue Bank since the early 70s and focuses on the preservation of kidneys for transplantation for clinical Regional Transplant Centre at Urological Department. The average of transplants that are made per year is 40 kidneys, which means, 20 donors. [22]

This department is oriented on kidney preservation by simple hypothermic storage as well as on continuous kidney perfusion on a machine. In either process, the organ must be in contact with a preservation solution. To control if the organ is viable it is needed to measure the perfusion pressure. If it is higher, it means that the organ is dying.

Human Milk Bank

Human Milk Bank is another part of The Tissue Bank. It was founded in 1958 and it focuses on the collection and storage of breast human milk. This milk is used for feeding immature babies that are hospitalized. The main work of this department consists in the pasteurization of breast human milk and storage for future use and it is done almost everyday. I had the opportunity to visit this bank with Mgr. Barbora Honegrová, who explained me the work that is done there. [22]

The milk is given by mothers who are on maternity, by mothers who have their babies in the intensive care unit or, also, it can be voluntarily brought by mothers who are breastfeeding, and in this case is given a small reward. The milk stored here is for internal use of the hospital, is allowed only for premature babies and for babies who are in intensive care units. In special cases, the pediatrician can prescribe this milk, however it is a very rare situation because of the limited stock of the bank and of the high price. The milk is collected everyday from the mothers that are in the hospital or it is given once a week to the bank by mothers who are at home (they store the milk in the freezer for maximum of one week).

The composition of breast milk changes during the time of a mother breastfeeding even during a single lactation. There are three types of breast milk: colostrum, foremilk and hindmilk. The main components of colostrum are antibodies and macrophages. This milk contains more proteins and vitamins and less fat in the comparison with mature breast milk. Colostrum does not contain much water – kidneys are not able to process it yet. It is rich in amount of vitamins A and E, which protect the child´s body against oxidative stress, so it is more yellow than the others are. After three days

from childbirth, breast milk begins to form. At the start of each breastfeeding, a newborn gets watery milk, which contains a little amount of nutrients and fat. This type of milk is called foremilk and is able to hydrate adequately baby and quench his thirst. Foremilk is followed by hindmilk. This milk is thick, creamy and rich in fat and proteins content and that is why banishing child hunger. Besides this, each every milk has different color and flavor because of the mother´s alimentation. [26]

The work starts with the pasteurization of the milk. It lasts for 30 minutes and it is done at 62.5°C. After this, the milk has to be cooled to 15°C. Then it is manipulated in laminar flow to divide in bottles of 100mL (identified), and to take samples to microbiological analysis (culture of sample to identify if there are any bacterial flora). The milk from women that take medicines has to have a special identification because their own baby can only use this milk; the rest of the milk can go to any baby in pediatrics. After cooling, the milk is put into a shocker to be frozen under control to -16°C during one hour. The final step consists in storage the milk on the freezer at -22°C. After all this steps the milk has an expiration date of three months.

Fig 26 – Process of the pasteurization of the milk. A: cooling after the milk being at 62.5°C; B: taking samples for analysis; C: store in the freezer

As we can see, the pathway of milk is not so simple, and it is one of the reasons that makes this milk expensive. The microbiological tests contribute a lot for the higher price of this milk.

6. III. Internal Gerontometabolic Clinic - Laboratory

The III. Internal Gerontometabolic Clinic - Laboratory makes part of one of the best hospitals in Czech Republic, known for the excellent conditions it offers. This department cooperates mainly with other departments such as Oncology, Surgery, Nephrology and with the Department of Analytical Chemistry of the Faculty of Pharmacy. This department has several functions like: acute diagnostics and treatment of patients with disorders of metabolism and nutrition (Diabetes Mellitus, liver or kidney insufficiency, obesity); support of patients that need enteral and parenteral nutrition, especially in complicated cases; diagnosis and treatment of acute illness of the aged patients, of disorders of metabolism and nutrition and of internal diseases with special emphasis on internal diseases in old age and the issue of premature aging. [27]

This department has different projects, but they are especially associated to degenerative diseases related to the biological phenomenon aging. Aging is a cause of concern since the health status decline. With aging, the population becomes more heterogeneous due to the increasing prevalence of chronic illness and disability, such as coronary heart disease, stroke and cancer. Many of the contributing mechanisms have not yet been identified. In attempt to identification and prognostic these disorders as soon as possible, the investigation teams are now focused in the vitamins and biomarkers of immunologic activation, like neopterin.

In this department, samples of amniotic fluid, ascites, exudates, urine, human breast milk and human serum are received and then is made the determination of important compounds, like vitamins. HPLC is one of the principle techniques performed in this department.

High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is one mode of chromatography that appeared in the middle of 1970s and is one of the most powerful tools in analytical chemistry. It enables the separation of complex mixtures into their individual components. This can be achieved by making use of different interactions of compounds in solution, with a mobile phase and a stationary phase, since they have different relative affinities in both phases. It is a process, which involves mass-transfer between stationary and mobile phase. By selecting a particular combination of these phases, it is possible to choose and optimize the mode of separation. The components of HPLC system are a solvent pump, a degasser, a communication model, a sample injector (autosampler), a column oven (with thermostat), a HPLC column, a detector and a computer data station. [28, 29, 30]

The HPLC system works as follows: the liquid mobile phase is in a reservoir and is pumped through an injector into a column and out to a detector. Firstly, the analytes are dissolved in a mobile phase or in another solvent compatible to the mobile phase and then this mixture is injected into a flowing mobile phase and reaches the chromatographic column. This column contains the packing material, called stationary phase that separates target analytes. After separation, the analytes are eluted to the detector and cause a signal that will be multiplied and sent to the data acquisition system. This system records the signal, required to create the chromatogram on its display and to identify and quantify the concentration of the sample constituents. [28, 29, 30]

A commonly used form of HPLC is so-called reversed phase HPLC. Here, a hydrophobic stationary phase is used and compounds are loaded under aqueous conditions. Consequently, hydrophobic compounds preferably interact with the stationary phase, rather than remaining dissolved in the aqueous phase. After loading, the conditions in the liquid phase are slowly changed from aqueous to organic. This results in the elution of compounds from the stationary phase in order of hydrophobicity. [28, 29, 30]

Because of sample compounds characteristics, different types of detectors have been developed such as UV-VIS detector, fluorescence detector, mass spectrometer (MS) detector, electrochemical detectors, refractive index (RI) and conductivity detector. [28, 29, 39]

I had the opportunity to analyze and quantify vitamin B in plasma, serum and urine, as well as in standards prepared by me. The biological samples first have to be prepared using sample preparation techniques and just then can be injected into the HPLC. Besides this, I also had the opportunity to test the reproducibility of the method developed to determine arginine and its metabolites (citrulline and ornithin) in wounds, with some standards. In both this works, derivatization is needed.

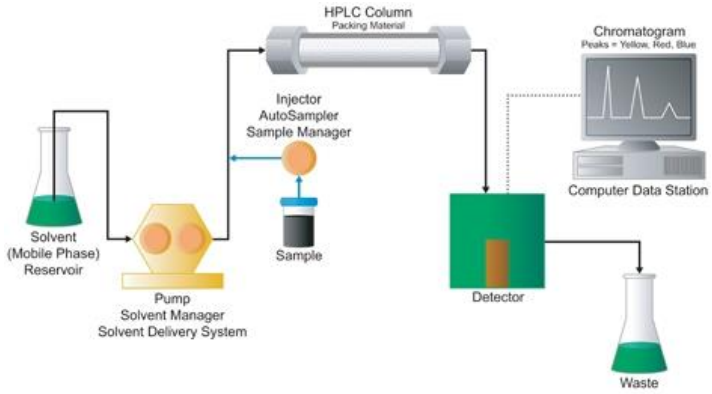

Fig 27 – Schematic representation of a HPLC system [29]

7. Department of Hematology

The department of Hematology provides comprehensive care for patients with blood diseases and hormonal disorders in the East Region, working as a central laboratory (24 hours open per day). I had the opportunity to visit this department and to learn about their work thanks to Mgr. Filip Vrbacký. This clinic performs routine and specialized examinations for University Hospital and East Bohemia and it is possible to collect samples, in a separately room, however just without puncture. [31]

The main work of this department is the count of white and red blood cells, the determination of blood viscosity and the measurement of adhesion and aggregation of platelets (this way it is possible to see if they are totally functional). They also can measure the fragility of red blood cells membrane by osmotic pressure.

It is possible to do differential count extremely fast, from the peripheral blood, which is a great advantage. However, if something is wrong in this count it is possible to smear the sample and then see in the microscope the cells. Another possible option is to use the Cell Image Analysis System (cellavision) that separates every cell present in the sample and then takes photos to be analyzed.

In relation to coagulation time, there are two different ways to determinate it. In one of them, it is used cuvettes, where the machine mix the sample with the reagent and, then, a light beam measures the time of coagulation. In the other way there is an instrument that uses a small ball that moves inside the sample when the blood is not coagulated and it stops moving when the blood is coagulated. This time between the movement of the ball and the stop gives us the coagulation time. The result of coagulation time is expressed in ratio between control and the patient sample. The time of the control has to be measured everyday.

I also had the opportunity to see at the microscope samples of bone marrow, from patients that have leukemia to see the difference in number and in shape of leukocytes in these patients when it is comparable to healthy people.

8. Biomedicine Center

The Biomedicine Center is a department of the University Hospital in Hradec Králové and I had the opportunity to see the work that they make and the importance of this Center thanks to Dr. Ondrej Soukup. This Center exists since 2011 and counts with 50 to 60 employees being most of them pharmacists. [32]

The main work of this Center is to help clinics with basic research in cooperation with the Hospital, the Faculty of Pharmacy and Medicine and with the Faculty of Military Health Science. This center produces, per year, around 1000 compounds mainly in areas of Alzheimer, cancer, infections and antidotes against organophosphates that are a poison. [32]

The work of this center can be divided in two main areas, Drug Development and Proteomics. In Drug Development, to have a result, it is needed to go through different steps and it all starts with in silico design in computers. Then it is needed to pass to in vitro screening tests and measure physico-chemical properties such as solubility (principal concern), log P/log D, pka, metabolites and is also important to see the Blood-Brain Barrier prediction that is performed by a PAMPA assay (this tell us how the drug crosses the lipophilic membrane). After this step, it is needed to check the cytotoxicity in cell lines and also the toxicity in animals by measuring the acute, sub-acute and chronic doses and how the local of administration changes these parameters. The fourth step is about pharmacokinetic tests where it is measure drug plasmatic levels, half-life time of the drug and clearance of the same. Finally, the last step is to do in vivo tests.

In Proteomics, the main goal is to measure proteins in biological products, in a quantitative or qualitative way. However, it is also done purification and protein profile by identifying thousands of proteins in nanoHPLC.

9. Department of Analytical Chemistry

In this department, I had the opportunity to learn about two different important techniques used in the field of Pharmacy. Capillary Electrophoresis, which I learned from Mgr. Klára Petrů, Ph.D. and Flow Injection that was explained to me by Dr. Burkhart Horstkotte.

Capillary Electrophoresis

Capillary electrophoresis is an analytical technique that separates ions based on their electrophoretic mobility with the use of an applied voltage. The electrophoretic mobility is dependent upon the charge of the molecule, the viscosity, and the atom's radius (electro-osmotic flow). The rate at which the particle moves is directly proportional to the applied electric field; however, neutral species are not affected, only ions move with the electric field. This happens because the neutral species do not have affinity to the cathode neither to the anode. If two ions have the same size, the one with greater charge will move fast. For ions of the same charge, the smaller particle has less friction and overall faster migration rate. Therefore, first migrate the smaller cations and then the bigger ones, after them migrates the neutral species and finally migrates the anions (migrates to cathode and not to anode, but the applied voltage makes them follow the direction of anode). For detection is possible to use conductivity, UV or fluorescence detector. To increase this signal and its sensitivity it is possible to use z-cell or a tube with a bubble. Capillary electrophoresis is used most predominately because it gives faster results and provides high resolution separation. [33]

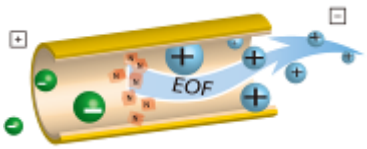

Fig.28 – Migration of particles in Capillary Electrophoresis [34]

Flow Injection

Flow Injection is a continuous flow technique. The sample is injected into a flowing carrier stream of reagent. As the injected zone moves downstream, the sample solution disperses into the reagent, and a product begins to form at the interface between the sample zone and the reagent. A detector (spectrophotometer) placed downstream, records the color or another parameter as it changes due to the passage of the sample material through the flow cell. The detector readout is therefore the result of two processes that take place simultaneously: sample dispersion and subsequent chemical reactions. It is important to apply flow programming, so that each step of an assay protocol is performed at an optimized flow rate. The advantage of flow programming is the increased sensitivity and detection limit of most reagent based assays, achieved by adjusting the flow rate and incubation time. Flow Injection is a valuable tool for studies in Pharmacology, Chemical Oceanography and Environmental Monitoring. 35

10. Conclusion and Acknowledgments

This internship was a great experience of life, mainly because I had the opportunity to learn about many different areas, that makes part of a hospital. I am sure that this experience made me richer in terms of knowledge and maturity and for all this, I owe an enormous thanks to Prof. Doutora Angelina Pena for being responsible for this opportunity and for all her support and availability during my training period in Czech Republic.

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