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University Hospital Hradec Králové – Czech Republic

Hospital Practice Report

Hospital Pharmacy

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Index

1. Introduction

This report was developed during my Erasmus Student Placement at the University Hospital Hradec Králové in collaboration with the Faculty of Pharmacy of Charles University in Prague, between January and March of 2015. It includes a description of the techniques that I performed or observed and also theoretical concepts that I learned in the different departments where I have been.

The first part of this report is a brief introduction of the Faculty of Pharmacy, the Charles University and the University Hospital Hradec Králové.

The second part is about the Hospital Pharmacy, its organization and mostly the visits to the Oncology Department, Geriatric Department and Pediatrics, where I could practice Clinical Pharmacy, reviewing the medication of patients with cancer, elderly people and children. Also, I have a description of my passage at the Cytostatics Department.

The third part concerns to my stay at the Tissue Bank, where I learned for the first time the concepts of cryopreservation based on cryotechnology and human breast milk donation.

The fourth part includes my passage at the Biomedicine Center, in particular its organization and the work that is performed in the present.

The fifth part of this report explains the work developed by the Department of Clinical Immunology and Allergology as well as some concepts that I have learned.

In the sixth part there is a description of the Department of Metabolic Care and Gerontology and some techniques performed by me there.

The seventh part is about the Department of Hematology, where I could see its organization and the different phases of hematological analyses.

The eighth part is about my visit to the Faculty of Pharmacy, specifically in the Department of Analytical Chemistry.

2. University Hospital / Charles University / Faculty of Pharmacy

The University Hospital Hradec Králové is one of the biggest health facilities in Czech Republic. The hospital is an important center of training for pharmaceutical and medical workers. In 2009, the University Hospital Hradec Králové took the prestigious first place in the University Hospitals of the Czech Republic Category.

Fig. 1 - University Hospital Hradec Králové

University Hospital Hradec Králové fulfills its historical legacy in close cooperation with Faculty of Medicine in Hradec Králové, Charles University in Prague and other partners such as the Military Medical Academy Association and the Faculty of Pharmacy. [1, 2]

Fig. 2 – Charles University in Prague

Charles University was founded on April 7th of 1348 by Charles IV, making it one of the oldest universities in Europe. It is also renowned as a modern, dynamic, cosmopolitan and prestigious institution of higher education. There are currently seventeen faculties at the University (in Prague, in Hradec Králové and in Plzeň). [3]

The Faculty of Pharmacy of Charles University is located in Hradec Králové and it was established in 1969. This Faculty of Pharmacy has continued in the old and long-time tradition of the education of pharmacy at Charles University. [4]

Fig. 3 – Faculty of Pharmacy

3. Hospital Pharmacy

The Hospital Pharmacy is composed by seventy employees: twenty pharmacists, twenty technicians and the rest are auxiliaries. The pharmacy is completely opened between 7h30 to 16h and after that the service to the public is made through a wicket by a pharmacist and a technician (in the total the pharmacy is on service 24h/day).

The responsible person for my stay here was PharmDr. Martina Maříková, who taught me most of the things I learned in this department. The Hospital Pharmacy has many different areas such as:

1 – Outpatients: Every person can go to the pharmacy and take the medicines or medical devices that are needed. The person doesn't have to be hospitalized in the hospital to get the medicines. The pharmacy provides basic and specialized pharmaceutical services for outpatients too, not only for inpatients. The most required medicines are for cardiovascular diseases.

Fig. 4 and 5 – Ambulatory facilities.

2 – Inpatients: The hospital has 1200 beds, but only about 1000 are occupied. The main goal of the pharmacy is to delivery in time, safe and correctly all the medicines to the different departments according to the prescription.

3 – Laboratory of Manipulation. Essentially, the main activities include the preparation of special dosage forms such as preparation of parenteral nutrition or individual preparation of sterile drugs. Besides, medicines to clinical trials developed in the hospital are produced here, namely clinical trials about multiple sclerosis. There are also different places for the preparation of pills, solutions (eye drops and disinfectants as well) and ointments.

Also, there are specific and sterile rooms for parenteral nutrition. Everyday about 20/30 bags of parenteral nutrition are produced in the hospital specifically for each inpatient according to his needs, his weight and the desired volume. For outpatients these formulas are

produced once a week. This is a high clean space with background grade B, where I could observe the preparation of parenteral nutrition for adults and for neonatal babies.

The other sterile room is used to the preparation of sterile solutions for use in hospital, such as ethanol 70% and 96% (V/V) and color solutions (for example, Panteblau) that are main used in gastroenterology (endoscopy) and radiology. These solutions are sent to a company that tests their sterility and only after that, the solutions return to the hospital to be used.

Fig. 6 – Parenteral nutrition preparation.

4 – Laboratory of Drug Control – High Quality Assurance Department. Here the organoleptic proprieties are tested and analytical analyzes are done on the drugs that are produced in large amounts in the hospital, principally solutions that are posteriorly divided and storage. I have been in these two types of laboratories inside the pharmacy building.

Fig. 7 – Laboratory of drug control.

5 – Clinical Pharmacy: to improve the pharmacotherapy and to provide scientific information about the drugs, the pharmacy has a cooperation (that started two years ago) with the Department of Social and Clinical Pharmacy Drug Information Center in Faculty of Pharmacy. [5]

Two types of information are given: how much the patients have to pay for the medicines and pharmacological information about the doses, how to take, interactions and special advices for each situation. These informations are given to the patients and also to the doctors and it works on demand. The critical care units are metabolic diseases (geriatric, kidney transplantation and diabetic diseases) and also some cooperation with gastroenterology and hematology. Furthermore, pediatrics and oncology are another two areas of special care.

I have been in the storage of medicines to in and outpatients. Also, I passed in the dispensing of drugs. The medication is requested once or twice a week, depending on the needs, for each department and not for each patient. Nevertheless, it's possible to go to the pharmacy everyday to ask for some forgotten medication or in case of urgency.

Before giving or sending the medicines, it's necessary to check if everything is in accordance with the prescription. Also, everyday all the medicines that were provided have to be reviewed and this process has to be done for two different pharmacists to minimize the occurrence of mistakes.

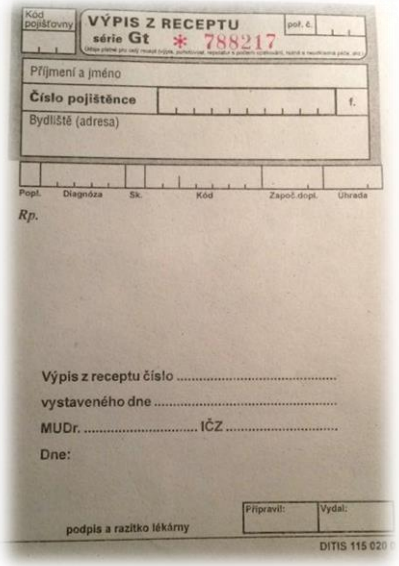


Fig. 8 – Prescription.

a) Clinical Pharmacy

i. Geriatric Department

The main work of the pharmacist in this department is to certificate that every medicine is correct for each patient. Before the visits to the patients, all the parameters have to be checked everyday: if the medicines are according to the disease, the doses, when and how to take, risks, complications and interactions. If there is an interaction, the medication has to be changed if there are some manifestations. Besides, it is important to check the compatibility between the chronic and the new medication. Essentially, the central activity is the review of all the medication.

Also, it's necessary to check biochemistry and hematology analyses. One important marker in this area is the C- Reactive Protein (CRP), which is an indicator of infection or cancer. And in case of infection, microbiology analyses have to be done and checked as well.

During my stay in this department, I had the opportunity to do visits with the pharmacist and the medical doctors, to every patient to see his state, how he feels, to hear his complains, reviewing the medication and checking if he is responding well to the treatment. Also, if it is needed the medical doctor has to ask some basic questions to the patient to realize his mental state such as the name, how old is he, where he lives. This way, it is very important the communication between the doctor and pharmacists and there is a dossier for each patient with all of this information.

There are only twenty-two beds and they are just for people with more than 78 years old. Because they are elderly people, it's required a special attention to the liver and kidneys to see if they are working correctly. If there are some problems, it's needed calculate and adjust the doses. Furthermore, taking account the age and the innumerous co-morbidities, the main goal is stabilize the patient until the previous state to his hospitalization.

There is no temporal limit to stay in the hospital; the patient only goes home when he is stabilized. However, the majority of the people that are in the Geriatric Department are acute situations. Because there are a lot of cases, there are no enough beds for all of them, so the patients with less serious complications also can stay in nurse's homes and in institutions of long term care.

Fig. 9 – Rooms in Geriatric Department.

The pharmacist has an important role in this cooperation with these institutions and with the family, with the central aim to keep the patient's well-being and comfort. The pharmacist has to explain to the patient's responsible all the care and ministration with the medication and what are the non-pharmacologic approaches.

Within this department there are many types of diseases, but the cases that I most contact were: broken arm, bronchitis, atrial fibrillation (edema, fever), urinary infection (with high CRP, high temperature and confusion), ischemic heart disease, thrombosis, pneumonia, pulmonary infection, gastrointestinal bleeding, *Helicobacter pylori* infection, chronic obstructive pulmonary disease and upper gastrointestinal *Candida* infection.

In accordance with these diseases, the more prescribed and reviewed medication was corticoids, anticoagulants (warfarin mostly), antibiotics, painkillers and the triple therapeutic for H. pylori (proton pump inhibitor plus amoxicillin plus clarithromycin).

ii. Pediatrics

Like in the Geriatric Department, my stay here was about medication review. Still, it's essential to be careful with the determination of the doses because the patients are babies and children, so every single dose of medicines for each patient has to be calculated based on the weight and sometimes based on the body surface area. The main sources that are used to calculate the doses were Lexicomp online and the book BNF for children and also UpToDate to consult the pathology of the disease.

This department offers diagnostic and therapeutic care to children from birth to 19 years of age and is divided in two parts: one of intensive care with high level of monitoring and one called standard wards where the cases are not so serious than the first part but with equal level of care. [6]

Before each visit, a multidisciplinary team constituted by medical doctors, pharmacists and nurses have a reunion to discuss every case and I had the opportunity to be present with PharmDr. Petra Thomson. In this meeting, the team reviewed what happened, why the children are in the hospital, his medical history and finally go through the prescription, not forgetting to check the correct doses, timetable and way of medical intake.

The cooperation between pharmacists and medical doctors started recently, but it has been a success and continues in development to improve more and more the life and well-being of these little patients, who deserve a special care.

I could contact with several cases such as fever and convulsions, thoracic insufficiency syndrome, *Pseudomonas aeruginosa* infection in child with Pierre-Robin syndrome, pneumonia caused by *Streptococcus pneumoniae*, acute obstructive pancreatitis, hypoglycemic crisis in patient with diabetes type 1, cystic fibrosis and hematuria.

Taking into account the above-mentioned diseases, the following medicines were reviewed: antibiotics (mainly cephalosporins and aminoglycosides), benzodiazepines (because some of them were very stressed), proton pump inhibitors, adrenergic β2 agonists, antihistamines, corticosteroids, painkillers (principally metamizol that is used a lot in Czech Republic), antipsychotics, insulin and vitamin D. Some of these patients had special alimentation or parenteral feeding to don't throw up.

iii. Department of Oncology and Radiotherapy

The Department of Oncology and Radiotherapy is ranked among the comprehensive cancer center and it is been leading to implement new methods of cancer treatment. Treatment of cancer is based on a system of standards treatment and interdisciplinary collaboration. The department is divided in two parts: ambulatory/outpatients and inpatients. Inpatients unit has a capacity of sixty-four beds and here are the patients with kidney and hematologic problems or the patients that are doing thirty days of radiotherapy. The patients that are doing chemotherapy treatments only stay six or seven days and then they go home. [7]

The use of chemotherapy or radiotherapy depends on the recommendations of guidelines that are updated every six months and that are based on clinical trials. There are different types of chemotherapy such as palliative, adjuvant, neo-adjuvant, exclusive or regional.

All the adverse effects of chemotherapy are reported to the National Institute in Prague. Vomiting and nausea are the main side effects of chemotherapy. The side effects of radiation depend on the affected area. For example, if the radiation is in the neck or face, there will be mucositis problems; radiation for breast cancer causes skin problems; radiation on the stomach causes nauseas and vomiting; radiation on anus origins diarrhea.

Besides the cytostatics, a lot of medicines are produced in the Hospital Pharmacy to relieve and prevent complications/pain: Morphine gel; Vitamin K for skin problems; Capsaicin crème and Lidocaine gel to alleviate pain; Benzocaine emulsion to put on the skin before the use of Qutenza patch, because it is very hot and also to help the pain during the feeding; Omeprazole suspension that is administrated through a tube in stomach for people with mouth problems such as mucositis; and also Caphosol with phosphorus and calcium to prevent mucositis. To xerostomia are used artificial salines and for pain the choice is Fentanyl and Buprenorphine.

During my passage to the Department of Oncology and Radiotherapy, I joined to the team that analyzes and discusses every case of inpatients. The team is constituted by the chief of chemotherapy, the chief of radiotherapy, the head of the department, medical doctors, pharmacist and senior nurse. The medical doctors review the actual medical history, predict what will happen in the future, what cytostatics are used (normally a combination of them with the aim to be more aggressive to the tumor), what are the effects that the patient will suffer and when it is the best time to end the treatment. The nurse, who is the person that contacts more with the patients, tells how they are, eat, sleep and so on. The pharmacist reviews all the medication and adjusts it to the kidney function of the patient, gives information to the doctors about new medicines and recommendations about changes in the

treatment that should be done. The pharmacist also recommends increase the dose if the medicine isn't taking effect or decrease the dose when it is too much, change the formulation or stop/start with some medicine.

During the visits that I did to the patients, the team asked about how they feel, how bad is their pain and it was possible to observe a lot of problems related to mucositis and skin injuries. I contact with different types of cancer such as gastric, in esophagus, kidney, brain, neck, utero, vulva, colon rectal, testicles, anus and pancreas. Also, I saw lung and triple negative breast cancers (which is the worst type of breast cancer) that are only treated with radiotherapy. There is a dossier for each patient with information about the following parameters: temperature, level of pain, medication, glycaemia, pressure and pulse, control of nutrition, results of the biochemistry and hematologic analysis. Hemoglobin level should be high because the tissues must have great levels of oxygen to radiotherapy works; if the levels are low, the patient has to do a blood transfusion. Moreover, if there is some infection, microbiological analysis are also done (antibiogram) to discover the microorganism's resistances to the antibiotics. However, it's necessary to be careful with the antibiotics administration because of the kidney function.

Neoplugen is a support treatment very used for people who are doing chemotherapy because the treatment decreases the number of leukocytes and this medicine increases the production of them and co-stimulates the production of granulocytes. Another class that is very used is anti-emetics drugs like Granisetron, Dexamed, Cetrone and corticoids in small doses. High doses of corticoids are used as anti-edemic for brain metastases; in this case, it's necessary to take omeprazole to protect the stomach and be careful with glycaemia and potassium levels. There are special cases when benzodiazepines are administrated as anti-emetic drugs, because of psychological effects, for example, when the treatment is done with doxorubicin which is red, patients start vomiting when they see red things. Antidepressants are very prescribed for patients who start developing psychological problems because all the suffering and pain with the treatments.

In the ambulatory, I saw the patients receiving the treatment. They spent all day there because they have to do blood tests to evaluate blood count and kidney function. Then they have to wait for the results and medical doctor's interpretation and decision about the treatment. After that, the patients have to wait for the cytostatics preparation and then they receive the intravenous transfusion. If the treatment is tablets, they can go home. Some drugs can destroy the small vessels with intravenous transfusion, so to prevent this situation most of the patients use a central line to inject the cytostatics directly into the vena julgularis.

b) Cytostatics

Annually, there are 67.000 of new cases. In particular, there is an increase of breast and cervical cancer in women and prostate cancer in men. In both groups there is a rise of cancer of the colon and rectum. [7]

Taking this into account, the Cytostatics Department has a fundamental role in the life of cancer patients. Besides, this department is also important in neurological, rheumatologically and ophthalmological diseases. With the explanations of Dr. Zuzana Ducháčová-Woidigová, I could better understand the complex job made by this department.

First of all, I had contact with the software of cytostatic's prescription, CATO. The medical prescription is made by the oncologist and the pharmacist has to do its validation, checking a lot of topics like the name of the patient, weight, surface area, name of the chemotherapy protocol, number of the chemotherapy cycle, the diagnosis, cytostatics and other prescribed drugs, time and way of administration and so on. The doses depend on the surface area (mostly), weight or renal clearance and the number of cycles of chemotherapy depends on the diagnosis, age and state of the patient.

After this, the cytostatics can be prepared but only by qualified staff like pharmacists or pharmacist's assistants. This preparation has to be done under aseptic and safe techniques, because of the risk of contaminating the operator. Firstly, all the medicines and preparation material are disinfected commonly with alcohol in a separate room and then they are transported to the isolators. The space of preparation itself is a closed system and has grade A with background of grade B and C, where the operator only has his hands inside of it. Besides, there is software inside the isolators that tells to the operator all the steps that he has to do.

Fig. 10 – Preparation of cytostatics inside the isolators.

The preparation of cytostatics involves sterile materials, cleaning rooms, air filtration, periodic monitoring, cleaning plan, periodic rotation of cleaning agents, because of the resistances, and special outfit (polystyrene) without emission of particles. The validation of

cleans rooms is done by a company once a year and the microbiological self-monitoring and sterile tests are done every months.

All these procedures are extremely important to guarantee the safety of preparation because of acute toxicity (for the patient) or delay toxicity (for the operator). Cytostatics could cause carcinogenicity, embryotoxicity or teratogenicity. To avoid these, workers have to use protective equipment such as respirators and protective garments. Waste disposal is made only by incineration to prevent contaminations and everyday after the work is done all the equipments and rooms are cleaned and sterilized. Every preparation has a unique number to prevent errors and the operators are required to have a break of 15 minutes every two hours.

The cytostatics can be solution or powder. If it's a powder, it has to be reconstituted before administration. And if it's a solution, it has to be diluted with physiological solution or glucose. After preparation the cytostatics are packed in plastic bags, put inside a closed box and identified. The photosensitive medicines are put inside black bags.

Fig. 11 – Packing of cytostatics in plastic bags.

Everyday are produced about two hundred and twenty cytostatics and the expiration date could be one hour or nine days. The most prepared cytostatics are 5-fluorouracil, rituximab, trastuzumab, doxorubicin and cyclophosphamide and the most used routes of administration are intravenous infusion and intravenous, subcutaneous or intramuscular bolus.

The department participates in international multicenter studies involving new drugs, mainly about breast carcinoma, lymphoblastic and acute leukemia and multiple myeloma. [7]

The role of the pharmacist in this process is critical since the establishment in collaboration with the clinical team of therapeutic protocols, validation of prescription, preparation and dispensing of preparations guaranteeing the correct composition, purity and asepsis, packaging, identification, transport and administration to the patient.

4. Tissue Bank

The Tissue Bank was established in 1952 and it is one of the oldest tissue banks in the world. It is a member of the European Association of Tissue Banks and the head of the tissue establishment is Pavel Měřička, M.D., Ph.D. He showed me the facilities and the work that is done by this department and also taught me some concepts of cryopreservation.

The Tissue Bank is a separate unit specialized in collecting, processing, testing, storing and distributing of cells and tissues of human origin for use in clinical transplantation. It includes, in addition to Tissue Bank, where the main activity is about cryotechnology, also the Organ Bank and the Human Milk Bank. [8]

My visit started outside of the main building where there is a big container with 10 tons of liquid nitrogen. This amount is enough to supply the entire department during one month. Inside of the building I had to take plastic shoe covers because all the area has to be clean as possible in order to decrease the number of particles in the environment. I saw many containers, where cells intended for clinical transplantation were stored at temperatures of the vapor phase of liquid nitrogen (-160 -195°C) to guarantee safe storage. Other solid tissues, such as skeletal tissue are stored at -80°C. Saving cryopreserved cell grafts at temperatures of liquid nitrogen allows their long-term storage for years or decades. [8]

For each type of tissue, The Tissue Bank has a license according to the European Union Directives 2004/23/EC, 2006/17/EC and 2006/86/EC. All the freezers have an emergency liquid nitrogen back-up cooling system to ensure that the level of nitrogen is between 50-100mm and to prevent damage in case there is a problem with the containers. When the temperature decrease increases to -160°C (warm set point), more nitrogen enter to restore the temperature to the normal values. Each freezer has to be controlled relatively to the temperature and double bagging is used to prevent cross contamination during storage. Also, in case of patients that have infections such as hepatitis or toxoplasmosis, the cells are preserved in a different freezer and/ or container to not contaminate other cells.

a) Cryopreservation

Cryopreservation is the process where living cells and tissues are preserved by coiling to deep subzero temperatures like -196°C, in presence of specific compounds preventing the freezing-thawing damage (cryoprotectans). At these temperatures, it is not possible to find any kind of biological activity, including biochemical reactions that could lead to the cell death are stopped. [9, 10, 11]

Cryoprotectives agents are used to prevent dehydration and the formation of intra and extracellular ice crystals, if they are not administered, the preserved cells can be damaged due the freezing process or warming to room temperature. [9, 10]

The first cryprotectant discovered was glycerol and it was used in sperm, bone marrow, tissues and skin. However, glycerol has some disadvantages like in the case of bone marrow cells, where it can't be used immediately in transplantation because of the osmotic effect of glycerol that could lead to the rupture of the cells. Thus, the deglyicerolization process has always to be done, removing the glycerol step by step with deglyicerolization solution and it takes much time (more than one hour). Because of that, the Tissue Bank uses glycerol only in sperm and skin cryopreservation. [10, 11]

Now, DMSO (dimethyl sulphoxide) is the most frequently used cryprotectant. It permeates more quickly into the cells and it is removed faster than glycerol. However, it is a toxic substance that has the capacity to accumulate in the organism, so the limit dosage per day is 1 gram/ kg of patient weight combined with normal saline and serum albumin. It is necessary to be more careful with patients 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. [9, 10]

Different materials can be processed and stored in the containers:

- Bone marrow cells, peripheral hematopoietic cells, umbilical cord blood which are used primarily in hematology and oncology. This cells and also sperm cells (in a different container) are stored at -196°C in the liquid nitrogen freezers. Some solid tissues like arteries, veins and heart valves are stored in liquid nitrogen as well;
- Other tissue grafts, e.g. bones, ligaments, cartilage, skin are stored at -80°C in the conventional mechanical freezers and are used during reconstruction surgery in neurosurgery, traumatology, orthopedics and burn medicine. [8]

Fig. 12 – The view inside a liquid nitrogen freezer.

b) Clinical Applications

- **Autologous vs Allogenic Transplantation**

Stem cells transplantation represents a critical approach for the treatment of many malignant diseases. There are two different types of transplant for malignant diseases: 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 immunosupressors since the graft is from them. Nevertheless, it is not possible to use this procedure for everything, it is used mostly in multiple myeloma and malignant lymphoma.

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.

- **Autologous Transplantation of Bones**

There are also some cases of autologous transplantation of bones. For example when people suffer a contusion in the brain that can lead to death or incapacity, a part of the skull bone has to be removed to decrease the intracranial pressure and cryoconserve it. Then when the situation is normalized, the bone will be put it in the place again.

An interesting fact is that the type of blood doesn't interfere with bone transplantation.

- **Deceased and Living Donors**

To obtain the tissues there are deceased donors and living donors. The last one can be the source of tissues usable for autologous or allogenic transplantation. In case of living donors the tissues are kept in the quarantine until all laboratory tests are done.

In living donors the first test is done after collection and then again after six months of quarantine. Also microbiological tests have to be done to prove that the method to conserve is

suitable and to see if there is some infection. Betadine is the agent used for decontamination, except of cardiovascular tissue where the option is a mixture of antibiotics.

After that the grafts are ready to be finally stored and ready to be used in the future.

- **Cancer Tissues**

Another type of tissues that are frozen belongs to breast and ovaries tumors. They are removed in the beginning of the disease to make a comparison in the future, after the treatment. Besides, these tissues can be also used to test new procedures and new drugs against cancer diseases.

- **Cord Blood**

Cord blood can also be preserved to be used in the future. However this a last option if there is no compatible donor and the probability of the use of cord blood from cord blood bank is about 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 exists now. As a result the most couples don't want to do the cord blood cryopreservation, whereby there are about ten cases per year. Besides, insurance companies only pay the cryopreservation of cord blood of the second child if the first one has an illness curable with cord blood transplantation.

- **Sperm and Oocytes/Embryo**

Part of cancer care for patients who are receiving chemotherapy or radiation therapy is the cryopreservation of sperm and its long-term storage in the event that they suffer from temporary or permanent loss of fertility. Posteriorly, if it is needed, it's possible to do *In Vitro Fertilization* (IVF) with the cryopreserved sperm.

In case of women, it is not recommended to do the freezing of oocytes. It's harder to do the IVF, because after the thawing the oocytes may be damaged, so there are lower rates of successful *In Vitro Fertilization*. The alternative is freezing the embryo and it´s implantation when the woman wants to have children. This results in a higher chance to achieve good pregnancy rates.

- **Blood Vessels**

Other functionality of the Tissue Bank is the cryopreservation of veins and arteries according to the type of blood (0, A, B, AB). These vessels are more used in cases of ischemic

disease of lower limbs, freezing and thawing must be done slowly in order to prevent their rupture.

Fig. 13 – Cryopreserved blood vessel.

c) Clean Rooms

The pharmacist can have different functions in this department and one of them is the control of quality of the procedures and of clean rooms. The processing of tissues and cells grafts for transplantation has to be done in aseptic conditions in these clean rooms with air lock system, restricted standards of cleanliness and appropriate system of filtered air. These three parameters are essential to avoid secondary contamination of samples before cryopreservation.

During my stay in The Tissue Bank, I could attend to the manipulation of a tibia bone to be deep frozen that was made in an area of grade A (with laminar flow) with background of grade B, where the operator wore sterile antiemission blue garments.

Fig. 14 – Preparation of a tibia bone to be cryopreserved.

In the tissue culture laboratory the main work is to prepare cultured tissue replacement for articular cartilage reconstruction. In this part of the bank, I could observe at the microscope some cultures of bone marrow cells for diagnostic purposes.

I also have been in areas of grade B (where are also used blue garments) for the culture of chondrocytes and areas with grade C for the manipulation of stem cells (where I wore yellow garment).

Inside these rooms there was a pressure difference of 30Pa in order to prevent the entrance of particles into the critical processing area and there was also a high efficiency filtration of the air able to remove 99% of the particles.

d) Organ Bank

The Organ Bank is part of the Tissue Bank since the early 70s and focuses on the preservation of kidneys for transplantation for clinical Regional Transplant Centre. The average is about twenty donors per year (forty kidneys). [8]

The Organ Bank is oriented on kidney preservation by simple hypothermic storage as well as on continuous kidney perfusion on a machine. The organ has to be in contact with a preservation solution. One way to control if the organ is viable is measuring the perfusion pressure, because if the organ is dying the pressure increases.

e) Human Milk Bank

The Human Milk Bank was founded in 1958 and it was the first milk bank in Czech Republic. It focuses on the collection and storage of breast milk for immature babies that are hospitalized at Department of Pediatrics. The Milk Bank was shown me by Mgr. Barbora Honegrová who explained me that main work of this department consists in the pasteurization of breast human milk and storage for future use and it is done almost everyday. [8]

The composition of breast milk changes during the time that a mother is breastfeeding or 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. 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 banishes child hunger. Besides this the milk has a different color and flavor depending on mother´s alimentation. [12]

The work starts with the pasteurization of the milk. This step takes about thirty minutes and it's realized at 62,5°C. Then it's necessary to cool rapidly to 15°C. After that, a sample of each bottle is collected to be analyzed at the Microbiology Department. Through the culture of the sample it's possible to identify if there is bacterial flora. The next step is the identification of all the bottles with 50 or 100mL of milk. The bottles from women that take medicines have a different identification and this milk only can be given to their children.

Fig. 15 – Collection of a milk sample from all the bottles.

The bottles are placed in the shocker where control freezing to -16°C is done during more or less one hour. The last step consists of storage of the milk in the freezer at least at -22°C. After this, the milk is ready to be used and has an expiration date of three months.

Fig. 16 – Milk stored in the freezer at -22°C.

The donors are mothers that have too much milk, mothers who are on maternity, mothers whose babies are hospitalized or voluntary mothers who are breastfeeding, and in this case, the hospital pays for the milk. The milk is not collected everyday. Mothers store the milk in the freezer at home during one week and then bring it to the Milk Bank.

5. Biomedicine Center

The Biomedicine Center makes part of the University Hospital Hradec Králové and it is a modern department that starts its work in 2011 and has about fifty employees, most of them are pharmacists. PharmDr. Ondrej Soukup, Ph.D. showed me the organization of the department and explained me the work that is developed there.

The center helps the clinics with basic research in cooperation with medical doctors, the Hospital, the Faculty of Medicine, the Faculty of Pharmacy and with the Faculty of Military Health Science. [13]

Per year, about one thousand compounds are produced. The main researched areas are Alzheimer, anti-cancer drugs, medicines to infections and organophosphorus pesticides poisoning antidotes. The medical doctors come to this department with an idea to treat some disease and they help building a project that makes this idea work. It focuses primarily on two areas: development of new drugs and proteomics. [13]

In drug development, the main tasks are drug discovery, chemical synthesis and toxicity tests. The first step is *in silico* design, using computers to find possible compounds that can be used like medicines in the future; the second part occurs *in vitro*, testing physico-chemical properties such as: solubility (main problem), log P/log D, pKa, metabolites (*in vitro* prediction), blood-brain barrier prediction (PAMPA assay to see how and if the drug crosses the lipophilic membrane); the third step is about making cytotoxicity tests in cell lines and toxicity tests in animals, measuring acute, sub-acute and chronic doses; the fourth is pharmacokinetics studies like plasmatic levels, half-life ($t^{1/2}$) and clearance; and the last one is *in vivo* tests to see if the compounds really can be used in humans with safety.

This process serves for identification of the most suitable drug candidates which are then recommended for preclinical testing or offered to the commercial partners. The synthetic chemistry lab is aimed to preparation of bioactive compounds via appropriate synthetic methods.

Relatively to proteomics, using modern proteomic technologies, qualitative and quantitative changes which occur during various pathological phenomena at the protein level can be studied. New protein biomarkers that allow early detection of disease are sought. Furthermore, evaluation of drug effects at the protein level is performed to better understand the mechanism of action and identify patients who can benefit from the pharmacotherapy. It's possible to look at the human protein profile and identify thousands of proteins with nanoHPLC.

6. Department of Immunology and Allergology

The department of Immunology and Allergology was founded in 1921 and provides specialized outpatient care in the field of clinical immunology and allergy and within of this place operates the National Reference Laboratory for Immunology. The Department of Clinical Immunology and Allergology provides specialized diagnostic and therapeutic activities for patients with disorders of the immune system such as immunodeficiency, infections like HIV, autoimmune diseases or fertility problems. [14]

During my stay in this department I learned a lot of concepts of immunological diseases and techniques which provides the diagnostic care in the field of Clinical Immunology and everything was explained to me by PharmDr. Doris Vokurková, Ph.D.

Despite the large number of analysis performed, almost all of them are based in the same principle: the connection binding of monoclonal antibodies associated with a specific fluorophore to the surface receptors of blood cells (the cluster of differentiation) detected by Flow Cytometry. This is the most used technique with a lot of applications like immunophenotyping and functional tests to lymphocytes and granulocytes.

In this department, it is used uncoagulated blood with heparin or EDTA and the measurement has to be done as soon as possible because the cells have to be viable.

a) The Cluster of Differentiation

CD stands for Cluster of Differentiation, which indicates a defined subset of cellular surface receptors (epitopes) that identify the cell type and stage of differentiation and which are recognized by antibodies labeled with fluorophores.

The CD system is commonly used as cell markers that are present on the surface of white blood cells, allowing cells to be defined and identified based on them. These markers are often used to associate cells with certain immune functions or properties such receptors, ligands or cell adhesion. [15, 16]

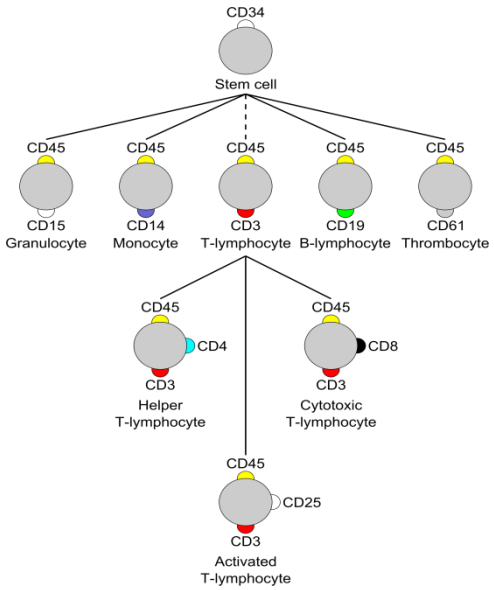


Fig. 17 – Some examples of CD molecules in leucocytes. [17]

Cell populations are usually defined using a + or a – to indicate whether a cell expresses or lacks a CD molecule. There are more than 300 markers. CD molecules are utilized in flow cytometry as a way of diagnosis of some allergic diseases and tumors.

Type of Cell	CD Markers
Stem cells	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+ and several different markers

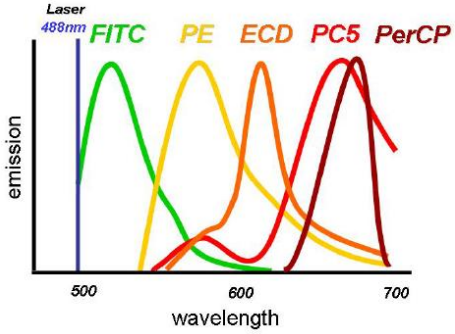
Table 1 – Different types of cells and respective CD markers.

b) Fluorophores

A fluorophore is a functional group in a molecule which absorbs energy on a specific wavelength and re-emits energy at a different but also specific wavelength. This involves the emission of a quantum of light that is possible to measure (fluorescence) after excitation with a laser beam.

Each antibody directed against each type of CD molecules is labeled with a different fluorophore and that way it's possible to see in each sample all the different types of cells present there. A particular type of cell based on the individual antigenic surface markers of the cell is identified by using a fluorescent dye conjugated to a monoclonal antibody. [18, 19]

To analyze the phenotype of the cells is important to use monoclonal antibodies marked with different fluorophores to recognize them. There are many types of fluorophores, but the most used were fluorescein isothiocyanate (FITC) and phycoerythrin (PE).



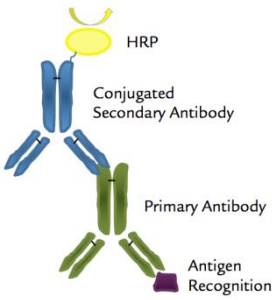


Fig. 18 – Emission spectra for some dyes used in labeled antibodies. Fig. 19 – Fluorophore-labeled antibody interacting specifically with the antigen of cell surface. [20]

c) Flow Cytometry

Flow Cytometry is a technique that allows the measurement of multiple physical characteristics of cells, as they flow in a fluid stream through a beam of light. The properties measured are particle's relative size, granularity, internal complexity and fluorescence intensity.

To detection and enumeration of cellular elements in a suspension is used an instrument named flow cytometer. 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 where the laser light is focused (cells are measured in 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, which is able to convert the detected light signals in electronic signals to be processed by the computer. [21, 19]

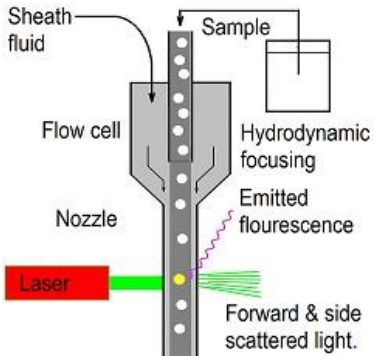


Fig. 20 – Scheme of how a flow cytometer operates. [22] Fig. 21 – Beckman Coulter flow cytometer.

In flow cytometry, cell suspensions are pumped 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. This scattering is measured by a photomultiplier tube (PMT) detector. This detector permits quantify the intensity of cell fluorescence that is correlated with the antigen density on the cell surface.

The scattered light and fluorescence of different wavelengths are then recorded. Typically, light scatter at two different angles is measured (side and forward scattered). Forward scatter is more sensitive to the size of the cell while side scatter is more affected by the optical homogeneity. Orthogonal light scatter or side scatter, defined as 90° light scatter with respect to the beam axis, correlates with cellular granularity and with the plasma/nucleus ratio of the cells. [21, 19]

The light scatter defines three different populations, which are granulocytes, monocytes and lymphocytes (G, M and L, respectively). This method allows the 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.

Data are usually shown either as single parameter histograms or as two parameter correlated plots, often called cytograms, where each point represents one cell. [21]

Before start the analysis, blood cells (after their isolation and purification) are incubated with the fluorescent-labeled monoclonal antibodies directed against the CD molecules during fifty minutes. After that, ammonium chloride is used to do the osmotic lysis of the erythrocytes during ten minutes (the reaction is stopped with saline) to not destroy the lymphocytes with the osmotic pressure.

The material that can be used is bone marrow, blood, suspension of cells from biopsies, cerebrospinal fluid and urine. Flow cytometry is a fast (more than 1000cells/second) and reliable technique that allows the analysis of thousands of cells in a small amount of sample (25µL).

d) Clinical Applications of Flow Cytometry

- **Cytocount**

The flow cytometer gives the result in %. So, to know the absolute number of cells using flow cytometry, it's necessary to use a suspension of small polystyrene beads with an exactly known number of them, usually 982 beads/µL. The following expression is used to know the exactly number of cells:

$$\frac{\text{number of lymphocytes}}{\text{number of beads}} \text{ x } 982$$

- **Immunophenotyping:**

Flow cytometry applied to Immunophenotyping is a very important tool of diagnostic (mainly for myelomas, lymphomas and leukemias), because it allows the analysis of cells population with the purpose of identifying the presence and quantities of the subpopulations of cells.

It is used to distinguish between healthy and abnormal cells. The normal cells express specific cell surface markers depending on the cell type and maturation degree. However, abnormal cells have a different expression of the markers, which can result in an over or under-expression of them.

There are many applications of immunophenotyping such as the diagnosis of leukemia or to get the proportions of CD4+ and CD8+. I did the analysis of the results of four patients relatively to the CD4+ and CD8+ count with the purpose to identify and to discriminate the T helper lymphocytes and T cytotoxic lymphocytes, respectively.

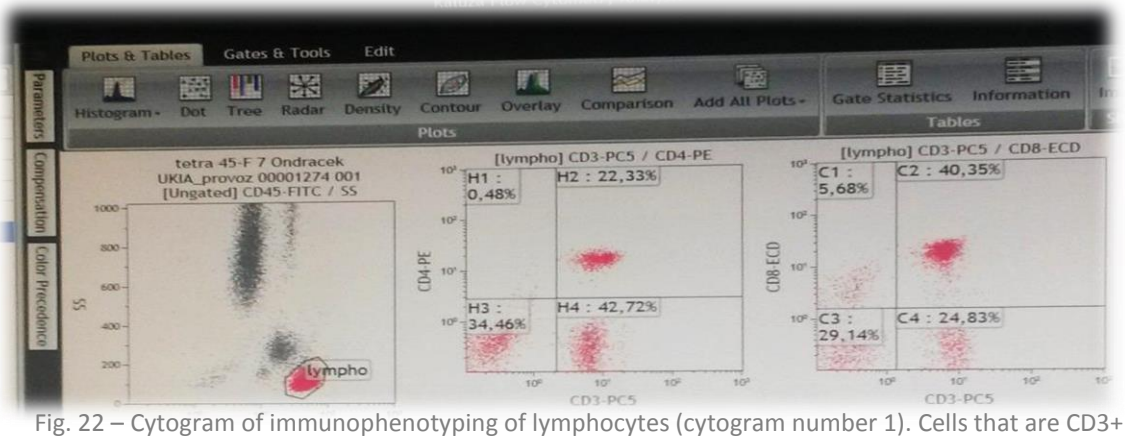


Fig. 22 – Cytogram of immunophenotyping of lymphocytes (cytogram number 1). Cells that are CD3+ plus CD4+ represent the number of T helper lymphocytes (cytogram number 2). Cells that are CD3+ plus CD8+ represent the number of T cytotoxic lymphocytes (cytogram number 3).

Normally, the number of CD4+ markers plus the number of CD8+ markers has to be equal to the number of CD3+. Nevertheless, sometimes the number of CD3+ markers are higher that the number of CD4+ and CD8+ together. It could happen because the subpopulation of T receptors γδ is increased and it could mean that the person has tuberculosis.

Immunophenotyping of Leukemia – Cancer Diagnostic

Leukemias are a group of neoplastic diseases in which precursor cells of the bone marrow become unable to differentiate and start to have high proliferative activity. In every stage of the development, cells can transform themselves in neoplastic cells.

After the clinical suspicion, a lot of analyses (for diagnostic and later for monitoring) are done in biological samples to confirm if there is leukemia and which type. The samples can be from peripheral blood, bone marrow or cerebrospinal fluid. Also it's possible to work with lung, brain and stomach tissue, but first the tissue has to be broken and then an emulsion of cells is made.

In this part, Mgr. Ondřej Souček elucidated me how the diagnosis of leukemia is done and once again flow cytometry is the chosen technique, however it's better for myeloma and acute leukemia diagnosis. The most important markers for the diagnosis are CD3+ (T cells), CD19+ (B cells), CD138+ and CD38+ (plasmatic cells), CD34+ (stem cells) and CD117+ (myeloid progenitors).

B cells produce antibodies that in the light chain of its constitution can be kappa or lambda. The higher number of kappa or lambda is related with B proliferation. Normally, the proportion is 3:1, but in patients with leukemia the proportion is very different: much more kappa or kappa negative. Because of that, it's possible to use as method of diagnosis antibodies against kappa and lambda and detect them by flow cytometry. In acute myeloid leukemia there is an increase of progenitor B cells, while in chronic myeloid leukemia there is an increase of mature B cells.

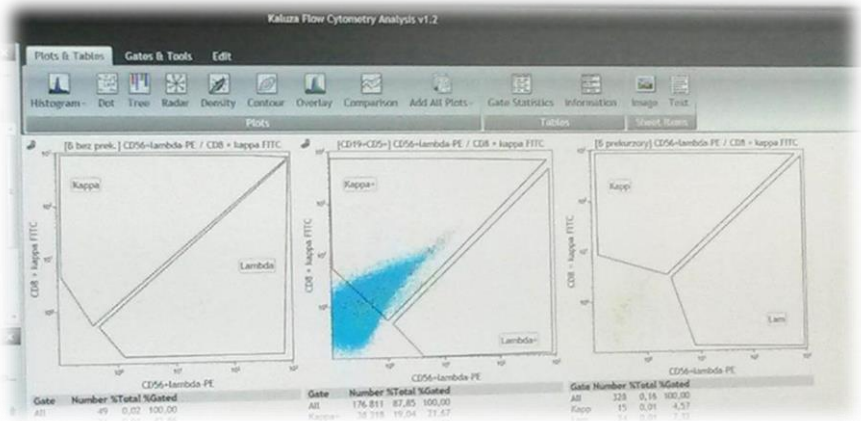


Fig. 23 – Immunophenotyping of leukemia. In this cytogram, it's possible to see that the population of kappa is very marked, while the population of lambda doesn't exist. This could be an indicator that the patient has leukemia.

Unfortunately, isn't possible to completely distinguish between different types of lymphomas/cancer. Cooperation with the Department of Pathology and with the Department of Hematology is needed, where new analyses to differentiate the several types of leukemias are done.

- **Viability of Stem Cells:**

To measure the viability of stem cells, it's used the CD34+ marker and not the CD45+ marker. They are immature cells, so they have a low amount of this marker and then low fluorescence.

Propidium iodide which has red fluorescence and can be excited at 488nm is used to realize the amount of viable cells, which aren't in apoptosis. It is an intercalating agent which is bound to double-stranded DNA and for that it has to get to the core of the cell. This is only possible if the cells are permeabilized. When the membrane is permeabilized and the cell is in apoptosis, there is a higher fluorescence comparatively when the cell is alive.

In case of transplantation at least 80% of the cells have to be alive. If the number is lower, could be danger for the patient because of apoptosis.

- **Functional Tests for Lymphocytes**

Blastogenic Transformation Lymphocyte Test (BTT)

The BTT is an *in vitro* test which 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 the measurement of the incorporation of fluorescent dye propidium iodide.

The fluorescent intensity of dye in each cell is a direct measure of the amount of nuclear DNA, because the propidium iodide is an intercalating agent of DNA. With this dye it's possible determinate the precise number of cells in each phase of the cell cycle (G0/G1, S and G2/M) and calculate the proliferative activity.

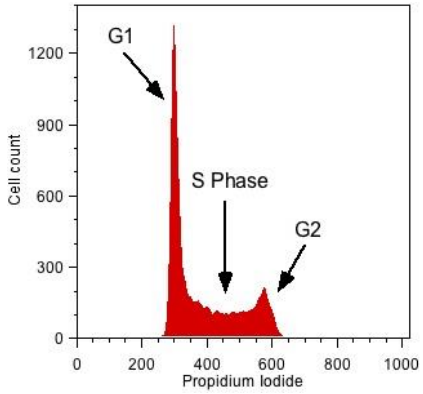


Fig. 24 – DNA histogram correlated with the phase of cell cycle. [23]

This is the graphic of a normal situation obtained by flow cytometry. However, if there is stimulation after the contact with an antigen, the G2 phase will increase after three days of incubation, because 72h is the time needed to form new DNA. There are an increase of the fluorescence, because more DNA was formed and more propidium iodide bound to it. This means that there is more proliferation of blast cells.

To do this procedure, Phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) are used to stimulate the T and B cells, respectively. This test is used in cases of immunodeficiencies and chemotherapy, where the proliferation of lymphocytes is low. As well, this test can be done in cases of organ transplantation, where higher values of proliferation could mean organ rejection.

Fast and Later Activation

This test has the same purpose of the previous test, but it takes less time. The test is divided in two parts. In the first one, the searched marker is CD69+. This is the marker of the

fast activation of lymphocytes. It appears 4h after the stimulation and decreases after 24h. This way, CD3 plus CD69 markers are searched to identify T lymphocytes and CD19 plus CD69 are searched to identify B lymphocytes.

The second part is realized after 48h and the following markers are searched with the antibodies directed against them and attached with different fluorophores: CD25-PE plus CD3-PC7 for T lymphocytes and CD23-F plus CD19-PC5 for B lymphocytes.

In this test, three tubes for each type of lymphocyte are needed - one tube with x-vivo (medium to dilute the blood) inside, acting as negative control; one tube with PHA to stimulate T lymphocytes, acting like mitogen; one tube with PWM to stimulate B lymphocytes. All the tubes contain blood as sample to analyze.

After the incubation during 24h or 48h, the antibodies are added and after 15 minutes, the lysis of erythrocytes has to be done with formic acid during 10 seconds. To stop the reaction the saline is added and then the flow cytometry is done.

- **Functional Tests for Granulocytes**

Granulocytes (neutrophils, eosinophils and basophils) are an important fraction of white blood cells (leucocytes) and they have a central role in the first line of immune defense, especially in the combat to bacterial and fungal infections. Their main activity is named phagocytosis and it is divided in four steps:

1 – Chemotaxis

2 – Intake/invagination

3 – Burst

4 – Bacteria digestion

It is possible to analyze the phagocytic capacity in three of these four steps. The step number two is observed in the microscope. For that, a suspension of *Candida albicans* is incubated with patient's blood during one hour, at 37°C and under agitation. This way, granulocytes will englobe the microorganism. Later, one drop of the suspension is smeared in a glass slide to observe it in the microscope. After that, the total number of granulocytes (100%) and the number of granulocytes that ingested at least three microorganisms are counted and then the percentage of phagocytosis is calculated.

Burst Test

Relatively to step number three, this test is based on the measurement of 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 hypochloride ions inside phagocytes that 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.

In each test are needed three tubes, all containing blood sample and DHR 123: One tube with saline that works as negative control and, as such, will have lower intensity of fluorescence; one positive control with PMA that will have the higher intensity of fluorescence; and finally, one tube with *E. coli* that will have intermediate intensity of fluorescence because it isn't such a stronger stimulant as PMA.

All the tubes are incubated during 45 minutes, at 37°C and with 5% of CO_2. After incubation, red blood cells have to be destroyed and the next step is to do the flow cytometry. The normal values are in the range of 75 to 100%.

If in the tube with *E. coli* there is no fluorescence, it could be a signal of 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 featuring deficiency of a downstream myeloperoxidase enzyme generating hydroxyl radical and hypochlorite, which is the most effective killing agent produced during a respiratory burst.

Finally, about the four and last step, it's possible to cultivate pathogens with blood, incubate it during 24h and observe if there is growth of colonies. The desired is that there is no growth of colonies.

e) Separation of Lymphocytes

Besides the applications of flow cytometry, others techniques are performed in this department like lymphocyte separation. For this it is necessary to use Histopaque 1077 that 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.

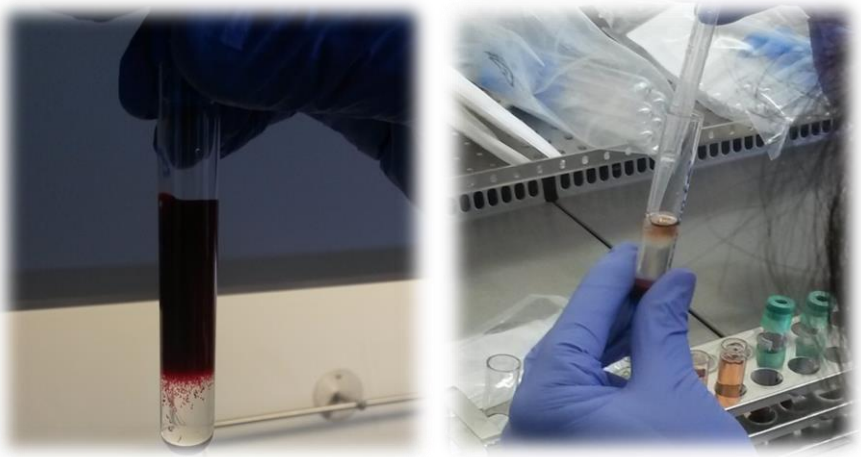


Fig. 25 and 26 – Separation of lymphocytes. In the first image, it's possible to observe the sedimentation of erythrocytes before centrifugation. The removal of plasma (first layer) to isolate the leucocytes, in the second image.

It is impossible to separate the lymphocytes in one single step. Actually, the Histopaque 1077 solution just makes the separation between granulocytes and mononuclear cells (lymphocytes and monocytes stay in the same layer).

The second step of the isolation process consists in separate these two kinds of cells by the ASPAS adherence method which consists in putting the mononuclear cells layer in a plastic wells plate and wait for the monocytes adhere to the plate and in the cells suspension there will be only 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. The separation of lymphocytes is a very common technique used for identify some pathologies and also for research for new signals pathways involved in specific disorders.

f) Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assays (ELISAs) are plate-based assays designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. The analyte is indicated by a color reaction between an enzyme and a substrate.

An antigen corresponding to the target antibody is immobilized in microwells and then it is incubated with an antibody present in the added sample (200µL) at room temperature. If this antibody is present in the test sample, it will bind to the antigen. An enzyme-conjugated secondary antibody (100µL) is added to the plate to bind to the target antibody. Between every addition of antibody, it's necessary to wash away the unbound material three times to make sure that there are no free antibodies present which could result in a false positive. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction. [24]

With the help of Mgr. Martina Koláčková, Ph.D., I performed an ELISA test to detect auto-antibodies against thyroid peroxidase to discover if the patient has Hashimoto's disease. For that I used a positive and negative control prepared in the same way as samples and four standards and I did the above-mentioned technique. After that, I used the spectrophotometer to measure the antibody-antigen interaction. It measures the absorbance, gives the concentration and the calibration curve (absorbance vs concentration) is calculated automatically by the equipment measuring the standards concentrations. The amount of color is directly proportional to the concentration of antibodies present in the sample.



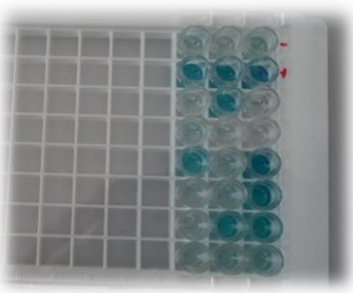


Fig. 27 – Addition of the enzyme-conjugated secondary antibody (blue). Fig 28 – Addition of the substrate (pink). Fig. 29 – The result of the antibody-antigen interaction (blue).

g) Reproductive Immunology

More and more, people are having problems with pregnancy and it could be caused by several factors. In Czech Republic, it is a concerned problem because the natality rounds 1,1 and lots of couples try to have a child unsuccessfully.

Some immune response can cause infertility and in this case the number of leukocytes and NK cells in sperm are very high. The vitality of sperm and the integrity of acrosome are also changed in pathological situations and they also can cause infertility.

Because of that, the Department of Immunology has a special attention to these cases and developed a series of tests to better understand the reasons why people can't have a child. Samples are blood from women and man and sperm. The tests have to be performed until 2h after the collection of sperm. The sperm sample is put in the thermostat to keep its temperature at 34°C. I have been present in the following three tests: Sperm Flow, Inhibition of Migration and Activation of NK cells.

Once again the used technique was flow cytometry because it permits the evaluation of some characteristics like sperm integrity, viability and function.

- **Sperm Flow**

With this test, it's possible to assess diverse parameters in the sperm sample, using flow cytometry, such as sperm count, leukocyte count, sperm viability, sperm acrosome integrity and presence of intra-acrosomal protein in sperm.

These parameters are important because the acrosome contains digestive enzymes that breakdown the outer membrane of the ovum, the so-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, four tubes with 10x diluted semen with saline (25µL in tube A and 100µL in tube B, C e D) are used in the following tests:

Tube A – Sperm Count and Leukocyte Count: addition of an internal standard (fluorescent beads with known concentration) after incubation of the semen sample. The detection of leukocytes is performed by staining with labeled antibody against human CD45 antigen.

To know the exact number of sperm cells the follow expression is used:

$$\frac{\text{number of sperm cells}}{\text{number of beads}} \text{ x } 1000 \text{ x } 10 \text{ (dilution factor)}$$

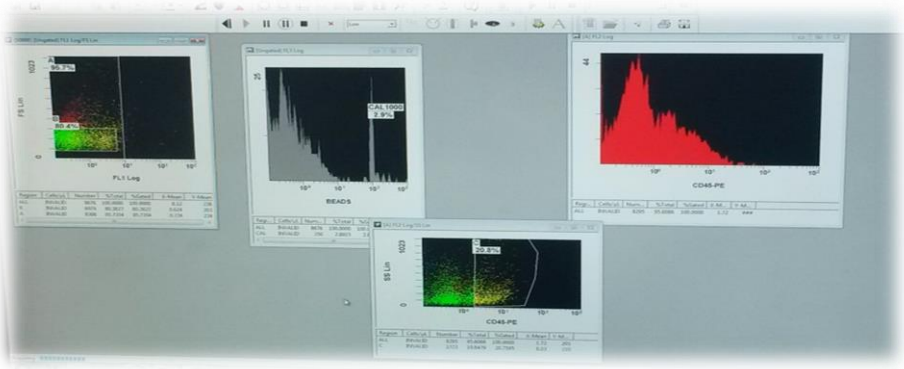


Fig. 30 – The first cytogram is the count of sperm (the green part is the mature sperm and the red part is the immature sperm); the second graphic is for calibration; in the third and fourth graphics are represented the leukocytes present in the samples that are identified by CD45+.

Tube B – Sperm Viability: it is examined using propidium iodide which permeates through damaged membranes of dead cells and binds to their DNA.

Tube C – Acrosome Integrity: it 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 of 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. Ethanol is used as permeabilization reagent and the expression of the protein should be more than 75%.

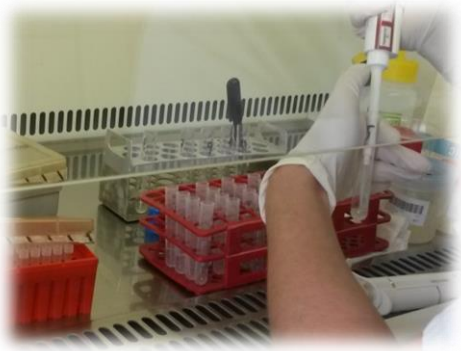


Fig. 31 – Addition of the sperm to the four tubes to perform the Sperm Flow tests.

- **Inhibition of Migration**

This test is applicable for man and for woman and to do it is necessary to use two plates with agarose with small holes.

Relative to the man, half of the plate is used to put a suspension of man's leukocytes plus medium (negative control) in the holes and half to put a suspension of man's leukocytes plus sperm. After that, the plate is incubated during 16h. The lymphocytes will produce cytokines that impede the migration when they are sensitive to the sperm antigen.

In relation to the woman, the plate is divided in three parts, all with a suspension of leukocytes from the woman: one to put the sperm; one to put trophoblastic cells, having the antigen of the embryo (these cells are obtained in cell banks where they are frozen); and one to put the medium (negative control). If the cells don´t migrate, the surface of the hole is smaller, so that means that the woman is positive for the sperm antigen of her partner.

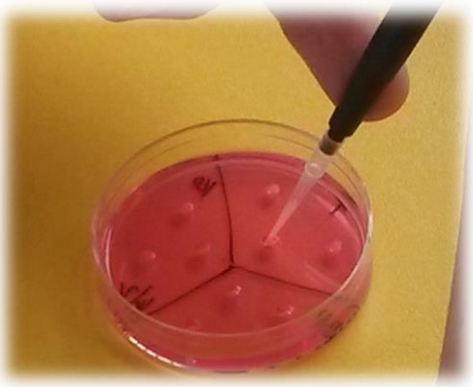


Fig. 32 – Addition of trophoblastic cells to the holes where is the suspension of leukocytes from the woman.

Woman's cells can have antibodies against the sperm antigen of the man and this could be a reason of infertility. The longer the woman is together with the same man, the greater is the development of antibodies against his sperm. Also, taking anti-contraceptive pills increase the production of antibodies.

To solve this problem, the treatment is based on corticoid therapy to decrease the activity of the immune system. Furthermore, it's recommended the use of condoms during three months to not make the woman's immune system more susceptible to the sperm. If none of these actions works, the best solution is to do *in vitro fertilization*.

In case of a man's problem, the situation is more complicated because there is no treatment. The preventive measure go through do not wear tight trousers, do not use the mobile phone in the trousers pocket, do not smoke, do not work with computer in the legs and be careful about feeding.

- **Activation of NK Cells**

Natural killer cells (NK cells) are a type of cytotoxic lymphocytes critical to the innate immune system. They are extremely efficient in recognize and eliminate the cells that show alterations. A higher number of NK cells could be a reason for not be pregnant, because these cells can act against sperm and trophoblast cells. The target is to see if there is activation of peripheral blood natural killer (NK) cells in infertile women.

To know the activity of NK cells, the woman's blood is incubated in a 96-well plate with medium (negative control), sperm and trophoblast cells. The incubation occurs during the night and in the next day the antibodies are added. Then, activated NK cells are identified by the detection of CD3-, CD56+ and CD69+ (early activation of leukocytes marker) markers by flow cytometry analysis.

If the result is more than 30%, it happens because there is activation of NK cells. This is a bad condition, because it means that the NK cells are attacking the sperm as well the trophoblast. On the other hand, the treatment with corticoids above-mentioned (that are used to decrease the woman's immune system) could lead to the increase of NK cells and this situation isn't good too.

7. III. Internal Gerontometabolic Clinic – Laboratory

The III. Internal Gerontometabolic Clinic is situated at the University Hospital Hradec Králové and is the base for clinical research on the Metabolism, Clinical Nutrition, Gerontology, Diabetology and Nephrology. The department cooperates mainly with the Department of Oncology, Surgery and Nephrology and with the Department of Analytical Chemistry in the Faculty of Pharmacy. It has several functions such as:

- Acute diagnostics and treatment of patients with disorders of metabolism and nutrition (e.g.: Diabetes Mellitus, liver or kidney insufficiency, obesity);
- Support of patients who need parenteral and enteral nutrition;
- Special diagnostics and treatment of disorders of metabolism and nutrition (diabetology, disorders of lipid metabolism);
- Diagnosis and treatment of acute illness of the aged patients;
- Diagnosis and treatment of internal diseases with special emphasis on internal diseases in old age and the issue of premature aging. [25]

This department has different projects related with the degenerative diseases associated with aging and is focused in early diagnostic and prognostic of diseases more prevalent in older people. Aging is a complex biologic phenomenon and with the increasing age there is a higher prevalence of illness and chronic diseases such as stroke and cancer. In this laboratory, the technique I performed was HPLC which I explain bellow.

High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) allows the separation of complex mixtures into their individual components. This technique can be used for all soluble compounds which are dissolved in a specific solvent and then separated. This can be achieved by making use of different interactions of compounds in solution with a stationary phase, since they have different relative affinities in both phases. The mode of separation can be chosen

and optimized by selecting a particular combination of a mobile and stationary phase. 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 computer data station.

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 and then this mixture is injected into a flowing mobile phase and reaches the chromatographic column. This column contains the stationary phase that separates target analytes. After separation, they are eluted to the detector and generate a signal that will be multiplied. Then it is sent to the data acquisition system that records the signal, required to create the chromatogram and to identify and quantify the concentration of the sample constituents.

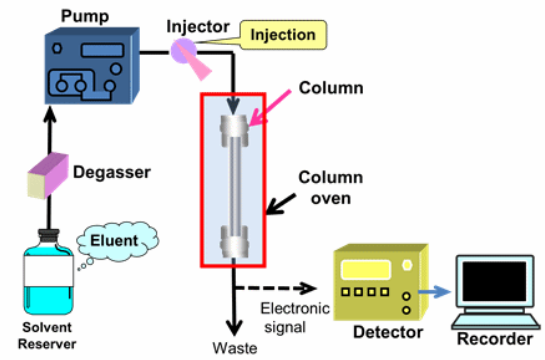


Fig. 33 – Schematic representation of a HPLC system. [26]

Detection of eluting compounds is expressed in a chromatogram. Numerous types of stationary phases are available differing in interaction type and/or strength and 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. HPLC coupled with sensitive detector can identify several compounds that are in low concentrations and it is used to analyze several samples such as pharmaceuticals, food, cosmetics, environmental matrices, forensic samples and industrial chemicals.

In this laboratory, I had the opportunity to measure biological materials and standards of Vitamin B with the purpose of the optimization of the method. The biological materials were urine, plasma and serum and before the injection in HPLC, they had to be processed by sample preparation procedure. Besides, I also measure the amino acid Arginine and its metabolites Citrulline and Ornithine to evaluate the reproducibility of the method that allows their determination. These are very important markers in the wound healing process.

8. Department of Hematology

The Department of Hematology supplies ample care for patients with hormonal disorders and blood diseases, it is opened 24h/day and it was showed me by Mgr. Filip Vrbacký. The visit started in the blood collection room and then we were passing for the different laboratories where I could see the analyzers where the following parameters are analyzed: blood cells (red and white cells and platelets) count, differential leukocyte count, erythrocyte sedimentation rate, viscosity of the blood, adhesion and aggregation of the platelets. [29]

Other parameters observed were the fragility of the membrane of the red blood cells that is measured by osmotic pressure and the coagulation time. There are two different ways to measure the coagulation time: using covets with samples and reagents and it is measured by laser beam or using a machine with a small ball that moves when the blood isn't coagulated. So when it stops moving it is because the blood is coagulated and this gives us the coagulation time. The coagulation section implements assays for monitoring of anticoagulant therapy.

If there is some problem with the cell count, one of the alternatives is to do a smear and observe it in the microscope. Another option is using the Cell Image Analysis System that takes pictures of all the cells present in the smear for they could be analyzed. Here it is also done the diagnosis of thalassemias, anemias and hemoglobinopathies, by gel electrophoresis of hemoglobin. And also antiplatelet therapy is monitored with optical and impedance aggregometer.

In this department I observed samples of bone marrow cells with leukemia in the microscope to see the difference in the number and shape of leukocytes in comparison to the normal ones.

9. Department of Analytical Chemistry

During my visit to the Faculty of Pharmacy, in the Department of Analytical Chemistry, I had the opportunity to learn two different techniques: capillary electrophoresis which was taught by Mgr. Klára Petrů, Ph.D. and flow injection that was explained 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 Electroosmotic Flow (EOF) is the movement of the particles in direction to the anode according with the charge and with the velocity imposed by the applied voltage. The smaller cations are the first that migrate; second, the bigger cations because for ions with the same charge, the smaller particle has less friction and overall faster migration rate; then, the neutral species don't have affinity to the anode neither to the cathode, so they only migrate thanks to the applied voltage; finally, the anions are supposed to migrate in the direction of the cathode, but the applied voltage makes this species follow the flow in direction to the anode.

Capillary electrophoresis is used most predominately because it gives faster results and provides high resolution separation. To improve the detection and increase the sensitivity the following materials are used: Z-cells (ten times more) or tube with bubbles (three times more).

In the Electrophoretic laboratory at the Department of Analytical Chemistry, I saw three different detectors: UV, florescence and conductivity detector. [30]

➢ Flow Injection

Flow Injection is a continuous flow technique where 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 placed downstream records the color of the product that changes due to the passage of the derivatized sample material through the flow cell.

By applying flow programming, each step of an assay protocol is performed at an optimized flow rate. The advantage of use it is the increased sensitivity and detection limit of most reagent, achieved by adjusting the flow rate and incubation time from the computer without need the reconfiguration of the system. FI is a valuable tool for studies in Pharmacology, Chemical Oceanography and Environmental Monitoring. [31]

10. Conclusion and Acknowledgements

This internship was a huge opportunity for me. I learned so many new concepts and techniques in a lot of different departments and because of that I think that it couldn't be more rewarding. In every department I contacted with the work that is done there and I got a completely new notion about all the things that are possible to do in the hospital.

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